Enzymatic fractionation of brewer's spent grain and bioconversion of lignin-rich fractions in a colon model in vitro

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Abstract

The objectives of this thesis were to produce lignin-rich fractions from brewer's spent grain (BSG) and to study the interactions of the lignin in these fractions with colon microbiota *in vitro*. Different milling pre-treatments were studied to enhance enzymatic hydrolysis of BSG carbohydrates. Ball-milling, which was the most efficient treatment, increased carbohydrate solubilisation from 23 to 45 %. Thus, milling notably improved enzymatic solubility of cell wall polysaccharides but was not effective enough to enable their total hydrolysis. Two lignin-rich fractions (24 and 40 % lignin content) were obtained by enzymatic fractionation of BSG using carbohydrases and proteases. In addition, a separate alkaline extraction provided BSG-derived material with low ferulic acid content. BSG and the fractions were used to study if lignin is degraded and metabolised by colon microbiota in a metabolic model and if lignin suppresses microbial conversions in the colon. A number of mono- and dimeric phenolic metabolites were formed upon digestion of BSG and the fractions by the microbiota. It appeared that many of them were structurally lignin-related indicating their release from lignin and conversion by colon microbiota. However, the extent of lignin degradation was estimated to be low. No notable suppression of microbial conversions was detected based on the formation of linear short chain fatty acids. In addition, experiments with pure strains of lactobacilli and bifidobacteria showed no inhibition of growth by a lignin-rich fraction. Association of lignin with carbohydrates or proteinaceous material may have reduced the possible antimicrobial effects of lignin. The results of the present study provide new information on the significance of lignin as part of dietary fibre indicating its partial metabolism by colon microbiota.

Tiivistelmä

Tämän väitöskirjatyön tavoitteena oli valmistaa ligniinipitoisia jakeita panimomäskistä sekä tutkia näiden jakeiden avulla ligniinin ja suolistomikrobien vuorovaikutuksia. Erilaisten jauhatusten vaikutusta mäskin hiilihydraattien entsymaattiseen hydrolyysiin tutkittiin, ja kuulamyllytys osoittautui tehokkaimmaksi parantaen hiilihydraattien hydrolysoituvuutta 23 %:sta 45 %:iin. Jauhatus ei kuitenkaan ollut riittävän tehokas esikäsittely hiilihydraattien kokonaishydrolyysin kannalta. Kaksi ligniinipitoista jaetta (24 ja 40 % ligniiniä) valmistettiin hiilihydraatteja ja proteiineja pilkkovien entsyymien avulla. Lisäksi emäskäsittelyllä tuotettiin jae, josta ferulahapot oli lähes kokonaan poistettu. Mäskin ja sen jakeiden avulla tutkittiin paksusuolen olosuhteita simuloivassa metaboliamallissa, pilkkovatko suolistomikrobit ligniiniä ja estääkö ligniini mikrobien aineenvaihdunnan toimintoja. Metaboliamallissa muodostui pieniä, fenolisia aineenvaihduntatuotteita, joista useat olivat rakenteensa perusteella todennäköisesti ligniiniperäisiä. Ligniinin pilkkoutuminen vaikutti kuitenkin vähäiseltä. Lyhyiden, suoraketjuisten rasvahappojen muodostumisen perusteella ligniini ei haitannut mikrobien hiilihydraattifermentaatiota. Ligniini ei myöskään estänyt laktobasillien tai bifidobakteerien kasvua. Ligniinin sidonnaisuus hiilihydraatteihin tai proteiiniperäiseen materiaaliin saattoi suojata mikrobeja ligniinin mahdollisilta antimikrobisilta ominaisuuksilta. Tutkimuksen tulokset antavat uutta tietoa ligniinin hajoamisesta suolistossa sekä sen merkityksestä osana ravintokuitua.

Acknowledgements

Contents

APPENDICES : Publications I–V

List of abbreviations

BSA   bovine serum albumin

BSG   brewer's spent grain

CFU   colony forming units

CHP   combined heat and power

DEFE   deferuloylated BSG fraction

DFRC   derivatisation followed by reductive cleavage

DF   dietary fibre

DM   dry matter

DP   degree of polymerisation

EFSA   European Food Safety Authority

FC   fold change

FPU   filter paper units

GC/FID   gas chromatography coupled with flame ionisation detector

GCxGC-TOFMS two-dimensional gas chromatography coupled with time-of-flight mass detector

GMD   GOLM Metabolome Database

HPLC   high performance liquid chromatography

INS   insoluble residue (after three-step enzymatic hydrolysis)

MRS   de Man-Rogosa-Sharpe (medium)

NMR   nuclear magnetic resonance

P-AEF   protease-alkaline extracted fraction

Py-GC/MS   analytical pyrolysis coupled with gas chromatography and mass detector

RCM   reinforced clostridial medium

RT   room temperature

SCFA   short chain fatty acid

UV   ultraviolet

List of publications

This doctoral dissertation consists of a summary and of the following publications which are referred to in the text by their numerals.

I   Niemi P., Faulds C.B., Sibakov J., Holopainen U., Poutanen K., Buchert J. (2012) Effect of a milling pre-treatment on the enzymatic hydrolysis of carbohydrates in brewer's spent grain. *Bioresour. Technol.* 116, 155-160. DOI: 10.1016/j.biortech.2012.04.043.

II   Niemi P., Martins D., Buchert J., Faulds C.B. (2013) Pre-hydrolysis with carbohydrases facilitates the release of protein from brewer's spent grain. *Bioresour. Technol.* 136, 529-534. DOI: 10.1016/j.biortech.2013.03.076.

III   Niemi P., Tamminen T., Smeds A., Viljanen K., Ohra-aho T., Holopainen-Mantila U., Faulds C.B., Poutanen K., Buchert J. (2012) Characterization of lipids and lignans in brewer's spent grain. *J. Agric. Food Chem.* 60, 9910–9917. DOI: 10.1021/jf302684x.

IV   Niemi P., Aura A.-M., Maukonen J., Smeds A., Mattila I., Niemelä K., Tamminen T., Faulds C.B., Buchert J., Poutanen K. (2013) Interactions of a lignin-rich fraction from brewer's spent grain with gut microbiota *in vitro*. *J. Agric. Food Chem.* 61, 6754–6762. DOI: 10.1021/jf401738x.

V   Aura A.-M., Niemi P., Mattila I., Niemelä K., Smeds A., Tamminen T., Faulds C.B., Buchert J., Poutanen K. (2013) Release of small phenolic compounds from brewer's spent grain and its lignin fractions by human intestinal microbiota *in vitro*. *J. Agric. Food Chem.* 61, 9744–9753. DOI: 10.1021/jf4024195.

Author's contribution

Publication I: Piritta Niemi had the main responsibility for preparing and writing the article and she is the corresponding author. She planned the study together with co-authors. From the experimental work she conducted enzymatic hydrolyses, wet millings in co-operation with technical staff, particle size analyses of the wet milled samples, and analysis of reducing groups as a function of hydrolysis time. Composition analysis, dry millings and the respective particle size measurements, HPLC analyses, microscopy (supporting material) and enzymatic activity assays were carried out by co-authors and technical staff. Piritta Niemi was mainly responsible for calculating the results and she carried out the interpretation of results with the aid of co-authors.

Publication II: Piritta Niemi had the main responsibility for preparing and writing the article. The study was designed together by the authors, and Duarte Martins conducted the experimental work as part of his Master's Thesis work. He was supervised by Piritta Niemi and the co-authors. The interpretation of results was carried out as co-operation by all authors.

Publication III: Piritta Niemi had the main responsibility for preparing and writing the article, and she is the corresponding author. The study was planned together by the authors. Piritta Niemi carried out the enzymatic fractionation of BSG. Lipid and lignan analyses were conducted by co-authors. Composition analyses and microscopy imaging were carried out by technical staff. Interpretation of the results was carried out jointly by Piritta Niemi and the co-authors.

Publication IV: Piritta Niemi had the main responsibility for preparing and writing the article, and she is the corresponding author. The study was planned jointly by the authors. Piritta Niemi prepared the studied sample and participated in the experimental work of the colon model experiment led by Dr. Aura. Lignan analysis as well as GCxGC-TOFMS analysis including identification and determination of chemical structures of metabolites were carried out by co-authors. Piritta Niemi and Dr. Aura prepared the heat map. Experiments with bacterial strains were conducted by Dr. Maukonen. Interpretation of the results was carried out jointly by the authors.

Publication V: The study was planned together by the authors. Piritta Niemi prepared the studied samples and participated in the experimental work of the colon model experiment led by Dr. Aura. Lignan analyses and GCxGC-TOFMS analysis including identification and determination of chemical structures of metabolites were carried out by co-authors. Piritta Niemi and Dr. Aura prepared the heat maps. The results were interpreted together with the co-authors.

1. Introduction

Brewing is an age-old technique and science. In fact, it is often said that brewing beer and baking are the world's oldest food technologies. As reviewed by Boulton and Quain (2006), brewing with malts was probably started in the Middle East sometime after the birth of agriculture in 6000 BC. Most likely the early fermentations were spontaneous reactions, where a natural source of sugar was contaminated with yeast in the presence of a sufficient amount of water. Furthermore, the discovery of malting is also believed to have occurred by accident. In ancient times, alcoholic beverages were not just part of human diet but presumably were associated with religion and rituals as well, due to the physiological effects of alcohol. Drinking beer instead of water also had its benefits in times when diseases, such as cholera, spread in contaminated drinking water. As described by Boulton and Quain (2006), the skill of brewing was further developed in Europe, where it was an important part of everyday life already in the medieval times, and taken to a larger scale production by the abbeys at least in the UK and Belgium. In addition to beer, brewing produces also spent grains, which are the insoluble residues of the malts containing a high amount of protein and cell wall polysaccharides.

1.1 From barley to beer and spent grains

1.1.1 Grain structure

Cereal grains are composed of the starchy endosperm, embryo (or germ) and several layers around them having different functions, such as protein storage and enzyme secretion (aleurone) and protection (testa, pericarp and husk). The different components and layers are presented in Figure 1 and a more detailed structure of the barley grain is shown in Figure 2.

   Husk and pericarp are the outermost, protective parts of the grain, shown as yellow in Figure 2. The colour is due to autofluorescence caused by phenolic components in the cell walls. Nearly all of cellulose in barley (96 %) is located in the husk (Duffus and Cochrane 1993). Below the pericarp, there is the testa or seed coat, which is a thin protective layer rich in hydrophobic cutin. The aleurone layer in barley usually consists of three layers of cells, which contain most of the storage proteins in the grain, as visualized by the red color in Figure 2. Together, pericarp, testa and aleurone layer are referred to as bran, which is rich in dietary fibre and several other nutrients. Bran is often removed in the milling of grains to flour. However, in malting and mashing husk and bran are not separated but they as well enter the process. Under the protective layers there is the endosperm, which is the largest part of the grain. Endosperm cell walls are composed of β-glucan (70 %) and arabinoxylan (20 %), whereas in the aleurone layer the ratio is almost the opposite: 26 % of β-glucan and 67 % of arabinoxylan (Duffus and Cochrane 1993). Endosperm stores energy in

the form of starch, which can be hydrolysed to sugars during germination providing energy for the growing plant. Also the embryo, where the new plant grows from, stores lipids and proteins to initiate growth.
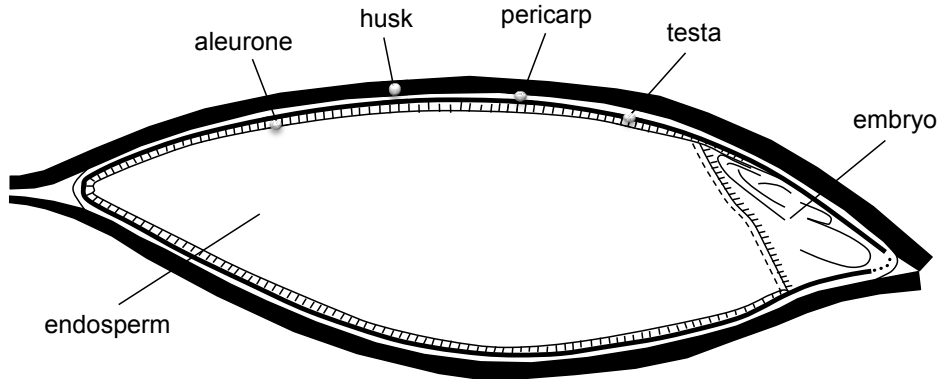
Figure 1. Structure of a barley grain. Courtesy of Oili Lappalainen, VTT.

1.1.2 Malting and mashing

The first step in the making of beer is malting of grains. The majority of beer is brewed from barley malts but other cereals, such as wheat, buckwheat or rice can be used as well (Depraetere *et al.* 2004, Nic Phiarais *et al.* 2010, Teramoto *et al.* 2002). This thesis is, however, focused on barley and other raw materials are not further discussed. In malting, the grains are first steeped in cold water to increase the moisture content of the grains to above 40 % (Bamforth and Barclay 1993). This initiates the germination process and induces the production of endogenous enzymes. The enzymes are synthesised from the protein storages in the aleurone layer and secreted to endosperm, where they start to hydrolyse carbohydrates and proteins to provide nutrients for the embryo. Amylases, β-glucanases and proteases are the most important enzymes considering the following mashing process. In addition to the endogenous enzymes a diverse microbial community originating from the field, storage and post-harvest processing is present in barley grains. Microbial interactions with the grains may affect safety, technological, nutritional and organoleptic properties of malts and beer (Laitila 2007). Germination process is stopped by kilning, which preserves the produced enzymes although they are inactivated. The enzymes are needed in mashing to further digest the endosperm cell walls and starch.

In mashing, ground malts are mixed with water, and the water temperature is raised stepwise to allow different hydrolytic enzymes to become activated and hydrolyse their substrate polymers (Briggs *et al.* 2004a). Mashing times and temperatures vary from brewery to brewery, but usually mashing is finished in approximately 2 h. Examples on mashing schedules and temperatures are given by Briggs *et al.* (2004a). β-glucanases are heat-labile and active at temperatures 30–40 °C. Proteolysis occurs at approximately 35–60 °C. Amylases are more heat-stable and become activated around 50 °C, and saccharification of starch occurs up to 70°C.
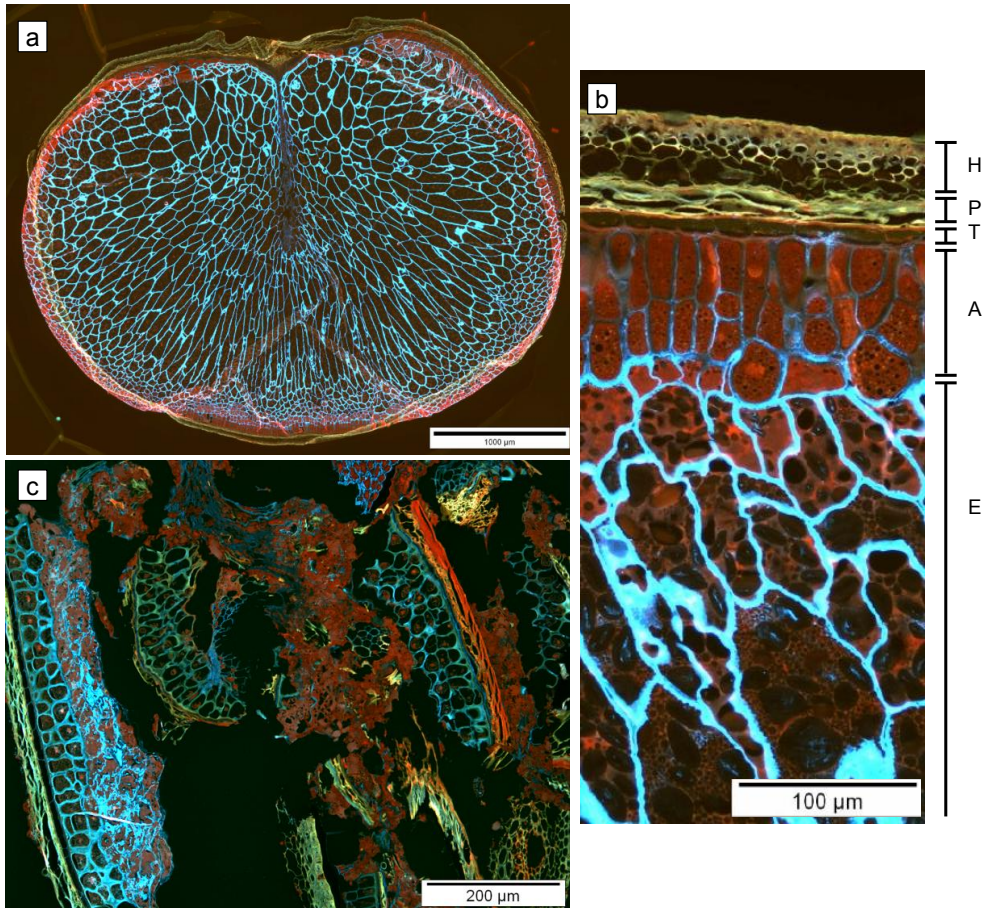
Figure 2. a) A microscopy image of a cross-section of a barley grain, b) a close-up of the different layers of the grain (H=husk, P=pericarp, T=testa, A=aleurone, E=endosperm), and c) spent grains recovered after mashing. In all images protein appears as red and aleurone and endosperm cell walls blue. Husk and pericarp cell walls show yellow and green autofluorescence due to their phenolic components. Images a and b are courtesy of Ulla Holopainen-Mantila, VTT. Image c is adapted from Paper I (supplementary material) and reprinted with permission of Elsevier B.V.

β-glucanases are the first enzymes to become activated in mashing and they are responsible for releasing the starch granules from the endosperm, as majority of endosperm cell wall is composed of β-glucan (Briggs *et al.* 2004a). This can be seen also from the intensive blue colour in Figure 2, as the dye Calcofluor stains β-glucan blue. In malting, a part of proteins is degraded to amino acids and peptides by proteases, and the proteolysis continues in mashing. Starch gelatinization of malted barley occurs at 64–67 °C (Briggs *et al.* 2004b), and after the mashing temperature reaches this level, amylases start degrading starch to fermentable sugars, mainly maltose. Starch is the most abundant component in the grain and accounts for 60–64 % of the total weight (Jadhav *et al.* 1998). The endosperm is almost completely solubilised as the result of malting and mashing, and the starch granules, which appear as black particles in Figure 2b, are no longer visible in the insoluble residues of the malts, i.e. brewer's spent grain (BSG) (Figure 2c). The applied temperatures and duration of mashing define the characteristics of the final wort, which is the sugar-rich liquid phase after mashing. After mashing, the wort containing all the solubilised compo-

nents is separated and BSG remains in the mash kettle (Figure 3). Hops are added to the wort, which is then boiled and fermented to beer.
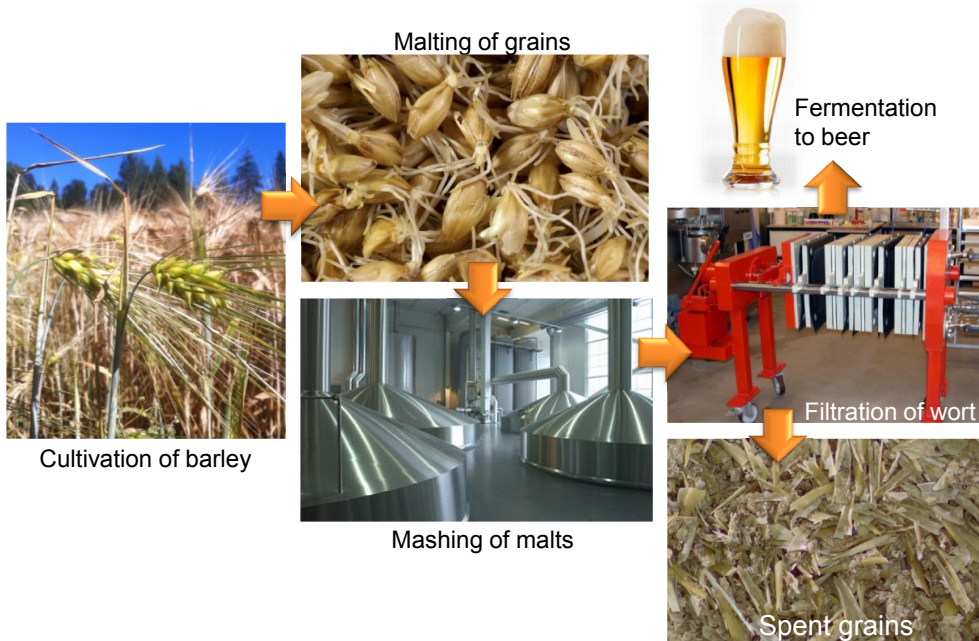
Figure 3. The steps from barley to beer and BSG.

1.1.3 Production and current uses of BSG

The brewing industry produces annually 1.9 billion hectolitres of beer from barley, worldwide (FAOSTAT 2013). Asia produces most of the beer (35 %), followed by the Americas (30 %) and Europe (27 %) (FAOSTAT 2013). Brewing generates also side-streams, such as BSG, spent hops and spent yeast, of which BSG is the most abundant. Currently, the side-streams are mainly utilised as cattle feed. Approximately 15–20 kg of BSG is generated per every hectolitre of beer, which converts to an annual production of ca. 33 million tons of BSG worldwide. The commercial value of BSG is low, and if a brewery produces more BSG than the local feed companies and farmers are able to buy, it may have to pay for the disposal of BSG. However, as BSG is a food grade material rich in protein and dietary fibre, it would have potential for more valuable applications as well, such as a food ingredient, if suitable processing methods are developed.

1.1.4 Properties and chemical composition of BSG

BSG is composed mainly of the husks and outer layers of the grain, which are not solubilised in mashing. These parts consist mainly of cell wall polysaccharides and lignin, which are arranged in the cell walls in a complex matrix with cross-links between the polymers. An illustration of the plant cell wall structure has been proposed by Sticklen (2008). Part of BSG protein is located inside aleurone cells. In addition to the outer layers, remnants of endosperm remain in BSG. The heterogenous nature of BSG is visible in Figure 2c. The chemical composition of BSG is presented in Table 1. As BSG has a water content of up to

80 %, it is not microbiologically stable without drying or chemical preservation (Robertson *et al.* 2010a), which hinders the storing of BSG.

Table 1. Chemical composition of BSG from barley. Nd = not determined.

Arabinoxylan	Glucan[1]	Lignin	Protein	Lipids	Ash	Ref.
28.4	16.8	27.8	15.3	5.8	4.6	Mussatto et al. 2005
23.9	14.4	18.9	26.9	6.8	3.4	Forssell et al. 2011
25.4	21.8	11.9	24.0	10.6	2.4	Kanauchi et al. 1997
26.5	19.4	20.1	17.6	5.2	Nd	Faulds et al. 2008
22–29	14–24	13–17	10–24	Nd	Nd	Robertson et al. 2010b

[1]Glucan in BSG is mainly cellulose but residual amounts of starch and β-glucan are also present.

Carbohydrates

The most abundant carbohydrates in BSG are arabinoxylan and cellulose (Table 1), which are cell wall polysaccharides. Cellulose is present as bundles or microfibrils, which are formed from linearly arranged cellulose chains and held together by inter- and intramolecular hydrogen bonds. The numerous hydrogen bonds make cellulose water-insoluble and resistant to enzymatic action. Cellulose microfibrils are surrounded by arabinoxylan, which is a branched polymer, composed of xylan backbone and substituted by arabinose and acetyl residues. Arabinoxylan is less rigid than cellulose, and its susceptibility to enzymatic digestion depends on the level of substitution. It is known for wheat bran that arabinoxylan in aleurone is less substituted than in pericarp, which makes aleurone more easily digestible for xylanases (Benamrouche *et al.* 2002). The arabinose residues may be further substituted with ferulic acid via ester-linkage (Ishii 1997). Ferulic acids may form dimers and cross-link arabinoxylan chains together (Bunzel *et al.* 2001). In the pericarp and husk, where lignin is present, ferulates can also cross-link arabinoxylan to lignin (Bunzel *et al.* 2004). These types of cross-links construct physical barriers for enzymes and thus protect the grain from the attacks of pathogens.

Starch is the main form of energy storage in barley grains. It is the most abundant polysaccharide in the grains but in mashing it is almost completely solubilised into wort. Some starch (2–13 %) and residual amounts of mixed linked β-glucan (0.5–1.1 %) remain in BSG as well (Robertson *et al.* 2010b). The starch content is usually lower in BSG generated in lager beer production (2–8 %) than in ale production (7–13 %) (Robertson *et al.* 2010b).

Lignin

Lignin is an important constituent of plant cell walls. It acts as a structural component providing rigidity to the cell walls, it is an important factor in water transportation of plants due to its hydrophobic nature, and it helps protect the plant from the attacks of micro-organisms, as lignified cell walls are resistant to enzymatic attacks (Sarkanen and Ludwig 1971). After cellulose it is the second most abundant polymer in nature. In plants lignin is present in highest amounts in wood, but it is also present in lower amounts in many foods that belong to our everyday diet, such as cereals, fruit and vegetables (Bunzel *et al.* 2005, Bunzel *et al.* 2006). The lignin content of foods is usually not very high; for instance, in whole grain wheat, kale and pear lignin contents have been reported to be 5 %, 7 % and 16 % of the dietary fibre fraction, respectively (Bunzel *et al.* 2011).

In barley, lignin is present in the husk and pericarp, as indicated in Figure 2a and b by the yellow autofluorescence. Other phenolics may also influence the autofluorescence, but being the most abundant phenolic compound, lignin contributes the most. Lignin content

is enriched in mashing and represents about 12–28 % of BSG (measured as Klason lignin) (Table 1). Lignin is formed from phenolic units or monolignols, *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Figure 4a), which in lignin form *p*-hydroxyphenyl, guaiacyl and syringyl units, respectively. During the synthesis of plant cell walls, monolignols are polymerised by radical coupling reactions (Boerjan *et al.* 2003), and therefore lignin structure is irregular and consists of different units and different types of linkages between them, as the bond formation can occur at various sites of the monolignol molecules. Depending on the plant origin, lignins vary in the ratio of the monomers. Recently, BSG lignin has also been characterised using analytical pyrolysis coupled with gas chromatography and mass detector (Py-GC/MS), NMR spectroscopy and DFRC (derivatisation followed by reductive cleavage) (Rencoret *et al.* 2015). Lignins from enzymatically treated BSG fractions have also been studied using Py-GC/MS (Ohra-aho *et al.* 2016) and the results were in agreement with the data of Rencoret *et al.* (2015). BSG lignin is predominantly composed of guaiacyl units, with ratio of syringyls to guaiacyls being 0.4–0.5, and it is associated with ferulic and *p*-coumaric acids. The major intramolecular substructures are β-*O*-4 aryl ethers (77–79 %) and β-5 phenyl coumarans (11–13 %) (Rencoret *et al.* 2015) (Figure 4b). Ferulic acids are mostly etherified to lignin whereas *p*-coumaric acids are esterified in γ position (Rencoret *et al.* 2015, Ohra-aho *et al.* 2016).
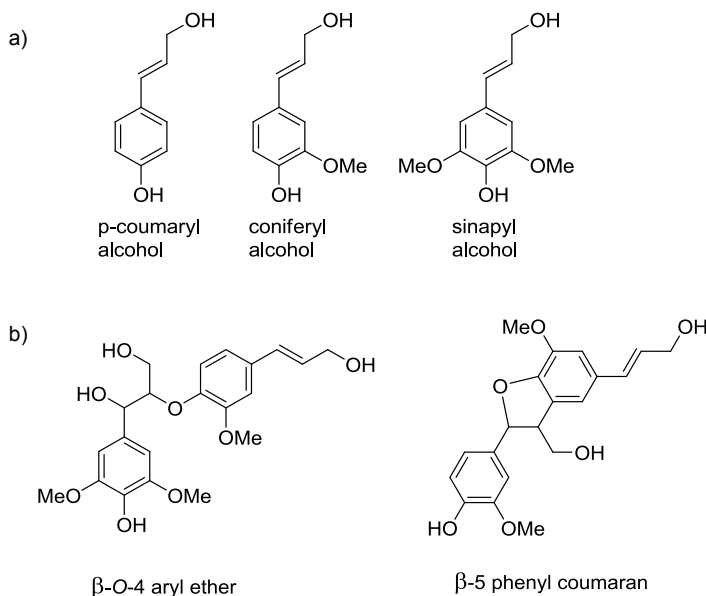
Figure 4. a) Monolignols, from which lignin is formed, and b) the most common substructures found in BSG lignin: β-*O*-4 aryl ether linkage and β-5 phenyl coumaran structure.

In the literature there are currently no published images of lignin in cereal grains. Wheat straw lignin is the most studied lignin related to cereals and a proposed structure of it is presented in Figure 5. However, based on the most recent findings by Rencoret *et al.* (2015) there are some inconsistencies between the proposed wheat straw lignin and BSG lignin. In Figure 5 α-*O*-4 ethers are abundant, whereas in BSG β-*O*-4 ethers are dominant (almost 80 %) and α-*O*-4 ethers were not detected. In addition, in BSG the 5-5 linked structure would be in the dibenzodioxocin form instead of the biphenyl form.

Lignin is usually assumed to have no nutritional value. Although it may not act as an energy source, lignin may induce other effects in the human gastrointestinal tract, such as adsorption of carcinogenic compounds (Funk *et al.* 2007), antioxidative and radical scavenging activity in the lumen (Dizhibite *et al.* 2004) and being a precursor of the mammalian lignan enterolactone (Begum *et al.* 2004). Lignin is known to be degraded by certain fungi and some insects, such as termites, but the digestion of lignin in humans and animals is not well known. There is some evidence of lignin digestion in humans (Kelsay *et al.* 1981) but more research is needed to enable better understanding of lignin metabolism.
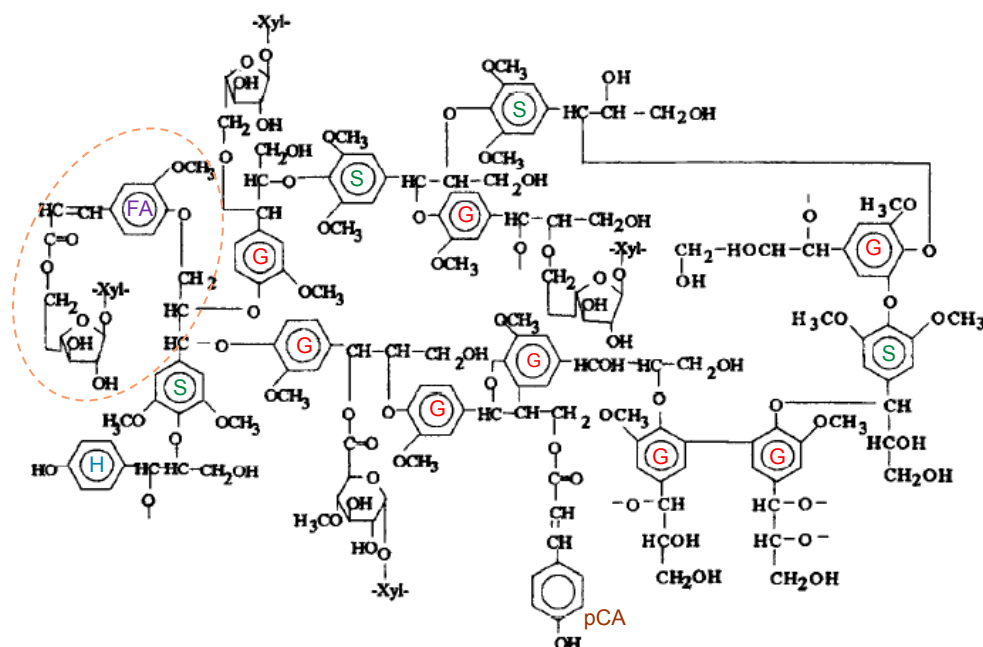
Figure 5. Proposed structure of wheat straw lignin. Modified from Sun *et al.* (1997), reproduced with permission from Elsevier B.V. The coloured markings signifying different components of lignin are added to the original figure. H=*p*-hydroxyphenyl unit, G=guaiacyl unit, S=syringyl unit, FA=ferulic acid, pCA=*p*-coumaric acid. The orange dotted circle points out a cross-link of lignin to carbohydrates via ferulic acid.

Proteins

The protein content of barley varies from 8–15 % (Shewry 1993). During malting, barley proteins are partially degraded to amino acids and small peptides by the endogenous barley peptidases (Jones and Budde 2005). However, most of malt proteins are not dissolved in mashing but 74–78 % of protein remains insoluble in the spent grains (Jones and Budde 2005, Celus *et al.* 2006). Due to the extensive endosperm solubilisation, the protein content is increased in mashing and may be up to 27 % in BSG (Table 1). In barley, there are four different types of proteins, which have been classified according to their sequential extractabilities by the procedure developed by Osborne (1909). Albumins, which are mainly enzymes, are water-soluble. Globulins may be enzymes or storage proteins and are extractable by salt-solutions. Hordeins are the main storage proteins in barley. Mashing causes disulphide bridge formation in hordeins, and therefore their extraction requires a reducing agent in addition to a high alcohol content in the extraction solvent (Celus *et al.* 2006). Glutelins, which are structural proteins, may be extracted with dilute acid or alkali or with detergents in the presence of a reducing agent (Celus *et al.*, 2006). The proteins in

BSG are mainly hordeins and glutelins, and albumins and globulins constitute only ap-proximately 10 % of BSG proteins (Celus *et al.* 2006).

The amino acid composition of BSG proteins has been determined by Treimo *et al.* (2008). BSG is rich in glutamic acid/glutamine and proline, which constitute 30 % of all amino acids. This is explained by the fact that hordeins are especially rich in these amino acids (Shewry 1993). The hordein content in barley is 35–55 % of all proteins (Shewry 1993) and is not significantly altered in mashing (43 % in BSG) (Celus *et al.* 2006).

Lipids

Most of lipids in barley are located in the endosperm and embryo, as their role is to provide nutrients and energy for the new seedling. The lipid content of unmalted barley varies from 2.0 to 4.6 % (Morrison 1993), and linoleic acid is the main fatty acid (55 %) followed by palmitic (22 %) and oleic acids (13 %) (Kaukovirta-Norja *et al.* 1993). During malting the amount of lipids is reduced by approximately 20 %, but the fatty acid composition is not significantly altered (Kaukovirta-Norja *et al.* 1993). During mashing the lipid content is increased due to the solubilisation of other compounds, and the lipid content in BSG varies from 5.2 up to 11 % (Table 1). The main lipid class in barley and malt is triglycerides (69 %) followed by polar lipids, but in mashing triglycerides are partially de-esterified by lipase activity releasing free fatty acids (Kaukovirta-Norja *et al.* 1993). A more detailed character-isation of BSG lipids and lipophilic extractives has been conducted by del Río *et al.* (2013).

1.2   Fractionation of BSG

BSG is a food-grade material rich in nutrients, such as protein, dietary fibre and antioxi-dant phenolic compounds. Therefore it would have potential for more valuable applica-tions than cattle feed, but this would require development of suitable fractionation and processing techniques. One possible option would be to integrate the fractionation directly into the brewery, and thus save time and energy on transportation. In addition, no preser-vation of BSG would be required, if it was processed immediately at the site of production.

The fractionation methods studied for BSG include mainly wet fractionation. Separation of different components from BSG is not always straightforward. For instance, the cell wall polysaccharides contain both 5 and 6 carbon sugars, and after enzymatic or acidic hydroly-sis of carbohydrates their efficient separation to different fractions may be difficult, unless they all can be utilised for the same end-use. In addition to the lignocellulosic cell walls, there is a considerable amount of protein and lipids. The more easily digestible parts of the barley grain have already been dissolved in mashing and the recalcitrant parts remain in BSG, which contains the husks and outer layers or the grain and only residual amounts of endosperm. The role of the outer layers is to protect the grain and its nutrients from other organisms, and thus they are designed to withstand the attacks of e.g. fungal enzymes. However, the different components in BSG might have the highest value as separate com-ponents, and several studies on fractionation methods have been published (Mussatto *et al.* 2005, Carvalheiro *et al.* 2004b, Mussatto *et al.* 2006a, Forssell *et al.* 2011, Treimo *et al.* 2008). Nevertheless, no large-scale applications have resulted from these studies and ru-minant feed is still the main use of BSG.

1.2.1 Chemical fractionation

Biomass components can be separated from each other with the aid of chemical, mechanical or biotechnical treatments. Chemicals may be less expensive and require shorter reaction times than enzymes, but on the other hand they are less specific and generation of unwanted side-products is possible. In addition, the suitability of chemicals for processing of food material has to be carefully considered, but for other end-uses than food and feed they may well be suitable methods. Acidic and alkaline treatments applied for BSG fractionation and the main results are summarised in Table 2, and the treatments are further discussed below.

Acid hydrolysis

For BSG, the most commonly applied chemicals are acids and bases. At elevated temperatures dilute acids depolymerise hemicelluloses without significant damage to cellulose. The main hemicellulose in BSG is arabinoxylan, and more than 90 % of arabinoxylan can be converted to monomeric sugars by a dilute sulphuric acid treatment (Carvalheiro *et al.* 2004a, Mussatto *et al.* 2005). The resulting sugar-rich hydrolysates contained some sugar degradation products (furfural, hydroxymethylfurfural, formic, acetic and levulinic acid), which were formed during the acid hydrolysis and could inhibit subsequent microbial fermentation of the sugars. Nevertheless, the amounts of the side-products were found low enough not to cause inhibition of yeasts (Carvalheiro *et al.* 2004a). After the acid treatment, cellulose is more accessible for further treatments, for example for enzymatic or acidic hydrolysis (Mussatto *et al.* 2008b).

A milder acidic treatment called autohydrolysis is based on acetic acid released from arabinoxylan. The arabinoxylan of BSG is substituted with acetyl groups, and when exposed to hot water or steam, these acetyl groups are released and form acetic acid, which partially depolymerizes xylan (Kabel *et al.* 2002, Carvalheiro *et al.* 2004b). In autohydrolysis, the sugars were mainly released as oligomers having a degree of polymerization above 9, but with prolonged reaction times the amount of smaller oligomers increases (Carvalheiro *et al.* 2004b). Due to the mild conditions, the generation of sugar degradation products is lower than with dilute acid treatments but on the other hand sugar yields are also lower (Carvalheiro *et al.* 2004b).

Alkaline extraction

Alkaline treatments, which are known to dissolve hemicelluloses and lignin, have been studied for the production of cellulose pulp from BSG (Mussatto *et al.* 2006a). For soda pulping process, BSG was first pre-treated with dilute sulphuric acid to remove most of arabinoxylan. The removal of hemicelluloses prior to pulping makes the material more accessible for pulping chemicals and thus reduces the amount of chemicals needed (Mussatto *et al.* 2006a). Under optimized conditions 90 % of lignin can be removed with negligible cellulose losses. The pulping process can be applied on untreated BSG as well, but the quality of the resulting pulp is not as good as after the acid pre-treatment (Mussatto *et al.* 2008a). Pulps can be further bleached with hydrogen peroxide to remove the residual lignin (Mussatto *et al.* 2008a). In addition to lignin, smaller phenolic compounds, namely ferulic and *p*-coumaric acids are also extractable in alkaline conditions (Mussatto *et al.* 2007a). Delignification enhances enzymatic hydrolysis of BSG cellulose due to modifications in BSG structure, which makes the cellulose fibres more susceptible to enzymatic attack (Mussatto *et al.* 2008b).

Arabinoxylan from BSG is also extractable with alkali (Mandalari *et al.* 2005). A sequential extraction with increasing alkali strength resulted in the solubilisation of 90 % of arabinoxylan and over 60 % of BSG. Mild alkali dissolved arabinoxylan with high molar mass, whereas strong alkali caused cleavage of the polysaccharides resulting in lower molar mass.

In addition to lignin and hemicellulose, proteins and lipids are also affected by alkalinity. BSG proteins are mainly water-insoluble hordeins and glutelins (Celus *et al.* 2006), and 40 % of BSG proteins are extractable in alkaline conditions (Celus *et al.* 2007). The ester-linkages of triglycerides may be broken by alkali resulting in saponified free fatty acids. When extracting with alkali to dissolve proteins Celus *et al.* (2007) reported that a notable amount of BSG lipids were released in the same conditions. However, studies on BSG lipids and their extractability are limited.

Table 2. Summary of chemical treatments of BSG.

	Chemical used and reaction conditions	Main results	Reference
Dilute acid hydrolysis	100 mg of H_2SO_4/g of DM, 120 °C, 17 min	93 % of hemicellulose was extracted.	Mussatto et al. 2005
Autohydrolysis	Hot water 150 °C, 120 min	49 % of xylose and 47 % of arabinose were solubilised.	Kabel et al. 2002
	Hot water 190 °C, 5 min	61 % of arabinoxylan was degraded to oligosaccharides, 70 % of which had DP ≥ 7.	Carvalheiro et al. 2004
Pulping	Pre-treatment with 100 mg of H_2SO_4 /g of DM, pulping with 2 % NaOH	Resulting pulp contained 72 % of cellulose and 10 % of residual lignin. The cellulose content was increased to 90 % with hydrogen peroxide bleaching.	Mussatto et al. 2006a, Mussatto et al. 2008a
Alkaline hydrolysis	2 % NaOH, 120 °C, 90 min	90 % of lignin was solubilised, and also ferulic and *p*-coumaric acid dissolved well in the applied conditions.	Mussatto et al. 2007a
Sequential alkaline extraction	Different concentrations of Na_2CO_3+$NaBH_4$ and KOH, 2 h, 4 °C or RT	Over 60 % of BSG dissolved. Ferulic acid and dimers present in the solubilised fractions. Strong alkali resulted in cleavage of arabinoxylan.	Mandalari et al. 2005
Alkaline protein extraction	Extraction with 0.1 M NaOH, 60 °C, 60 min	17 % of BSG dissolved and the solubilised fraction contained 41 % of proteins present in the starting material, and also a notable amount of fat.	Celus et al. 2007
Extraction of antioxidant compounds	Various solvents, such as: methanol, ethanol, acetone, hexane, ethyl acetate, acetone-water	Acetone:water 60:40 (v/v) mixture was efficient in releasing compounds with good antioxidant properties	Meneses et al. 2013
	Supercritical CO_2 extraction	Extract yield was 5.5 g/100 g. Antioxidativity of the extracted residue was higher than that of the extract.	Kitryte et al. 2015

Extraction of antioxidant compounds
Organic solvents have been studied to extract antioxidant compounds such as phenols and flavonoids from BSG. Acetone, ethanol and methanol and their mixtures with water were

found to produce extracts with higher phenolic concentrations and antioxidant potential than less polar solvents such as hexane and ethyl acetate (Meneses *et al.* 2013). Extraction of lipophilic antioxidants with supercritical CO_2 was found to be ineffective (Kitryte *et al.* 2015). The antioxidant capacity of the extract was significantly lower than that of the original BSG suggesting that the compounds with highest antioxidant capacity were not extractable with CO_2 but remained in the residue. On the other hand, unextracted BSG seemed to be a potential antioxidant material as such, without any treatments.

1.2.2   Enzymatic fractionation

Enzymatic methods, which can be carried out in mild conditions and are often suitable for food processing, have been studied for BSG protein and carbohydrate solubilisation (Table 3). The benefit of enzymes is their specificity and that they can function in moderate temperature and pH. The downsides of enzymatic treatments are longer reaction times and a need for a high amount of water in the process, which further requires large reactors and concentration of the product solutions. The cost of enzymes may be a limiting factor for bulk products such as bioethanol (Kumar and Murthy 2011), but for more valuable applications the enzyme cost can be compensated in the price of the final product. Efficient recycling of enzymes, if possible, would significantly reduce the costs.

It has been shown that BSG proteins can be solubilized to a large extent with proteases without any pre-treatment (Treimo *et al.* 2009). Alcalase 2.4, which is an alkaline subtilisin protease from *Bacillus licheniformis*, has been found the most effective for BSG protein solubilisation (Treimo *et al.* 2008). However, the optimal pH for Alcalase 2.4 is 9–10 (Faulds *et al.* 2008), which likely affects the solubility of other non-proteinaceous components as well, resulting in a mixture of solubilised compounds instead of a pure peptide solution. The use of proteases significantly decreases the molecular size of proteins to peptides (Treimo *et al.* 2008) and in certain applications, such as food ingredients, this may create problems such as bitter taste. Therefore non-enzymatic fractionation methods might suit some applications better than hydrolytic approaches.

BSG carbohydrates are not easily hydrolysed with commercial enzyme preparations. Several commercial carbohydrase preparations including Depol740, Depol686, Econase and Celluclast, have been studied for hydrolysis of BSG carbohydrates (Treimo *et al.* 2009, Forssell *et al.* 2008, Mussatto *et al.* 2008b). Nevertheless, without any pre-treatments, only about 30% of the carbohydrates can be enzymatically removed (Treimo *et al.* 2009). This is not, however, surprising considering that most of BSG carbohydrates are part of the outer grain layers, whose function is to protect the grain.

It has been shown on wheat bran that xylan in the outer bran is so highly substituted with arabinose residues (xylose to arabinose ratio 0.98) that it is resistant to xylanase action, although the same enzyme was able to release 80 % of carbohydrates in the aleurone and 50 % in the inner bran (Benamrouche *et al.* 2002). The improved solubility of arabinoxylan from the aleurone and inner bran was strongly related to a lower degree of substitution in those tissues, but according to the authors other factors such as the presence of diferulate cross-links and cutin in the bran, are likely to also contribute to the enzyme resistance. Indeed, the cross-linking of cell wall polymers with ferulates has been shown to hinder enzymatic and microbial cell wall degradation (Grabber *et al.* 1998a, Grabber *et al.* 1998b, Grabber *et al.* 2009). Ferulic and *p*-coumaric acids can be released from the cell walls using esterases (Faulds *et al.* 2002, Bartolomé and Gómez-Cordovés 1999), which could im-

prove cell wall digestibility. In addition, another study on wheat bran showed that the pore sizes in the bran are too small for a xylanase to diffuse in without first disassembling the cell wall (Beaugrand *et al.* 2005). As barley is assumed to have a similar bran structure than wheat, these observations support the previous findings by several authors that BSG is a recalcitrant material and its carbohydrates are not easily solubilised with enzymes.

Lignin is another important factor limiting enzymatic cell wall degradation. It constitutes a physical barrier for enzymes preventing them from accessing their substrate. Lignin also adsorbs enzymes by hydrophobic interactions (Ooshima *et al.* 1990, Palonen *et al.* 2004). It has been demonstrated that conversion of cellulose from delignified BSG was four times higher than from untreated BSG (Mussatto *et al.* 2008b). The same study showed that hemicelluloses as well hinder cellulose hydrolysis, but to a lower degree compared to lignin. It is generally acknowledged that a pre-treatment of some kind is required for lignocellulosic biomasses to obtain the highest carbohydrate solubilities (Agbor *et al.* 2011).

Table 3. Summary of enzymatic treatments of BSG.

	Pre-treatment	Enzymes used	Main results	Reference
Total solubilisation of BSG	None	Hydrolysis with carbohydrases and proteases over a wide pH range	Alkaline pH improved total solubility. Solubility was highest (36 %) at pH 9 by Depol740.	Faulds et al. 2008
Carbohydrate hydrolysis	Coarse milling	Various carbohydrase preparations	Maximal solubility of carbohydrates was 26-28 % corresponding to 13-14 % of total BSG.	Forssell et al. 2008
	Untreated Dilute acid Dilute acid+alkali	Celluclast1.5, 96 h 45 FPU/g	Cellulose conversion was improved from 22 % to 78 % after acid treatment and to 92 % and after acid+alkali treatment.	Mussatto et al. 2008b
Protein hydrolysis	Coarse milling	Various protease preparations	Alcalase was the most effective solubilising 30 % of BSG and 77 % of protein at pH 6.8	Treimo et al. 2008
Sequential treatments with carbohydrases and proteases	Coarse milling	Various carbohydrase and protease preparations	Sequential treatments with Depol740 and Alcalase2.4 resulted in solubilisation of 83 % of protein, 39 % of carbohydrates and 42 % of total BSG.	Treimo et al. 2009
	None	Econase and Alcalase2.4	First step with carbohydrase solubilised 14 % and second step with protease solubilised 36 % of total BSG.	Forssell et al. 2011
Solubilisation of phenolic acids	Extraction with hot ethanol	Ultraflo from *Humicola insolens*	80 % of ferulic 9 % of *p*-coumaric acid released, dimeric phenolic acids remained mostly bound to carbohydrates.	Faulds et al. 2004

1.2.3   Pre-treatment methods to improve enzymatic fractionation

One way to improve enzymatic digestibility of BSG carbohydrates would be to use a pre-treatment step. Pre-treatments that decrease particle size, open up the cell wall structures and reduce cellulose crystallinity improve enzymatic digestibility by making the material more accessible for enzymes (Hendriks and Zeeman 2009). Pre-treatments can be physical, such as milling and grinding, chemical such as acid, alkali or organic solvent pre-treatment or physico-chemical, such as steam explosion and liquid hot water pre-treatment, as reviewed by Agbor *et al.* (2011). Biological pre-treatments with fungi are also possible, but the required treatment times are usually too long for industrial purposes (Agbor *et al.* 2011).

The reported pre-treatment methods for enzymatic BSG fractionation include coarse milling using a 0.5 mm (Forssell *et al.* 2008, Beldman *et al.* 1987) or 1 mm (Treimo *et al.* 2009) sieve. However, these techniques are not sufficient to affect micrometre scale cell wall structures or crystallinity (Beldman *et al.* 1987). Other types of pre-treatments that have been studied are extrusion, homogenisation with an Ultra Turrax (Beldman *et al.* 1987, Macheiner *et al.* 2003), autoclaving, microwave radiation (Macheiner *et al.* 2003) and thermo-mechanical pre-treatment using high pressure and temperature (Pierre *et al.* 2011). Nevertheless, only minor improvements for the subsequent enzymatic hydrolysis were detected except for the thermo-mechanical pre-treatment, which significantly enhanced the hydrolysis of BSG cellulose. In addition, a hot water pre-treatment called autohydrolysis has been applied for the removal of hemicelluloses from BSG (Carvalheiro *et al.* 2004b). However, the conditions of autohydrolysis cause conversion of some of pentoses to furfural (Carvalheiro *et al.*, 2004b), which can be undesirable in certain processes.

1.3   Applications of BSG and its fractions

1.3.1   Current use of BSG

Currently, BSG is mainly utilized as cattle feed. Since it contains a high amount of cellulose, it is best digested by ruminants, but can also be fed to other animals as part of their diet, as reviewed by Westendorf and Wohlt (2002). BSG cannot be fed to animals as such due to its low energy content, but should be added only as a supplement. For instance, at a level of 15 % of the diet, BSG increased the production of colonic short chain fatty acids and had beneficial effects on the intestinal mucosa in piglets (Martins *et al.* 2010). Basically, there is no reason, why BSG could not be used as feed, but currently it is not bringing much money to the breweries. In addition, if there are not enough cattle in the proximity to the brewery, in the worst case the produced BSG could end up as landfill. BSG is also suitable for human consumption, which is a more valuable application than feed. In addition, BSG has been suggested as raw material for chemicals, materials and energy. With the aid of different refining techniques, such as mechanical, enzymatic of chemical treatments, new applications for BSG could be developed making it a more profitable side-stream for breweries.

A challenge with storing of BSG is that due to its high water content (70−80 %) it will start to deteriorate within a week at +4 °C or within two days at room temperature (Robertson *et al.* 2010a). Therefore it should be consumed almost immediately after production, if no preservatives are added. Traditional hot-air drying of BSG is costly because of the high amount of energy needed (Tang *et al.* 2005), and is nowadays rarely used

(Westendorf and Wohlt 2002). Drying of BSG with superheated steam is significantly more energy-efficient, and has also other advantages, such as a reduced risk of fire and explosion, sterilization, deodorization and faster drying rates (Stroem *et al.* 2009). The nutritional value of BSG is mostly not affected by drying, but the residual starch may be altered by the drying process due to formation of amylose–lipid complexes or resistant starch (Tang *et al.* 2005).

1.3.2   Potential applications and functionalities as food ingredient

As BSG is rich in proteins and dietary fibre, several studies on its utilisation in foods have been reported. For instance, dried and milled BSG has been added as dietary fibre supplement to frankfurtes (Özvural *et al.* 2009), and baked (Ktenioudaki *et al.* 2013) or extruded snacks (Stojceska *et al.* 2008) without affecting the sensory parameters or physico-chemical properties too much. The nutritional value of both products was improved by the high protein and dietary fibre content of the added BSG. In addition to scientific research some practical applications of BSG food uses have been described including a protein-rich food material (Kishi *et al.* 1991, Gannon 1993), dietary fibre additives (Erasmus 2009, Chambers 1994) and separation of bran from BSG for an additive in breadmaking (Dreese and Hoseney 1983). Xylitol, which is a commonly used sweetener, can also be produced from BSG xylan (Mussatto *et al.* 2007).

Peptides prepared with controlled enzymatic proteolysis have shown potential as emulsion-forming, foam-forming and foam-stabilizing agents (Celus *et al.* 2007), which could be used in foods or other products such as cosmetics. The hydroxycinnamic acids in BSG, namely ferulic and *p*-coumaric acid possess antioxidant properties (Meneses *et al.* 2013), and these compounds could be used in variable applications, such as in drinks or cosmetics to improve their antioxidant capacity (Gupta *et al.* 2013, Mathew and Abraham 2004).

Food additives derived from BSG have been demonstrated to possess health-promoting functionalities. For instance, a fraction with increased protein content (46 %) obtained from BSG by milling and sieving was shown to relieve constipation and colonic inflammation in rats (Kanauchi and Agata 1997, Kanauchi *et al.* 2003). Peptides from BSG have also been claimed to possess bioactivities, such as lowering glycemic response (Li *et al.* 2012). *In vitro* studies on xylo-oligosaccharides isolated from BSG showed indications of prebiotic properties by enhancing the growth of lactobacilli and bifidobacteria *in vitro* (Moura *et al.* 2008). In addition, enzymatically extracted insoluble dietary fibre from BSG promoted binding of bile salts *in vitro*, which may have an effect on lowering cholesterol levels in blood (Fu *et al.* 2010).

1.3.3   Potential non-food applications

Utilisation of BSG in several non-food applications has also been studied. These applications include adding BSG to bricks to increase porosity and strength and to lower the density (Russ *et al.* 2005), using BSG as a biofilter medium for groundwater denitrification (Benyoucef *et al.* 2013), producing lactic acid from BSG carbohydrates (Mussatto *et al.* 2005), using BSG as a growth substrate in the production of mushrooms (Wang *et al.* 2001) and making activated carbon for adsorbent materials from BSG lignin (Mussatto *et al.* 2010). Fermentation of BSG carbohydrates to bioethanol has also been described (Xiros and Christakopoulos 2009, Birkmire *et al.* 2012). More extensive reviews on the possible

applications for BSG have been written by Xiros and Christakopoulos (2012) and Mussatto *et al.* (2006b).

One alternative for BSG use is to generate energy and heat from it, and examples of combustion and gasification processes for BSG have been described (Larson *et al.* 2013, Kepplinger *et al.* 2001). Furthermore, two CHP (combined heat and power) plants burning a mixture of BSG and forest residues have been built for Scottish and Newcastle breweries in Manchester and Tadcaster (UK) by MW Power (Power-Technology 2015, Greenpeace 2007). Due to the high amount of water, a drying step using a belt press is first needed to reduce the water content of BSG from 80 to 60 %. The power plants provide heat and energy for the breweries, and in addition, the excess energy is sold to the local electricity network.

1.4 Dietary fibre

Dietary fibre (DF) consists of the indigestible parts of plant-based foods and is important to the health and wellfare of humans. According to the European Food Safety Authority (EFSA) DF includes all non-digestible carbohydrates (EFSA 2010). This contains non-starch polysaccharides, resistant starch, resistant oligosaccharides with three or more monomeric units and other non-digestible, but quantitatively minor components that are associated with the DF polysaccharides, especially lignin. Lignin is considered as part of DF when associated with carbohydrate polymers of plant origin but not as an isolated compound added to food (Commission Directive 2008). Consumption of foods rich in DF is essential for normal gastrointestinal function and health (Schneeman 1998). Whole grain cereal foods are an important source of DF but also of energy and nutrients, such as protein, vitamins and minerals (Slavin 2003, Slavin 2004). The daily dosage for DF intake recommended by the National Nutrition Council of Finland is 25–35 g (VRN 2014).

1.4.1 Physiological functionalities of dietary fibre

DF has several physiological functionalities in the digestive tract, and their roles in protection of health are under extensive investigation. DF can be classified as soluble or insoluble in water, for example β-glucan and pectins form viscous gels in water whereas cellulose remains insoluble. The hydration properties of DF such as water-holding capacity and swelling properties may have many functionalities including increasing and prolonging satiety, lowering post-prandial glycemic response and preventing absorption of potentially harmful compounds, for example cholesterol, from ingested food (Schneeman 1998, Raninen *et al.* 2011). As DF is non-digestible, it passes through the small intestine but is partially fermented by intestinal microbiota in the large intestine, increasing faecal mass. Water holding capacity and increased faecal mass contribute to bulking effect, which induces bowel movement and thus shortens the transit time (Raninen *et al.* 2011).

Sufficient intake of DF has been shown to protect from several chronic diseases, such as obesity, cardiovascular diseases and type 2 diabetes (Smith and Tucker 2011, WHO 2003). DF is also of vital importance for the well-being of the gut and gut microbiota. In the fermentation of DF polysaccharides by gut microbiota the main metabolites are short chain fatty acids (SCFA) such as acetic, propionic and butyric acid, but also hydrogen and carbon dioxide are formed (Cummings and MacFarlene 1997). The formation of SCFA in the gut is important, as they are likely to provide several positive effects. For instance, butyric acid is

the major energy source for colon epithelial cells, and the formation of butyric acid in the colon enables the proliferation of these cells, which can help protect from colon cancer (Comalada *et al.* 2006). There are also signs that SCFA can be used to treat diseases, such as ulcerative colitis (Vernia *et al.* 1995). In addition, SCFA provide a source of energy for the host. The amount of energy obtained from SCFA may account for up to 10 % of a person's total energy need (McNeil 1984).

1.4.2 Lignin as part of dietary fibre

Currently, the effects of lignin as part of DF or interactions between lignin and human colon microbiota are not well characterised. Lignin is generally assumed to be an inert part of DF and resistant to microbial degradation. However, there is also some contradictory evidence demonstrating partial lignin digestion in human and animal *in vivo* studies (Kelsay *et al.* 1981, Williams *et al.* 1936, Silanikove and Brosh 1989). Polymeric lignin is not absorbed but remains in the gut lumen, and could thus interact with other components of food. For example, lignin-enriched DF can adsorb carcinogenic compounds in the conditions of upper intestine and colon (Funk *et al.* 2006, Funk *et al.* 2007). The adsorption of carcinogens by lignin may prevent their absorption from the gut into circulation and thus reduce the risk of cancer. Phenolic compounds within the insoluble DF are able to quench soluble radicals formed in the gastrointestinal tract (Vitaglione *et al.* 2008), and due to its polyphenolic structure also lignin possesses such antioxidative and radical scavenging activity (Dizhbite *et al.* 2004, Lu *et al.* 1998) in the lumen.

Alternatively, lignin could have effects on gut microbiota or its conversion activities, as has been demonstrated for isolated, condensed apple and grape tannins (Bazzocco *et al.* 2008, Aura *et al.* 2013). Tannins are also polymeric polyphenolic compounds and in isolated form they have been found to suppress carbohydrate fermentation to SCFA (Bazzocco *et al.* 2008, Aura *et al.* 2013). Tannins have been shown to bind proteins and thus inhibit enzymes (Scalbert 1991), which may explain the suppression of SCFA formation.

Although research on lignin degradation and metabolism in humans or other animals is limited, some studies can be found in the literature. As a component of DF lignin has been demonstrated to inhibit microbial carbohydrate fermentation in a ruminal model (Grabber *et al.* 2009). This would indicate that lignin may suppress microbial conversions instead of being degraded along with other cell wall components. The results of human studies have provided variable results. According to Holloway *et al.* (1978) lignin is not degraded in the human digestive tract. In fact, in this study the amount of lignin in faeces was more than in the ingested food indicating difficulties in lignin analytics. Opposite results were obtained by Williams and Olmsted (1936) and Kelsay *et al.* (1981), who detected lignin degradation in humans. However, none of the aforementioned studies analysed degradation products from lignin but the measurements were based only on lignin quantitated as an acid-insoluble residue.

Animal studies with both ruminants and monogastrics have demonstrated lignin degradation. In goats, lignin isolated from wheat straw was metabolised based on gravimetric analysis and a notable increase in hippuric and benzoic acid concentrations in urine (Silanikove and Brosh 1989). Due to the insufficient amount of other possible phenolic precursors in the lignin fractions Silanikove and Brosh (1989) concluded that the origin of the detected aromatic acids was lignin. Similarly, Csonka *et al.* (1929) observed an increase in the urinary hippuric acid concentration in cows and dogs. In addition, they measured the

content of methoxyl groups in lignin before and after digestion. Based on the loss of lignin methoxyls occurring in the digestive tract and the increased production of hippuric acid, they as well described that lignin was metabolised. A more recent study showed lignin degradation in rats (Begum *et al.* 2004). Rats were fed isotope-labelled synthetic lignin and formation of labelled degradation products was monitored. Dimeric units i.e. dilignols were cleaved from lignin and converted to enterolactone by the rats' intestinal microbiota. In several studies, higher concentrations of enterolignans have been associated with a lower risk of cancers (Vanharanta *et al.* 1999, Ingram *et al.* 1997, Adlercreutz 2002) indicating that lignin-related metabolites could have beneficial effects.

Preliminary results of lignin degradation and metabolism *in vivo* have been demonstrated. However, the analytical methods vary in each study and especially the gravimetric quantitation of lignin from faecal material may not be totally accurate. More research is required to better understand the interactions of lignin and gut microbiota and to identify the metabolites originating from lignin.

2. Aims and hypotheses of the study

The objectives of this doctoral study were to produce lignin-rich fractions from brewer's spent grain and to investigate their interactions with colon microbiota *in vitro*. The specific aims were as follows:

- ➢ To study enzyme-aided fractionation of brewer's spent grain as a means to separate lignin-rich fractions
    - To evaluate the efficiency of milling pre-treatments in enhancing enzymatic hydrolysis of cell wall polysaccharides
    - To produce different types of lignin-rich fractions for *in vitro* studies with colon microbiota

- ➢ To study interactions of lignin with colon microbiota *in vitro*
    - To assess, if lignin is degraded and metabolised in a metabolic colon model
    - To study, whether lignin suppresses colon microbial conversions

The hypotheses of the study were as follows:

- ➢ Brewer's spent grain can be fractionated using enzymatic methods to produce lignin-enriched fractions
- ➢ Lignin is at least partially metabolised by human intestinal microbiota

3. Materials and methods

3.1 Materials

BSG was kindly donated by the Sinebrychoff brewery (Kerava, Finland). The BSG used in the present study came from an all-malt lager mashing, where no adjuncts (additional enzymes or other starch sources) had been added. BSG was taken directly from the process after filtering the wort away with a Meura filter, and it was stored frozen at -20 °C until used. This BSG did not contain any spent yeast or precipitated protein from wort boiling, which are often mixed into BSG that goes to feed. BSG from two different mashings were used.

The carbohydrase enzymes used were Depol740L from *Humicola insolens* (Biocatalysts Ltd., Cefn Coed, Wales, U.K.), Celluclast1.5L from *Trichoderma reesei* (Novozymes, Bagsvaerd, Denmark), Novozym188 from *Aspergillus niger* (Novozymes, Bagsvaerd, Denmark). The activities of the enzymatic preparations are presented in Paper I. Proteases used were Alcalase 2.4L from *Bacillus licheniformis*, (Novozymes, Bagsvaerd, Denmark), Promod 144GL from papaya fruit (*Carica papaya)* (Biocatalysts Limited, Cefn Coed, Wales, U.K.), and Acid Protease A from *Aspergillus niger* (Amano Enzyme USA, Elgin, IL, USA).

3.2 Analytical methods

3.2.1 Composition analysis

For composition analyses, BSG was hot-air-dried overnight at 60 °C. After drying, BSG was milled with a 0.3 mm sieve (Hosokawa Alpine AG, Augsburg, Germany). The principles of the composition analysis methods are briefly described below and more detailed information and parameters are given in Papers I–III. The analysis methods for BSG fractions were the same as for BSG. Two replicate analyses were carried out for each component and the average of the two was calculated.

The content of lipophilic extractives was measured gravimetrically after extracting the dried and milled BSG in a Soxhlet apparatus with heptane for 5 h. The carbohydrate content of BSG was measured from the heptane-extracted BSG by high performance liquid chromatography (HPLC) after acid hydrolysis. The material was first incubated in 70 % sulphuric acid at 30 °C for 1 h, after which the acid content was diluted to 4 % with water, and the sample was autoclaved at 121 °C for 50 min. Remaining solids were separated from the liquid by filtration. Monosaccharides were analysed from the filtrate using Dionex CarboPac PA-1 column in a Dionex ICS-3000 system (Dionex Corp., Sunnyvale, CA) with electrochemical detection, and Klason lignin was measured gravimetrically from the dried acid-insoluble residue. Acid-soluble lignin was measured from the filtrate based on UV ab-

sorbance at 203 nm and calculated using absorptivity of 128 l/g (Paper I), or at 215 and 280 nm and calculated according to Goldschmid (1971) (Papers II and III).

Nitrogen was measured from dried and milled BSG with the Kjeldahl method (Paper I and II) or by total nitrogen analysis at Analytische Laboratorien Prof. Dr. H. Malissa and G. Reuter GmbH (Lindlar, Germany) with a standard method ASTM D-5291 (Paper III). In the Kjeldahl method, proteins were degraded with 98 % sulphuric acid and 30 % hydrogen peroxide in the presence of a catalyst. The mixture was burned at 420 °C for 35 min and the formed ammonium sulphate was converted to ammonium hydroxide with NaOH in a Kjeltec 2300 system (Foss Tecator, Höganäs, Sweden). The ammonium hydroxide was distilled and reacted with boric acid to form ammonia, which was titrated using 0.1 M HCl. The nitrogen content was calculated from the amount of HCl consumed. With both analysis methods, the nitrogen content was converted to protein by multiplying with a factor of 6.25.

The content of inorganic material was measured gravimetrically after burning of all organic material of the sample in a muffle furnace at 550 °C overnight.

Starch and β-glucan contents were determined with Megazyme kits Total starch (amyloglucosidase/α-amylase method) and Mixed-linked β-glucan according to the manufacturer's (Megazyme, Bray, Ireland) instructions.

Phenolic acids were extracted from BSG with 2 M NaOH. The samples were then acidified with HCl and extracted with ethyl acetate. The organic phase was collected, and the solvent was evaporated. The dried residue was dissolved in a 50:50 mixture of methanol and water. Phenolic acids were analysed with HPLC and UV detection at 324 nm. Quantitation was based on external standard (*p*-coumaric acid).

3.2.2 Particle size distribution analysis

Particle size distributions of the milled BSG materials were measured using Coulter LS230 (Beckman Coulter, Miami, FL, USA), which was able to measure both dry and wet samples. The average of two measurements was calculated.

3.2.3 Microscopy imaging

Epifluorescence microscopy was carried out as described previously (Van Craeyveld *et al.* 2009). In brief, samples were embedded in hydroxyethyl methylacrylate matrix from which 2 µm thick sections were cut. Prior to imaging with a microscope, the sections were treated with chemical dyes Calcofluor and Acid Fuchsin to enable visualisation of different components of BSG. Acid Fuchsin stains protein red, and Calcofluor stains β-glucan blue. The autofluorescence of lignin and other phenolics are seen as yellow and green. The excitation and emission wavelengths used were 400–410 nm and >455 nm, respectively. It should be noted that although specific dyes were used the method is not accurately quantitative, but provides information on the structural characteristics and locations of different components in the studied material. Samples were also imaged with UV light without staining. For this purpose the excitation and emission wavelengths used were 330–385 nm and >420 nm, respectively.

Stereomicroscopy was used to image dried but otherwise untreated BSG. The colour and surface features of the samples were examined with Zeiss SteREO Discovery.V8 stereomicroscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) and imaged using an

Olympus DP-25 single chip colour CCD camera (Olympus Life Science Europa GmbH, Hamburg, Germany) and the Cell^P imaging software (Olympus).

3.2.4 Analyses of colon model metabolites

Short chain fatty acids (quantitative analysis)
For the short chain fatty acid analysis (Papers IV and V) fermented samples were extracted with diethylether as described previously (Schooley *et al.* 1985). The diethylether extracts were analysed with gas chromatography with flame ionisation detector (GC/FID) (Agilent 6890 Series, Palo Alto, CA). Helium was used as the carrier gas. Both the injector and FID were kept at 250 °C. The temperature program started at 50 °C with 3 min holding time, then increased 25 °C/min up to 100 °C, finally increasing 10 °C/min to the final temperature 240 °C where kept at for 10 min. Compounds were quantitated with corresponding standards.

Metabolomics (quantitative and non-targeted analysis)
Phenolic metabolites were extracted from the colon model samples with ethyl acetate. 1 mL of 2 % NaCl solution was first added to the thawed fermentation samples to break the emulsion formed, especially in the P-AEF fermentation samples due to the high lipid content. 50 µL of 6 M HCl was then added to lower the pH near to 1. *Trans*-2-hydroxycinnamic acid (Aldrich St. Louis, USA) was used as the internal standard and 15 µL of it (123 mg/L in MeOH) was added to the fermented samples. The samples were extracted twice with 3 mL of ethylacetate. The organic phases were collected and combined, and evaporated under nitrogen. The dried samples were stored under a nitrogen atmosphere at -20 °C until analysed.

The analysis was performed using a two-dimensional gas chromatography coupled with time-of-flight mass detector (GCxGC-TOFMS). Sample derivatisation was done automatically by Gerstel MPS autosampler and Maestro software. External standards (listed in Papers IV and V) were used to quantitate certain phenolic metabolites. N-Methyl-N-trimethylsilyl-trifluoracetamide (Sigma, St. Louis, MO) and methoxyamine (Thermo Scientific, Bellefonte, PA) were used as the derivatisation reagents.

The data processing of GCxGC-TOFMS responses has been described by Aura *et al.* (2013). Briefly, the peaks were identified by ChromaTOF software, which matches deconvoluted spectra against NIST05 mass spectral library. The compounds in different data sets were aligned and normalised using an in-house developed software Guineu (Castillo *et al.* 2011) for further analyses. Alignment of the data was performed on the basis of retention indices, second dimension retention times and spectra. Metabolites were filtered according to the difference in responses between the fraction and the faecal control, i.e. the fold change (FC). FC was calculated as a ratio of the response of lignin sample to the faecal control at the maximally responding time point. Metabolites with FC value >2 and relevant structure were selected for further identification.

The identity of each selected, relevant metabolite (with FC>2) was checked by comparing the recorded mass spectra with those found in the GOLM Metabolome Database (GMD) (GMD 2012), NIST05 library, in-house database, and relevant literature (e.g. Niemelä and Sjöström 1986, Niemelä 1990). This way, a number of lignin-related metabolites and other aromatic compounds could be either fully identified or partially characterised. Several dilignol-type compounds were, however, only partially characterised due to the lack of ref-

erence spectra. Also, in many cases their molecular weights could not be reliably confirmed.

The visualisation was performed by calculating 2-based logarithmic fold changes of the relative peak areas from GCxGC-TOFMS analysis against the faecal control. The profile of an individual metabolite was visualised as colour intensities (red as over-expression and blue as under-expression) and the time point specific significances (t-test *p*-values) as asterisks against the corresponding control. The non-targeted metabolite profiling was semi-quantitative. Clustering of the metabolites was performed according to the similarity of the time profiles.

3.3   Experimental

3.3.1   Milling experiments

Both dry and wet milling techniques were studied (Paper I). The mills used in the dry millings were a pin disc mill, a TurboRotor and a ball mill. For the wet millings a Masuko grinder and a microfluidizer were used. In pin disc milling the dried material was milled at a rotor speed of 17 800 rpm using a Hosokawa Alpine 100 UPZ-lb Fine impact mill with stainless steel pin disc grinders (Hosokawa Alpine AG, Augsburg, Germany). TurboRotor treated material was provided by Mahltechnik Görgens GmbH (Dormagen, Germany) and the milling was carried out using TurboRotor type G-55 with a rotor speed of 113 m/s. The air flow was set to 1200 m^3/h and the gap between the rotor and the inner liner was 3 mm. For ball milling, the dried BSG was first coarse milled with the pin disc mill as above. 33 g of sample was milled with 500 g of stainless steel balls and rotation speed of 300 rpm in a Fritsch Pulverisette 5 planetary ball mill (Fritsch GmbH, Idar-Oberstein, Germany) under argon atmosphere.

For the wet milling experiments, the mills used were Masuko Supermasscolloider MKZA10-15J, (Masuko Sangyo Co. Ltd., Kawaguchi, Japan) and Microfluidizer Processor M-110Y (Microfluidics, Newton, MA, USA). BSG was suspended in tap water with 0.02 % sodium azide to ensure microbiological stability. In Masuko milling a 6 % BSG suspension was passed through MKGA10-80 grinding stones 7 times with a grinding speed of 1500 rpm. For microfluidizer experiments, the BSG suspension was first pre-treated with the Masuko grinder. The pre-ground suspension was passed through the microfluidizer 6 times using 1000 bar pressure and chamber sizes 100 and 200 μm.

3.3.2   Effect of milling on enzymatic carbohydrate hydrolysis

Enzymatic hydrolyses of the milled BSG samples were performed in test tubes using 3 % solids content (w/w) at 50 °C and pH 5.0 for 5 h (Paper I). Three replicate hydrolyses were performed for each sample. Depol740 (dosage of 5000 nkat of xylanase activity per 1 g of substrate) was used to study the effects of milling on enzyme action. For cellulase addition experiments, the dosages were 50 FPU/g of substrate for Celluclast1.5 and 500 nkat of β-glucosidase activity per 1 g of substrate for Novozym188. Reactions were stopped by boiling the samples for 10 min. After centrifugation the amount of solubilised sugars in the supernatant was determined with HPLC (see 3.2.1 for details). Prior to the analysis, all soluble carbohydrates in the supernatant were hydrolysed to monomers with acid hydrolysis

by adding 200 μL of 70 % sulphuric acid into 4 mL of diluted (sample/water 1:3) sample solution and autoclaving the samples for 50 min at 121 °C.

3.3.3 Effect of pH on protein and total solubilisation

The effect of pH on protein and total biomass solubilisation from BSG was studied using both undigested and carbohydrase treated BSG as substrates (Paper II). BSG was dried and coarse milled using 0.3 mm sieve (Hosokawa Alpine AG, Augsburg, Germany). The carbohydrase pre-treatment was carried out with Depol740 (1700 nkat of xylanase per g of BSG) using 10 % solids content at 50 °C for 5 h. After the hydrolysis, the solids and liquid were separated by centrifugation and the solids were washed with distilled water and used as substrate to study the effect of pH on non-enzymatic solubilisation and the enzymatic protein degradation.

The following buffers (100 mM) were used to study the effect of pH on non-enzymatic solubilisation of BSG: sodium citrate (pH 3.5 and 4.0), McIlvaine's buffer (pH 5.0–6.5), sodium phosphate (pH 7.0–8.0), Tris–HCl (8.5), and sodium carbonate (pH 9.0–10.0). After the reaction (5 h, 50 °C), solids were separated by centrifugation, washed with distilled water and lyophilised. After lyophilisation the amount of remaining solids were measured to determine the total solubilisation. In the protease experiments the hydrolysis was carried out at the optimal pH of the enzymes (3.5, 6.5 or 9.5), at 50 °C for 5 h. The protease dosage (170 nkat/g of DM) was based on casein substrate. The amount of solubilised protein was determined from the supernatants with the Lowry method using the Bio-Rad DC Protein Assay. Bovine serum albumin (BSA) was used as the standard protein.

3.3.4 Enzymatic preparation of lignin-rich fractions

BSG was suspended in tap water and a 6% BSG suspension was milled with Masuko. After milling a portion of the water was removed by centrifugation. Enzymatic hydrolysis consisted of three steps (Figure 6) (Papers III and IV) and was carried out in a 10 L reactor using a 10 % (w/w) solids content. The amount of BSG in the first hydrolysis step was 1000 g and the enzymes used were Depol740 (5000 nkat of xylanase activity per g of BSG) and Celluclast 1.5 (50 FPU per g of BSG). The hydrolysis was carried out at 50 °C for 5 h. After the reaction supernatant was separated by centrifugation, and the solid residue was washed with water. The second step was carried out with Alcalase 2.4 (200 nkat/g) at 60 °C and pH 10 for 4 h. The decrease in pH during the hydrolysis was adjusted back to 10 with 10 M NaOH. The residue was again separated by centrifugation and washed before the third step. The final hydrolysis was a repetition of the first one with Depol740 and Celluclast 1.5.

Precipitation of the soluble fraction from the second hydrolytic step was carried out by lowering pH to 2.5 with 5 M hydrochloric acid. The precipitated material was separated by centrifugation and washed twice with acidic water (pH 2.5). The precipitate was referred to as protease-alkaline extracted fraction (P-AEF). All the insoluble fractions and samples taken between different hydrolytic steps were freeze-dried for further analyses.

3.3.5 Preparation of deferuloylated fraction of BSG

To release ester-linked hydroxycinnamic acids (namely ferulic and *p*-coumaric acids and ferulic acid dimers) BSG was incubated in 2 M NaOH at room temperature for 2 h with

continuous stirring (Paper V). After the incubation, solids were separated by centrifuga-
tion, washed thoroughly with distilled water and neutralized with HCl. Finally the solids
were lyophilized, and the dried material was denoted as deferuloylated BSG (DEFE).
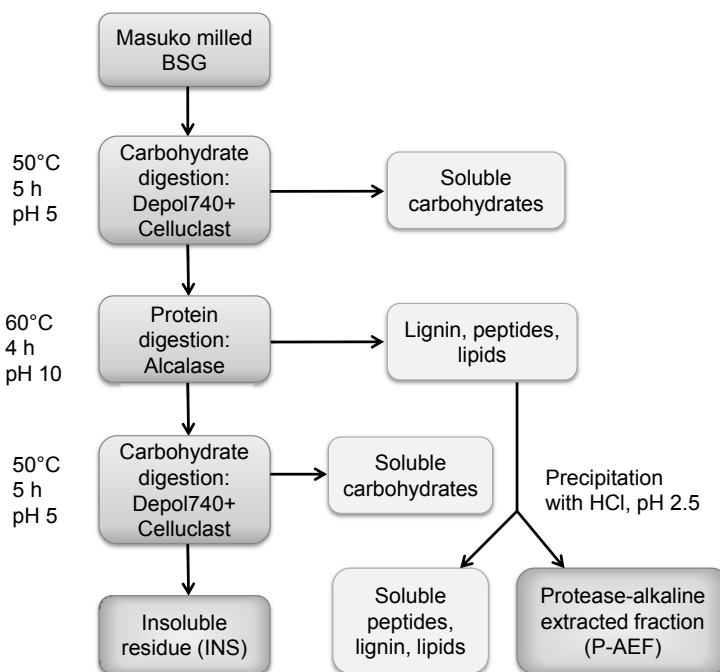
Figure 6. The scheme of the three-step enzymatic hydrolysis of BSG.

3.3.6 Colon model fermentation

Lignin-rich fractions from BSG were subjected to an *in vitro* fermentation by human faecal
microbiota (Papers IV and V). To obtain the microbiota, human faeces were collected from
5 healthy volunteers. Freshly passed faeces were immediately placed in an anaerobic
chamber, pooled and homogenised. The slurry was diluted to 20.8 % (w/v) by adding the
culture medium, filtered through a 1-mm sieve, placed on ice and used immediately as an
inoculum in the experiment. Each sample (200 mg) was weighed into bottles (50 mL) and
suspended with 2 mL of the culture medium 1 day before incubation to reduce the lag in
fermentation rate due to the hydration of the samples. Pre-hydrated samples were inocu-
lated with 8 mL of faecal suspension and a 16.7 % (w/v) final concentration of fresh faecal
matter was obtained. Bottles were tightly closed and incubated in a water bath at 37 °C
with continuous stirring (250 rev/min) for 0, 2, 4, 6, 8 and 24 h. Faecal controls were pre-
pared similarly but they did not contain any of studied fractions. Fermentations were car-
ried out in triplicate. After incubation, the samples were rapidly cooled using an ice water
bath and the pH was measured. The samples were then rapidly frozen using liquid nitrogen
and stored at -20 °C for further analyses. Averages and standard deviations were calculated
from triplicate measurements at each time-point.

For statistical analyses of the phenolic metabolites and short chain fatty acids, Two-Way
ANOVA was used to test significance between samples. The statistics were performed using
MatLab Version R2008b. Significantly different response levels between lignin fraction

and the faecal background within a time point were indicated with asterisks (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

3.3.7 Lignin-rich fraction as growth substrate for lactobacilli and bifidobacteria

Four lactobacilli strains (*Lactobacillus rhamnosus* VTT E-97800, *L. rhamnosus* VTT E-97948, *L. paracasei* VTT E-97949 and *L. salivarius* VTT E-981006) and three bifidobacterial strains (*Bifidobacterium adolescentis* VTT E-981074, *B. breve* VTT E-981075, *B. longum* VTT E-96664) were used to study the effects of a lignin-rich fraction on bacterial growth (Paper IV). The fraction used in the experiment was the protease-alkaline extracted fraction (P-AEF), which was extracted with heptane to remove lipids. Lactobacilli were grown on the following culture media: 1) unmodified MRS medium (de Man-Rogosa-Sharpe medium, Oxoid, Basingstoke, UK) as a positive control medium; 2) MRS medium without carbohydrates and P-AEF as a negative control medium; 3) MRS medium without carbohydrates as a basal medium with 1.0 % (w/w) addition of P-AEF. Bifidobacteria were grown on 1) unmodified *Bifidobacterium* medium (DSMZ medium 58) as a positive control medium; 2) *Bifidobacterium* medium without carbohydrates and P-AEF as a negative control medium; 3) *Bifidobacterium* medium without carbohydrates as a basal medium with 1.0 % (w/w) addition of the fraction as test media for bifidobacteria. Incubation was performed in anaerobic conditions at 37 °C and bacterial growth was monitored by serially diluting and plating on MRS agar (lactobacilli) or RCM (Reinforced Clostridial medium) agar (bifidobacteria). The plates were incubated in Anoxomat WS8000 anaerobic jars (Mart Microbiology, Lichtenvoorde, Holland) containing 10:5:85 H_2:CO_2:N_2 for 2 d (lactobacilli) and 3 and 7 d (bifidobacteria) at 37 °C.

4. Results

4.1 BSG properties and composition

BSG contained both the husks and residues of the grains, and the heterogeneous nature of the material is visualised in Figure 7. Although the malts had been coarse milled prior to mashing, the particle size of BSG was still up to ca. 5 mm.

Figure 7. A stereo microscope image of dried BSG.

BSG was obtained from two different batches, and they are referred to as BSG I and II. The two BSGs were very similar in composition as well as in their dry matter content, as presented in Table 4. Almost half of BSG was composed of polysaccharides, which are the major constituents of plant cell walls. The most abundant carbohydrates were arabinoxylan and glucan, which in BSG is mainly cellulose. Only residual amounts of starch (1.3 % for BSG I and 2.8 % for BSG II) and β-glucan (0.32 % for BSG I and 0.36 % for BSG II) remained after mashing. BSG was also rich in protein and lignin. Lipids accounted for approximately 10 % or less, and there was some difference in the lipid content between the two BSGs. In addition to the main components, the amount of hydroxycinnamic acids was determined. The amount of ferulic and *p*-coumaric acids were 2.7 and 1.3 mg/g for BSG I and 3.0 and 1.1 mg/g for BSG II. Sinapic acid was not detected.

Table 4. Compositions of the two BSGs used in the present study. BSG I was used in Paper I and BSG II in Papers II-V. *Dry matter contents of BSG I and II were 32.0 % and 31.6 %, respectively.

Component (% of dry weight*)	BSG I	BSG II
Carbohydrates	46.7	42.2
Arabinoxylan	22.9	22.2
Glucans	20.9	17.1
Lignin	19.4	19.3
Protein	23.3	22.8
Lipids	7.8	11.0
Ash	4.9	4.7

4.2   Milling of BSG

4.2.1   Effect of milling on particle size

The original BSG contained particles up to ca. 5 mm in size, but with milling the particle size was decreased down to µm scale (Figures 8 and 9). As shown in the microscopy images, after pin disc milling and TurboRotor treatment there were still large particles present in the material, whereas Masuko and microfluidizer treated samples appeared very uniform in size. After one hour of ball milling, there were both large cell fragments and fine particles, but after 24 h ball milling BSG appeared as fine dust. In the 24 h ball-milled sample colours seemed to blend together forming an overall brown colour. Aggregate formation was also detectable in the microscopy image of the 24 h ball-milled sample.

Pin disc milling, TurboRotor milling and ball milling were performed using dry BSG, whereas Masuko grinding and microfluidization were performed at over 90 % water content. Ball milling produced the smallest particles but the size distribution was not very uniform, ranging from only 1 µm to 50 µm (Figure 9). Pin disc milling was the least effective of the studied milling methods, and most of the particles produced were in the range of 100 to 1000 µm. The size distribution of TurboRotor milled BSG was wide ranging from 3 to 200 µm, but the majority of the particles were 10-100 µm in size. Both wet milling techniques produced distributions with a peak at 17 µm but the size distribution of Masuko milled BSG was somewhat wider than the that of microfluidized BSG. The distributions of the wet-milled BSGs were more uniform than those of the dry samples. In Figure 9, the distributions present the percentage of the total sample volume.

It was observed that the original, undried BSG sedimented rapidly when it was diluted with water prior to the milling with the wet milling techniques. However, only after one pass through the Masuko grinder the suspension became notably more homogenous and sedimentation was remarkably slowed down. The wet-milled samples produced a homogenous slurry in the course of the milling process, whereas the dry milled samples sedimented more quickly when resuspended in water.
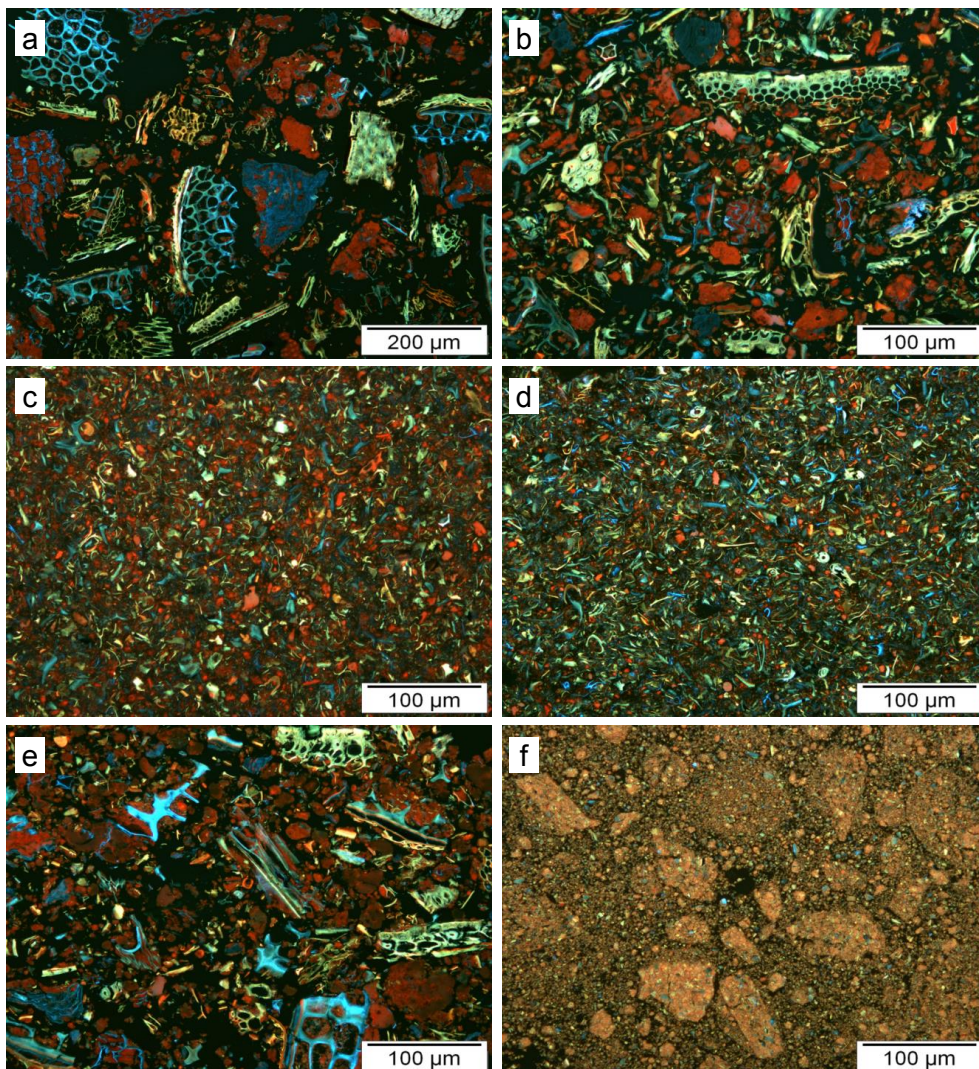
Figure 8. Microscopy images of BSG milled with different types of mills. BSG treated with a) pin disc mill, b) TurboRotor, c) Masuko, d) microfluidizer, e) ball mill for 1 h and f) ball mill for 24 h. Protein appears red (Acid Fuchsin), aleurone and subaleurone cell walls blue (Calcofluor) and lignified cell walls yellow and green (autofluorescence). Modified from Paper I (supplementary material) with permission of Elsevier B.V.

Particle size reduction by the different kinds of millings significantly improved the enzymatic hydrolysis of BSG carbohydrates (Figure 10). When a milling pre-treatment was applied, carbohydrate solubilisation increased from 23 up to 45 % at best. The highest solubility was obtained with 24 h of ball milling followed by the wet milling techniques (35–37 %) and TurboRotor, which was almost equally efficient (34 %). Pin disc milling, which was also used as a pre-treatment for ball milling, was the least effective (28 %). The yield of hydrolysed carbohydrates correlated with the particle size (Figure 9): the smaller the particle size, the better the hydrolysis yield. In addition, milling affected the amount of water-soluble carbohydrates as a significant increase in yield was detected also in the reference samples without the Depol740 enzyme preparation.
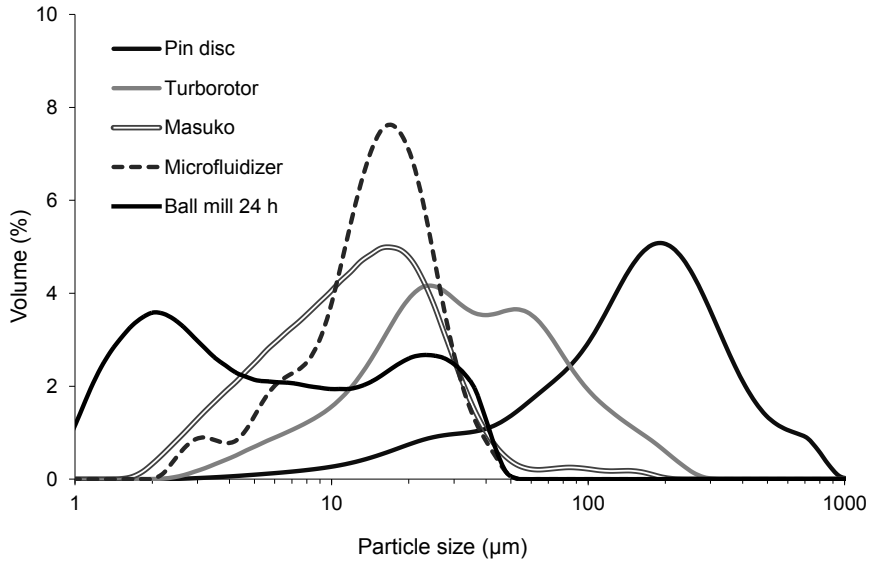
Figure 9. Particle size distributions of milled BSG samples. Data obtained from Paper I.

As Depol740 contained only low cellulase activity (Paper I, Table 1), the addition of enzyme preparations high in cellulase and β-glucosidase activities, Celluclast1.5 and Novozym188 respectively, was studied (Figure 11). Although ball milling produced the smallest particle size and highest amount of soluble carbohydrates, it was not chosen as the pretreatment for further studies. Instead BSG milled with Masuko was used because Masuko enabled the processing of significantly higher amounts of sample than the ball mill, and when using Masuko BSG did not need drying but could be milled in high water content.
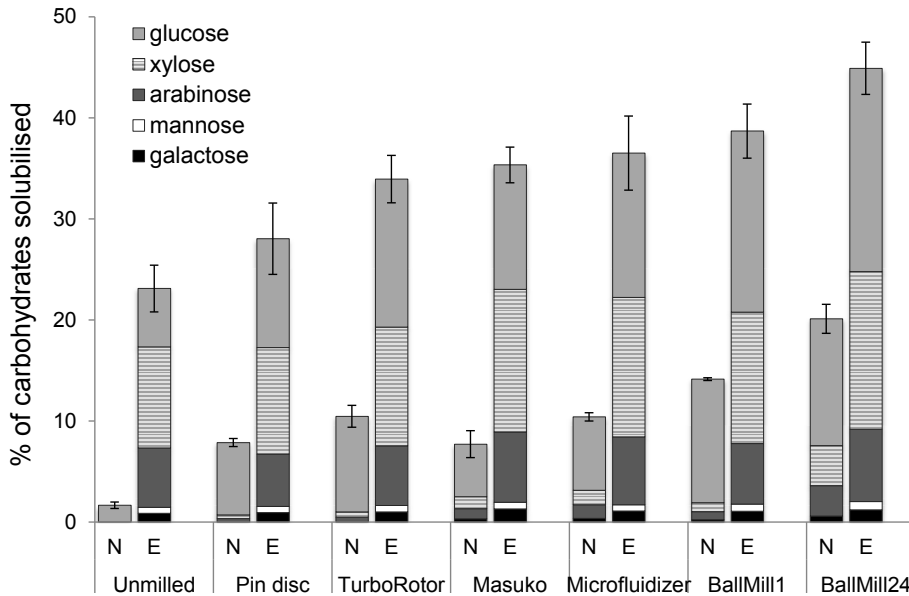
Figure 10. Solubilisation of different carbohydrates from BSG by Depol740 enzyme preparation after millings. N = reference, no enzyme; E = with enzyme. Data obtained from Paper I.

After the Masuko pre-treatment, 8 % of BSG carbohydrates had become water-soluble and Depol740 was able to release 36 % of the carbohydrates (Figure 11). The addition of Cellu-clast1.5 increased the solubilisation to 48 %, and it was not improved by a further supple-mentation with Novozym188. Compared to Depol740, Celluclast was almost equally effi-cient in xylose and arabinose hydrolysis and more efficient in cellulose hydrolysis.
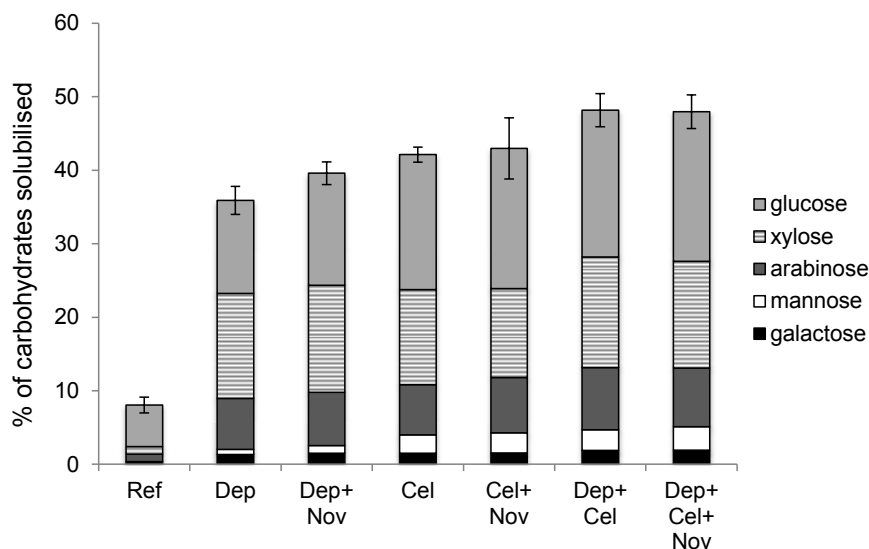
Figure 11. Solubilisation of different carbohydrates from Masuko-milled BSG by Depol740 (Dep), Celluclast1.5 (Cel) and Novozym188 (Nov). Ref = no enzyme. Data modified from Paper I.

4.3   Effect of carbohydrase treatment and pH on BSG solubility

pH is an important factor in biomass solubilisation, and therefore the impact of pH on sol-ubility was studied with both undigested and carbohydrase treated BSG without adding any enzymes. After the carbohydrate hydrolysis with Depol740 BSG solubility was im-proved 2–3 fold (Figure 12). In addition, an alkaline pH had a significant impact in increas-ing BSG solubility after the carbohydrate digestion, as 15 % of BSG was dissolved at pH 10 by the effect of alkalinity alone compared to the 5 % dissolved at pH 3.5. The increase in solubility was not as pronounced with unhydrolysed BSG, although some improvement, from 1.8 to 5.5 % was detected there as well.

BSG contained a significant amount of water-insoluble protein, and therefore the effects of pH and proteases on BSG protein solubility were also investigated (Figure 13). Three proteases with different pH optima were selected and their capability to release protein from carbohydrase-treated BSG was studied. Protein solubility was the highest with the alkaline protease (Alcalase 2.4) reaching 88 % at pH 9.5. The alkalinity had a significant effect on protein solubility as also the non-enzymatic solubility (pH 9.5 control) was 31 %. Acid Protease released 39 % and Promod144 24 % of the total protein.
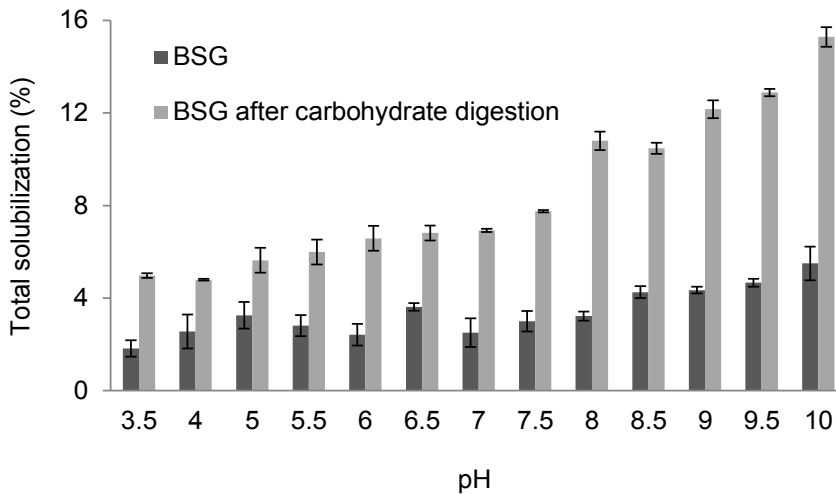
Figure 12. Effect of a carbohydrase treatment and pH on solubility of BSG (without enzymes). Data obtained from Paper II.
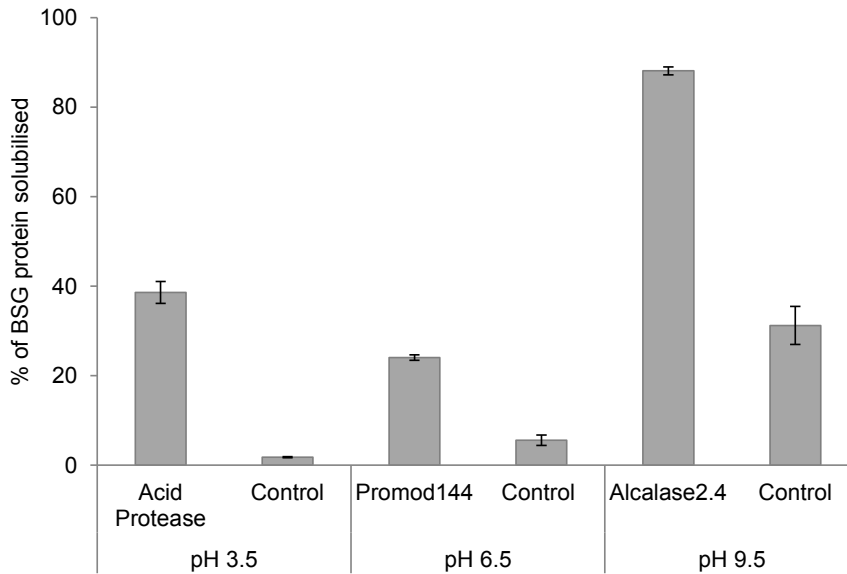
Figure 13. Effect of pH and protease on BSG protein solubility after a carbohydrate digestion. Previously un-published data.

The effect of pH on protein solubility was also investigated using optical microscopy (Figure 14). The undigested BSG was rich in protein as visualised by the red colour (Figure 14a). After it had been treated with the carbohydrate-degrading enzymes (Depol740), there was still a significant amount of protein present (Figure 14b). However, some changes in the proteinaceous material had occurred, as the red colour was somewhat less bright compared to the original BSG (Figure 14a). After the protease treatment at pH 9.5, protein had almost completely disappeared (Figure 14c), whereas after the non-enzymatic treatment at alkaline pH a notable amount of protein still remained in the residue (Figure 14d).
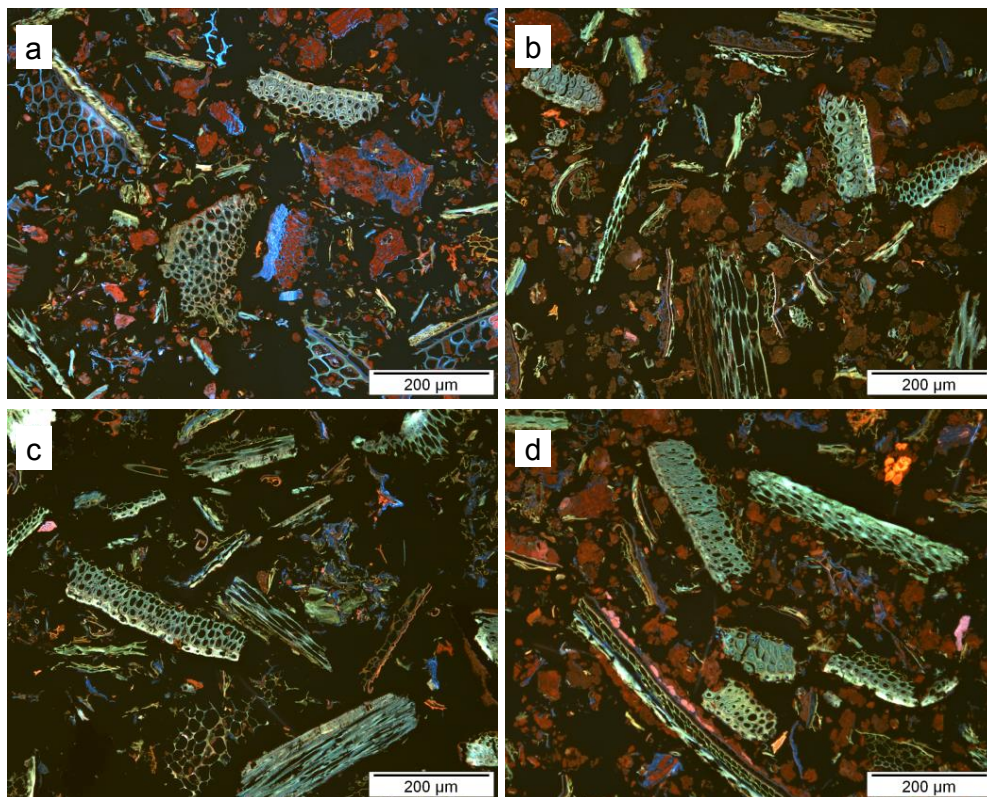
Figure 14. Optical microscopy images of a) Alpine-milled BSG, b) BSG after carbohydrase treatment, c) BSG after carbohydrase and Alcalase 2.4 (pH 9.5) treatments and d) BSG after carbohydrase and non-enzymatic alkaline (pH 9.5) treatments. Reprinted from Paper II (supplementary material) with permission of Elsevier B.V.

4.4 Preparation of lignin-rich fractions for the colon model study

4.4.1 Three-step enzymatic hydrolysis of BSG

To enrich the lignin content of Masuko-milled BSG, carbohydrates and proteins were hydrolysed by sequential enzymatic treatments (Figure 6). The compositions of the obtained fractions and the amounts of material solubilised are presented in Tables 5 and 6. In the first step 26 % of BSG was dissolved. The solubilised material was mainly carbohydrates, and their content was decreased from 42 to 22 % in the residue after the first hydrolytic step. In addition, 6 % of protein was released. The contents of lignin, protein and lipids increased in the residue as a consequence of the carbohydrate digestion.

In the second step, which was a protease treatment in alkaline conditions, most of the protein was solubilised, but in addition a significant amount of lipids and lignin were released. After the second hydrolysis, 93 % of protein, 48 % of lignin and 87 % of lipids had been solubilised. Practically no carbohydrates dissolved in the alkaline proteolytic step.

In the third hydrolytic step, the first carbohydrase treatment was repeated. This time, not only carbohydrates were solubilised but part of the remaining lignin (19 %) and lipids (7 %) were released as well (percentages are from the total amount in original BSG). The composition of the insoluble residue after the third hydrolysis (INS) is shown in Table 5. The

amount of the recovered residue was 19 % of original BSG. The INS fraction consisted mainly of the recalcitrant carbohydrates and lignin.

As lignin is soluble in alkaline pH but precipitates in acidic conditions, it was precipitated from the liquid phase with hydrochloric acid after the alkaline proteolysis. The obtained precipitate was denoted as protease-alkaline extracted fraction (P-AEF) and its composition is also shown in Table 5. However, it was not only lignin that precipitated but lipids and peptides as well. Furthermore, it appeared that only 30 % of the lignin released in the second hydrolysis precipitated and the rest remained in the liquid phase. The amount of recovered precipitate (P-AEF) was 11 % of the original BSG and the amount of lignin in the precipitate was 14 % of the lignin present in the original BSG.

Table 5. Compositions of the fractions obtained in the three-step enzymatic hydrolysis of BSG and after chemical extractions. Data modified from paper III, IV and V.

Component	Initial BSG	Hydrolysis residue 1st	Hydrolysis residue 2nd	INS	P-AEF	Extract. P-AEF	DEFE
Carbohydrates	42.2	22.2	49.4	39.2	4.0	6.7	53.4
Arabinoxylan	22.2	15.9	31.2	25.7	2.3	3.8	20.3
Glucan	17.1	4.5	15.9	11.2	0.7	1.2	30.9
Lignin	19.3	29.1	33.5	40.3	24.4	40.7	21.9
Protein	22.8	28.6	4.8	6.6	27.2	45.2	7.6
Lipids	11	15.5	4.1	3.1	39.9	0	2.9
Ash	4.7	4.6	8.1	8.7	4.5	7.4	6.0

Table 6. The amount of material solubilised by the three enzymatic hydrolysis steps. The solubilities are given for each step and as a total solubilisation after all steps. Data modified from Paper III and V.

Hydrolysis step	Enzymes used in the hydrolysis	Solubility in the step %	Cumulative solubilisation %
First	Depol740, Celluclast1.5	26.2	26.2
Second	Alcalase2.4	54.5	66.4
Third	Depol740, Celluclast1.5	47.3	81.3

To visualise the phenolic compounds in INS and P-AEF by their autofluorescence, the samples were imaged under UV light (Figure 15c and d) and also using the chemical dyes Calcofluor and Acid Fuchsin (Figure 15 a and b). Some blue colour was detectable in INS indicating presence of aleurone cell walls even after extensive treatments with hydrolytic enzymes (Figure 15a). For P-AEF the dyes showed that protein seemed to cover the precipitated lignin, as only very fine green particles were visible from the red mass (Figure 15b). (Please note that the green particles may not be clearly visible in the printed image due to the reduced quality of the reprint compared to the original microscopy image). In P-AEF the particles seemed to have formed aggregates in the precipitation, as mostly very large entities up to hundreds of μm were seen. The UV image confirmed the presence of phenolic material in INS (Figure 15c). Interestingly, for P-AEF hardly any autofluorescence was seen (Figure 15d). This finding supports the idea of protein covering lignin.
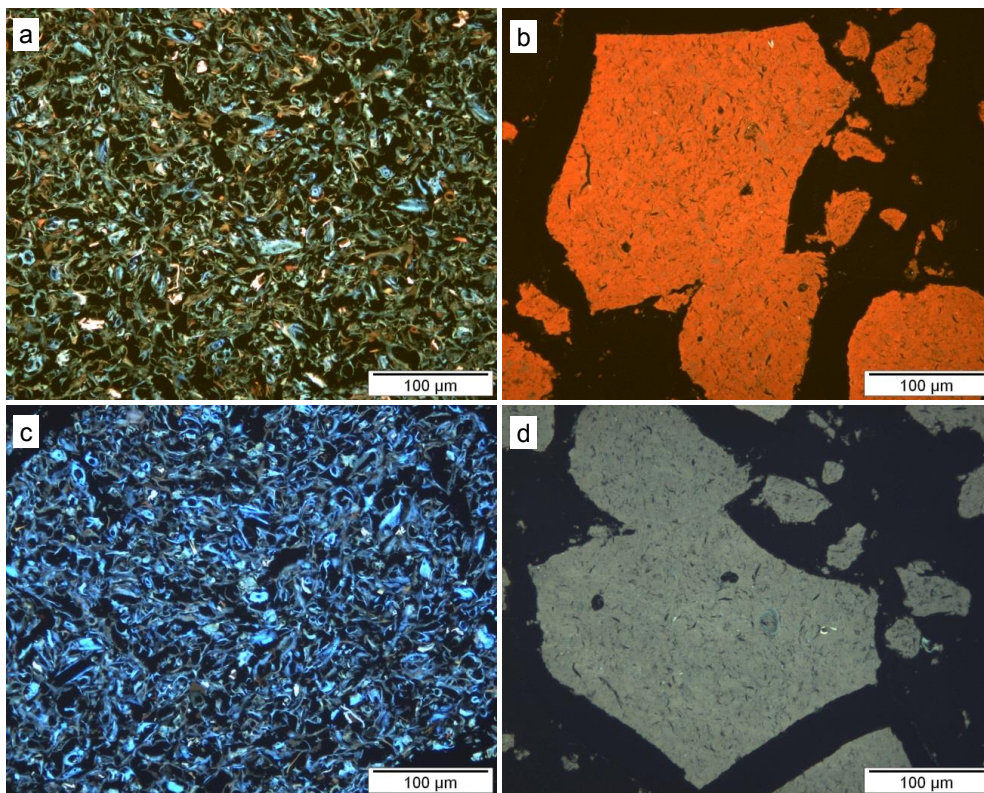
Figure 15. INS and P-AEF imaged with optical microscopy using chemical dyes (a: INS, b: P-AEF) and with autofluorescence due to UV light (c: INS, d: P-AEF). Previously unpublished figures.

4.5 Interactions of lignin-rich fractions with colon microbiota *in vitro*

In the *in vitro* colon model, a wide range of metabolites was detected for each studied fraction: 1800 for P-AEF, 2400 for INS and 2500 for BSG and DEFE. The metabolites with FC<2 were excluded. For each fraction there were several hundred metabolites with FC>2, and from those compounds the ones with structural similarity to lignin were selected. For some compounds it was not possible to identify the exact chemical structure, but they were indicated with a group-specific name, e.g. dilignols. The heat maps showing the profiles of aromatic metabolites are presented in Figure 16. Blue colour represents under-expression and red colour over-expression of the metabolite compared to the faecal background. The asterisks denote statistical difference between the sample and the control. The clustering on the left side of the heat maps groups similar time course profiles of different metabolites.

Eight mono- and dimeric phenolic and aromatic metabolites were formed from BSG (Figure 16a) during the *in vitro* colon model fermentation, and out them three compounds, 4-methylcatechol, a dilignol (compound 2) and ferulic acid, showed significant difference from the faecal background at several time points. Another dilignol (compound 6) with statistical difference from the background also appeared at the end of the fermentation. An unidentified guaiacyl compound and 4-hydroxymethylcatechol showed a higher response in the beginning of the fermentation, after which their responses notably decreased.

A)

	0h	2h	4h	6h	8h	24h	
		**	**	**	*		1. 4-Methylcatechol (2.8)
				**	*	*	2. Dilignol (5.5)
	**	**	**	*	*		3. Ferulic acid (3.9)
	*			*			4. Guaiacyl compound (7.0)
	*	*					5. 4-Hydroxymethylcatechol (2.2)
						*	6. Dilignol (2.7)
							7. 4-Aminobenzoic acid (2.1)
						*	8. Benzeneacetic acid (2.9)

B)

	0h	2h	4h	6h	8h	24h	
					*		1. Catechol derivative (11.9)
		**					2. Dilignol (17.3)
		*		*			3. Dilignol (11.2)
		*					4. Guaiacyl compound (48.0)
		*					5. Dilignol (52.5)
	**	**	*				6. cis-Ferulic acid (31.5)
		*					7. Guaiacyl compound (45.9)
		*					8. Vanillin (5.4)
							9. Phenolic acid (4.2)
	*	*			*		10. Homovanillic acid (34.9)
							11. Coniferyl aldehyde (2.4)
							12. Catechol derivative (5.1)
		**					13. Dilignol (5.9)
							14. Homovanillin (2.3)
							15. Aromatic compound (5.4)
							16. 2-Hydroxymandelic acid (2.5)
							17. Guaiacyl compound (3.0)
							18. 3-Syringylpropanoic acid (2.3)
							19. Benzeneacetic acid (7.3)
	*	**					20. Aromatic acid (2.3)

Color key

-1   -0.5   0   0.5   1   1.5   2

Figure 16. Heat maps of the aromatic metabolites found in the metabolomes of the *in vitro* colon fermentations, A) BSG, B) P-AEF, C) INS and D) DEFE. The number in parentheses after the name is the fold change (FC) of the compound. The colour key is the 2-log value of the FC. A blue colour indicates an under-expression and red colour represents an over-expression of the metabolite compared to the faecal control. The asteriks denote statistical difference between the sample and the control. Data obtained from Papers IV and V.

The lignin content in P-AEF (Figure 16b) was only 5 % units higher than in BSG but a notably greater amount of aromatic metabolites was released from P-AEF (20) compared to BSG (8). The P-AEF metabolic profile contained several lignin-type compounds with guaiacyl structure (compounds 8, 10, 11 and 14) and one with syringyl structure (compound 18). Two unidentified guaiacyl compounds (compounds 4 and 7) had especially high FCs (48 and 46), and responses of both increased towards the end of the fermentation. Formation of four dimeric metabolites (dilignols) was also detected. It seemed that most of the aromatic metabolites were formed at 6 h or later.

Lignin content of INS was the highest of all studied samples (Table 6), which also correlated with the number of low molecular weight aromatic metabolites (26) (Figure 16c). Both mono- and dimeric compounds were detected including catechols, phenolic acids and dilignols, and several compounds were statistically different from the faecal background at more than one time point. Compounds 2, 5, 6, 10 and 26 contained guaiacyl or syringyl structure, which are characteristic of lignin.

For DEFE 10 low molecular weight aromatic metabolites were found in the metabolome (Figure 16d), of which dilignols and catechols could possibly originate from lignin. C*is*-ferulic acid (compound 6) was still detected, although the sample had been treated with strong alkali to release ester-linked hydroxycinnamic acids. 2-Aminobenzoic acid (compound 4) was unlikely to be formed from lignin. Similarly as with P-AEF (Figure 16b), aromatic metabolites mostly started to form at 6 h.

Certain phenolic metabolites were also analysed quantitatively using authentic standards. The most important of these are presented in Figure 17. Ferulic acid, which was present in P-AEF at a high concentration, was almost completely metabolised to other products during the fermentation. The content of ferulic acid in the other studied samples was significantly lower. Some ferulic acid was released from BSG due to microbial activities but in INS and especially in DEFE there were no notable changes in the ferulic acid concentrations during the 24 h incubation period (see Table 7 for the statistical significances).

4-Methylcatechol was formed from P-AEF, BSG and INS at significantly higher levels than from the faecal control, and its formation started at the very beginning of the fermentation suggesting that it was a primary metabolite. 3,4-Dihydroxyphenylacetic acid, which is a metabolite from dehydrodiferulic acid, was formed at the highest concentration from P-AEF, but also in BSG and INS the levels were significantly above the faecal background.

Short chain fatty acid (SCFA) formation was measured during the fermentation in order to study possible inhibitory effects of lignin-rich samples on microbial conversions (Figure 18). The initial formation rate (0–2 h) was the fastest for P-AEF (17.7 mM/h) followed by DEFE (13.0 mM/h). The initial SCFA formation rate was on a similar level in BSG (8.2 mM/h) and the faecal control (10.3 mM/h in the case of P-AEF and 8.2 mM/h for the others, as fermentations were carried out at different times and with different inocula). The lowest rate (7.3 mM/h) was observed for INS. There was no indication that lignin in the samples suppressed SCFA formation, as the levels of the studied samples were above those of the faecal control and the carbohydrates originating from the faeces and BSG fractions were partially fermented. It should be pointed out though, that in first two hours the SCFA levels of BSG were lower than for the faecal control. However, this does not seem to be due to inhibition, as the SCFA levels quickly rose after the first two hours.
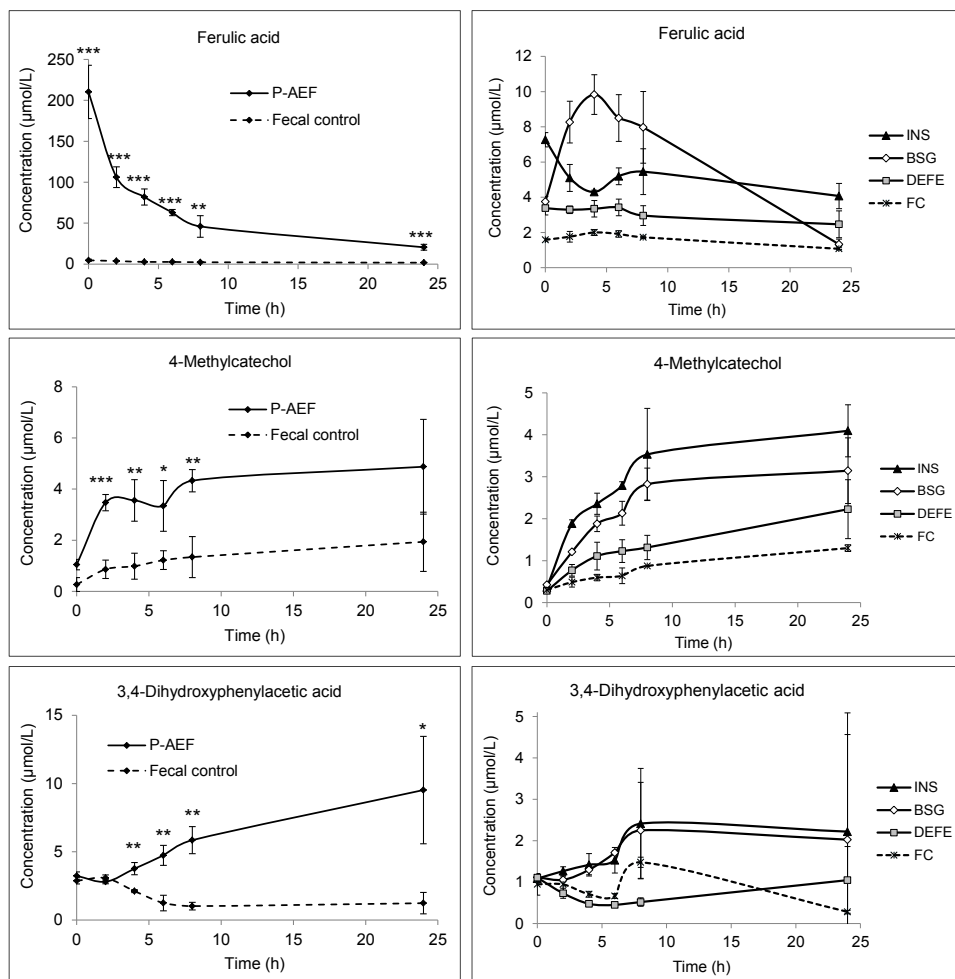
Figure 17. Concentrations of quantitated metabolites formed in the colon model fermentation. Left: metabolites formed from the fermentation of protease-alkaline extracted fraction (P-AEF), the asterisks denote statistical difference between the sample and the faecal control. Right: metabolites formed from BSG, insoluble hydrolysis residue (INS) and deferuloylated BSG (DEFE). The statistical differences between the samples and faecal control (FC) are presented in Table 7. Data obtained from Papers IV and V.

Table 7. Statistically significant differences between quantified phenolic metabolites from BSG, INS, DEFE. Statistical difference ($p < 0.05$) is represented with small letters (a, b, c, d). Data obtained from Paper V.

	Ferulic acid					4-Methylcatechol			
Time (h)	BSG	INS	DEFE	Faecal control	Time (h)	BSG	INS	DEFE	Faecal control
0	ab	a	ab	b	0	–	–	–	–
2	a	b	bc	c	2	b	a	c	c
4	a	b	bc	c	4	a	a	b	b
6	a	b	bc	c	6	b	a	c	c
8	a	ab	bc	c	8	a	a	b	b
24	b	a	ab	b	24	ab	a	bc	c

	3,4-Dihydroxyphenylacetic acid			
Time (h)	BSG	INS	DEFE	Faecal control
0	–	–	–	–
2	ab	a	b	b
4	a	a	b	b
6	a	a	b	b
8	–	–	–	–
24	–	–	–	–

39
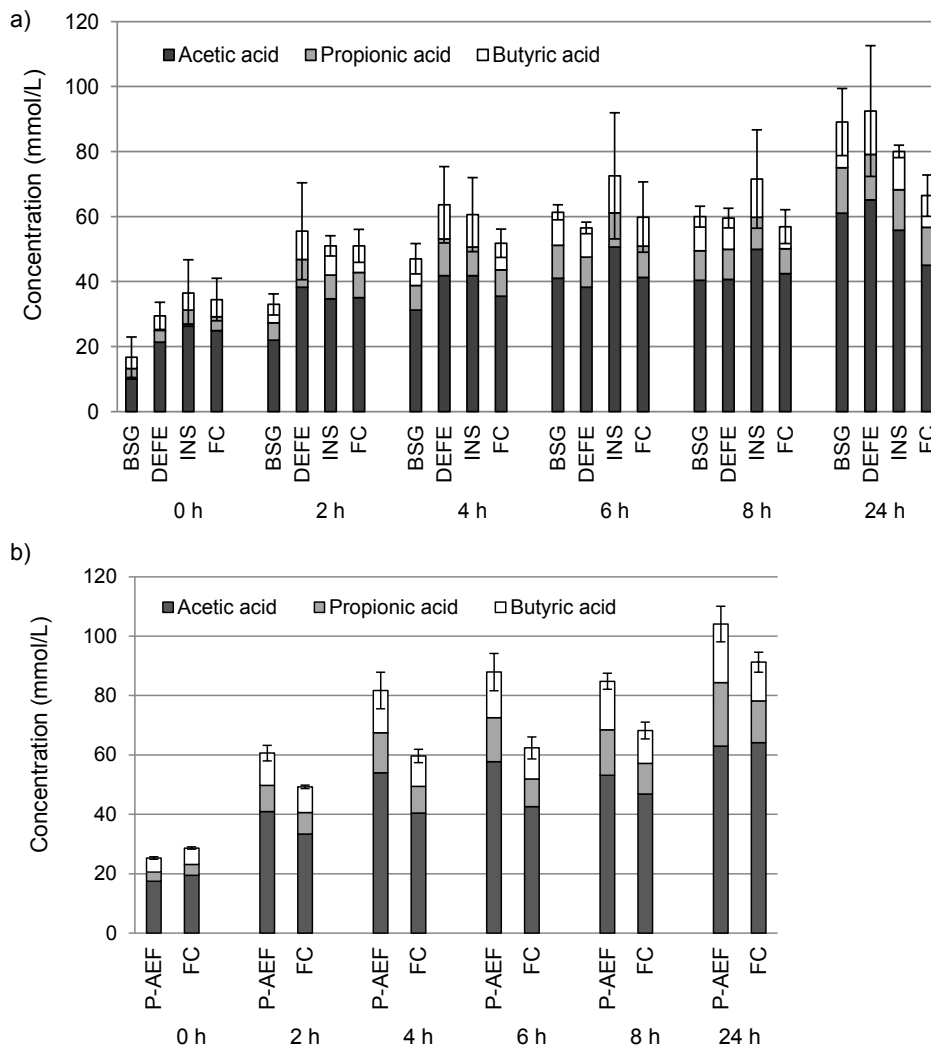
Figure 18. Short chain fatty acid formation in the *in vitro* colon model. BSG, INS and DEFE are presented in the chart above (a) and P-AEF in the chart below (b). Data obtained from papers IV and V.

4.6 Effects of lignin on lactobacilli and bifidobacteria

No inhibitory effects on the growth were observed after two days of incubation of different strains of *Lactobacillus* with P-AEF (lipids removed by extraction, Table 5) as the growth substrate (Figure 19). Both strains of *L. rhamnosus* were able to grow on P-AEF, as their colony counts (10^8 CFU/mL) were nearly equal to the positive controls (0 % P-AEF + glucose). For *L. paracasei* the positive control was 0.5 log units and for *L. salivarius* 1.5 log units higher than P-AEF. The colony counts of all strains grown on P-AEF were above the negative control (no added glucose or P-AEF).

No inhibition was seen with the three *Bifidobacterium* species either. After three days the growth was low (10^3 CFU/mL) in the positive controls of *B. adolescentis* and *B. longum*, as most of the cells were no longer culturable. With P-AEF as the growth substrate, the colony counts were above the negative control also for bifidobacteria. Moreover, after seven days of incubation, there was still notable growth (10^6-10^7 CFU/mL) in the presence of P-AEF

and some growth (10^5 CFU/mL) was also observed in the negative control. In the positive controls most of the cells were not culturable after 7 days.
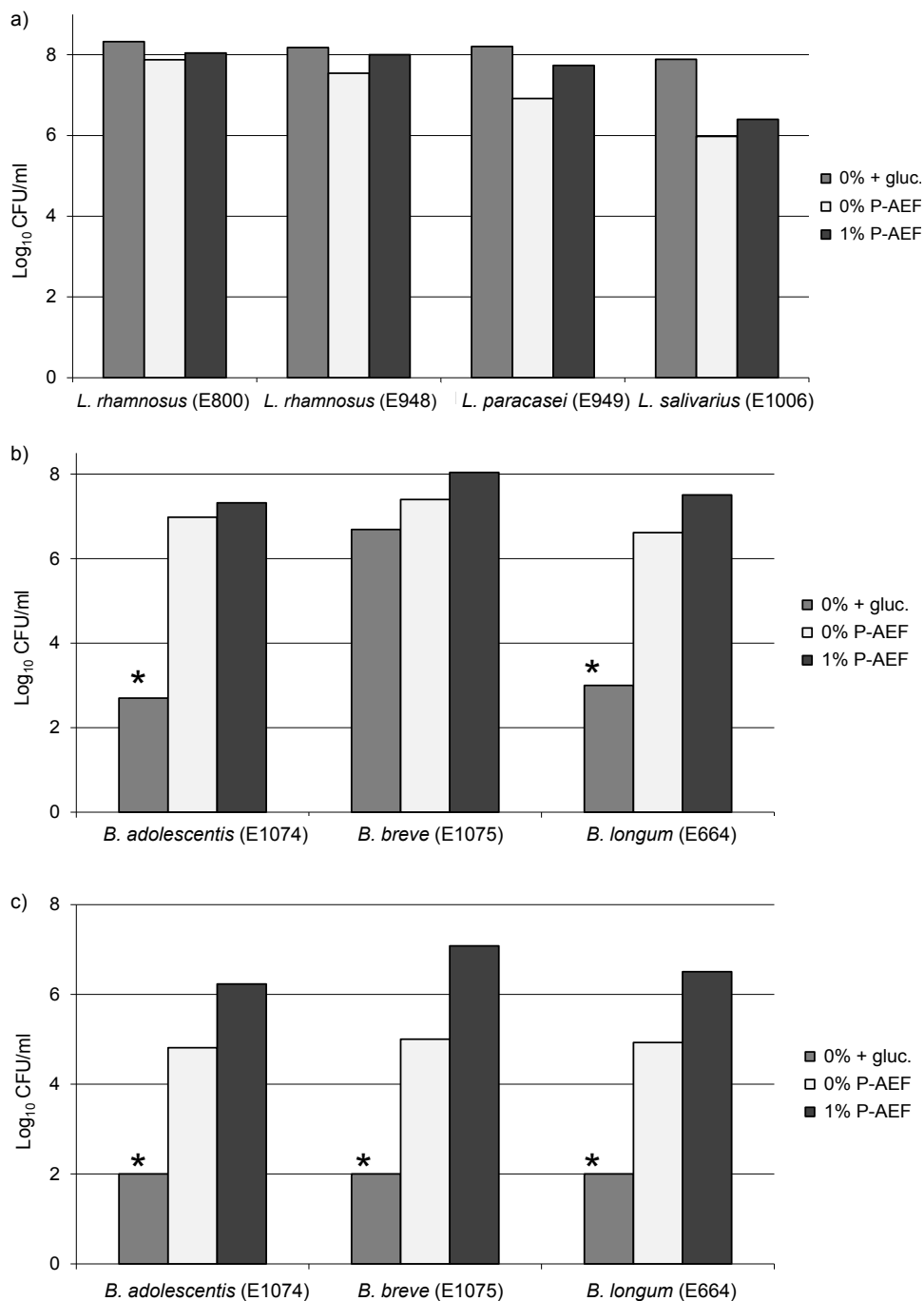
Figure 19. Figure 19. Bacterial counts of lactobacilli and bifidobacteria grown in the presence and absence of glucose (positive and negative control) or in the presence of the protease-alkaline extraction (P-AEF, lipids removed). a) Lactobacilli strains after 2 days, b) bifidobacterial strains after 3 days and c) bifidobacterial strains after 7 days of incubation. The asterisks denote too long an incubation time, as most of the cells were already dead. Previously unpublished figure.

5. Discussion

BSG consists mainly of the cell wall materials of husks, pericarp and aleurone, and the residual protein inside the aleurone cells. In mashing, malts have passed an extensive hydrolytic treatment by a variety of enzymes resulting in the solubilisation of the starchy endosperm, and the resilient lignin-carbohydrate matrix along with the insoluble protein remain in the solid residue, i.e. BSG. The results of this thesis showed that the enrichment of BSG lignin by enzymatic fractionation was limited by the recalcitrant cell wall matrix even when the material was pre-treated by milling. The separation of lignin was further complicated by co-solubilisation of lignin with protein. Several low molecular weight phenolic metabolites were formed from the lignin-rich fractions by the activities of faecal microbiota in the *in vitro* colon model indicating at least partial degradation of lignin. Lignin did not suppress microbial carbohydrate fermentation in the colon model or inhibit growth of lactobacilli and bifidobacteria, which is contradictory to the common conception that lignin is an antimicrobial compound.

5.1 Preparation of lignin-rich fractions from BSG

5.1.1 Limited enzymatic solubilisation of cell wall carbohydrates

It has been noted previously that BSG carbohydrates are poorly hydrolysed by commercial enzyme preparations, if no pre-treatments to open up the cell wall structures are applied (Forssell *et al.* 2008). Therefore, a milling pre-treatment to improve the hydrolysis yield was studied. Milling is a mechanical treatment, which does not affect the food grade status of BSG, but enhances the hydrolysis by reducing the particle size and degrading cell wall structures and therefore increasing the surface area accessible for enzymes.

The yield of enzymatically solubilised sugars from BSG was low (23 %), when no pre-treatment was applied (Figure 10). Milling improved the yield up to 45 %. Ball milling was the most effective followed by the wet milling techniques. However, even with the ball milling pre-treatment more than half of the carbohydrates still remained resistant to enzymes suggesting that other factors restricting the hydrolysis still remained. When looking at Figure 2b, it can be observed that the cell wall thickness in BSG is in the low micrometre range. As hardly any submicron particles were detected (Figure 9), it would suggest that cell walls remained at least partially intact even after the most effective of the studied milling techniques. In other words, although milling markedly reduced particle sizes, the cell wall structures were not completely broken down and the particles were not entirely digestible by the enzymes. Cell wall degradation might have been enhanced by prolonging the milling, but this was not further studied. The effect of a prolonged hydrolysis time was shown to provide only minor improvements (Paper I, Figure 3).

The monosaccharide profile of solubilised sugars (Figure 10) showed that hydrolysis of unmilled BSG by Depol740 resulted in solubilisation of mainly arabinoxylan and a smaller amount of cellulose-derived carbohydrates. Arabinoxylan is expected to be derived mostly from the aleurone cell walls as they contain 67 % of arabinoxylan and 26 % of β-glucan (Duffus and Cochrane 1993). The changes in BSG after the enzymatic treatment were also visualised by epifluorescence microscopy (Figure 14a). After the hydrolysis with Depol740 aleurone cell walls (shown as blue) had almost completely disappeared and only the ligni-fied cell walls remained. After mashing the β-glucan content of BSG was very low (<1 %) and thus the liberated gluco-oligosaccharides were expected to arise from cellulose. Milling increased the solubilisation of glucose and glucose-based oligosaccharides, whereas a smaller increase was observed with arabinoxylan. This implies that milling affected the cellulose-rich husk and pericarp more than the arabinoxylan-rich aleurone. This was seen for both enzymatic and non-enzymatic (control) solubilisation.

Previously ball-milling has been shown to be such a powerful treatment that it markedly increased the amount of water-soluble arabinoxylan in wheat bran by breaking covalent bonds inside arabinoxylan (Van Craeyveld *et al.* 2009). Moreover, the authors observed that ball-milling affected the solubility of pericarp-enriched (thus cellulose-enriched) bran more than the solubility of the original bran. It has also been reported that wheat bran is fractured more rapidly by milling than isolated aleurone layers, because pericarp is much more friable than aleurone (Antoine *et al.* 2004). These observations support the results of the present study, which showed that cellulose rich parts of BSG, i.e. the husk and pericarp, were more susceptible to milling. This effect was especially pronounced in the reference samples without the addition of Depol740. However, the presence of residual enzymatic activity remaining from malting and mashing cannot be ruled out, and this could have in-creased the carbohydrate solubility especially in the reference samples.

The enzyme dosage used in the Depol740 treatment was very high (Paper I, Table 1), so increasing that would most likely not further improve the yield. However, Depol740 is low in cellulase activity, and therefore the enzyme cocktail was supplemented with additional cellulases. Masuko-milled BSG was used as the raw material in this experiment, as Masuko was found to be a more feasible technique compared to ball milling; large quantities of sample could be handled and the milling could be performed in high water content thus avoiding the drying of BSG. Supplementing the enzyme mixture with cellulases increased the yield only moderately, from 36 to 48 % (Figure 11), indicating that the limited carbohy-drate solubilisation had not been due to the low cellulase activity in Depol740 but was more dependent on the physical restrictions of the material. Thus, irrespective of the en-zyme mixture used after the milling only half of the cell wall polysaccharides could be hy-drolysed.

The cell wall pore sizes in wheat kernels have been reported to be too small (<10 nm) to allow free penetration of cell wall degrading enzymes (Chesson *et al.* 1997). Beaugrand *et al.* (2005) demonstrated with an inactivated xylanase that cell wall deconstruction is a pre-requisite for enzymes to penetrate testa or pericarp, but accessibility to aleurone is not as restricted. According to Chesson *et al.* (1997) the differences in the pore sizes between dif-ferent plant species are likely to be small, so it can be assumed that the small pore size causes steric hindrance for enzymes also in the hydrolysis of BSG. Another factor impeding the hydrolysis is the presence of lignin, as lignin is known to adsorb and inhibit cell wall degrading enzymes (Ooshima *et al.* 1990, Palonen *et al.* 2004). Lignin is also cross-linked to arabinoxylan through ferulic acid (Bunzel *et al.* 2004, Ishii 1997) further restricting en-

zymatic accessibility. Similarly, diferulate cross-links between arabinoxylan molecules (Bunzel *et al.* 2001, Hatfield *et al.* 1999) negatively affect cell wall degradability (Grabber *et al.* 1998b). A lower degree of ferulate cross-linking has been shown to result in improved hydrolysis of maize cell walls, and the impact was more pronounced for xylan than cellulose (Grabber *et al.* 1998a). Although the extent of arabinoxylan cross-linking is lower in barley compared to maize (Bunzel *et al.* 2001), it can still be assumed to contribute to the limited carbohydrate hydrolysis observed in this study. Furthermore, hydrophobic cuticularised layers in testa and husk may act as barriers for enzyme penetration (Fincher and Stone 1993).

The outer layers of cereal grains provide a protective shield keeping other organisms apart from the starch and other nutrients inside the grain, and the low extent of enzymatic hydrolysis of BSG carbohydrates is likely due to the resilient nature of the outer layers. Although the enzyme mixture contained the most relevant cell wall degrading activities, cellulases, xylanase and arabinosidase, the hydrolysis was restricted by physical barriers or the lack of some important enzymatic activity, or both. As ferulate cross-links are known to be a key factor limiting cell wall hydrolysis (Grabber *et al.* 1998b, Grabber *et al.* 2009), feruloyl esterases should enhance the hydrolysis. Depol740 is a preparation from the fungus *Humicola insolens*, which has been reported to produce feruloyl esterases acting on BSG ferulic acid (Faulds *et al.* 2002). However, in the present study this activity was obviously not capable to effectively cleave the cross-links or the dosage was significantly lower than would have been needed. The observations discussed above as well as the results of the milling pre-treatment study suggest that either an improved enzyme mixture or another kind of pre-treatment, e.g. chemical or physico-chemical, or a combination of these, would be required for a more complete enzymatic hydrolysis of BSG carbohydrates.

5.1.2 Effect of pH and protease on BSG and protein solubility

Depending on the source of BSG, protein makes up 10–27 % of BSG (Table 1). Previously it has been shown that even without any pre-treatment alkalinity improves the total solubilisation of BSG (Faulds *et al.* 2008). In the present study the effect of alkaline conditions on BSG solubility was shown to be even more pronounced after a carbohydrate digestion (Figure 12). As aleurone cells are rich in protein and their non-lignified cell walls are not as resistant to enzymatic digestion as pericarp and husk cell walls, it seems likely that the Depol740 treatment made proteins in aleurone more susceptible to the following alkaline treatment. Alkaline conditions can also partially dissolve lignin, which in part contributes to the total solubilisation.

Three different proteases with different pH optima were compared for the hydrolysis of protein from carbohydrase treated BSG in acidic, neutral and alkaline conditions (Figure 13). Protein solubility was the highest with Alcalase 2.4 at pH 9.5 as almost 90 % of protein was hydrolysed after the two enzymatic treatments. Alcalase 2.4 has previously been found to effectively release proteins from BSG at pH 6.8–9 (Treimo *et al.* 2008, Treimo *et al.* 2009), although the relative activity of Alcalase 2.4 is only approximately 30 % of the maximum at pH 9, and reaches maximum between pH 9.5 and 10 (Faulds *et al.* 2008). Almost one third of the protein was released by the alkali alone (control) suggesting that the high pH was also an important factor in protein solubility. The acidic and neutral proteases were not as effective in protein release, and especially the non-enzymatic solubilisations at pH 3.5 and 6.5 were very low.

The results are in line with the observation that water-solubility of barley proteins is the lowest from pH 4 to 6 and the highest from pH 10 to 11 (Yalçın and Çelik, 2007). Based on the findings of Yalçın and Çelik (2007) it would seem likely that pIs of BSG proteins are in the range of pH 4–6, which makes them less water-soluble in this pH range. Furthermore, presumably the increased net charge of proteins at pH 9.5 enhanced both enzymatic and non-enzymatic protein release from BSG. However, the effects may have been different for hordeins and glutelins, which are the two classes of water-insoluble proteins in BSG. Most likely, alkalinity affected glutelins more as they are known to be soluble in mild alkali (Linko *et al.* 1989), whereas hordein solubilisation is enhanced by a reducing agent (Osborne 1909).

5.1.3 Co-solubilisation of protein and lignin in alkaline conditions

A three-step enzymatic hydrolysis of BSG was carried out to enrich the lignin content of the residue by the removal of proteins and carbohydrates (Figure 6). First, a carbohydrase treatment solubilised 26 % of Masuko-milled BSG (Table 6), and this was for the most part carbohydrates, although a minor amount of protein was co-solubilised. Secondly, a proteolytic treatment resulted in the release of almost 90 % of proteins and almost half of lignin. Finally, a carbohydrase treatment similar to the first step was repeated. In total, 81 % of BSG was dissolved by the three hydrolyses, and two different types of lignin-rich fractions were obtained.

40 % of BSG was released in the proteolytic treatment due to both the alkaline conditions and the presence of a protease. As part of the lignin was known to dissolve in the alkaline proteolysis, the aim was to separate the released lignin from the peptide solution by precipitation with acid (precipitate denoted as P-AEF). This type of lignin would be almost free of carbohydrates and thus more accessible for the gut microbiota, which would make it an interesting substrate for the further *in vitro* studies. As a result of the protease treatment, it was assumed that protein would have been degraded to such small peptides that they would not co-precipitate with lignin. According to previous research, peptides obtained from BSG using Alcalase 2.4 are mostly less than 1 kDa in size (Treimo *et al.* 2009). However, the precipitation did not occur as planned, as only one third of lignin precipitated and the rest remained in the soluble phase. Furthermore, despite the anticipated low molecular weight of the peptides, part of the soluble proteinaceous material (17 %) co-precipitated with lignin (Table 5). Most of the peptides still remained water-soluble, as was expected. The improved water-solubility of lignin and the co-precipitation of proteinaceous material raise the question of interactions between lignin and proteins, as lignin is normally water-insoluble at low pHs. Such interactions, e.g. hydrophobic, electrostatic and/or hydrogen bonding could possibly contribute to the water-solubility of lignin released by the alkaline protease treatment and thus explain the low amount of precipitated lignin.

Thus far, there is only a limited amount of studies showing protein-lignin interactions, and the interactions are not yet fully understood. In research related to nutrition, polyphenol-protein interactions are somewhat more studied, but they are usually limited to tannins (Bennick 2002) or flavonoids (Papadopoulou and Frazier 2004), which both are known to bind to proteins resulting in soluble or insoluble complexes. Several studies concerning enzymatic hydrolysis of lignocellulose have shown that lignin interacts with cellulase enzymes and adsorbs them on its surface (Ooshima *et al.* 1990, Eriksson *et al.* 2002, Palonen *et al.* 2004, Rahikainen *et al.* 2013). The adsorption can be reduced by pre-

treating lignin with another protein, which acts as a "lignin-blocker" (Yang and Wyman, 2006). When analysing lignin isolated from BSG with three well-known methods (milled wood lignin, dioxane lignin and cellulolytic lignin), Rencoret *et al.* (2013) discovered that all three lignin preparations contained a significant amount of protein (20–30 %), implicating that protein-lignin interactions likely occur in BSG as well. This was also supported in the present study by the microscopy image of the precipitate (P-AEF) showing only a protein-rich surface and hardly any autofluorescence (Figures 15b and d). Salas *et al.* (2013) described strong, nonspecific interactions between soy proteins and lignin model films. Furthermore, interactions with the soy proteins increased the hydrophilicity of the lignin films. This would be in line with the results of the present study, according to which lignin was made more water-soluble by the presence of a high concentration of peptides. The lignin-protein/peptide interactions were not, however, studied in more detail in this work.

In addition to lignin and protein, the precipitate contained a significant amount of fat. The ester-linkages of triglycerides were probably hydrolysed by the alkali, as the amount of free fatty acids had increased in the P-AEF compared to BSG (Paper III, Figure 3b). A part of the fat co-precipitated with lignin and peptides, whereas a part of it formed a layer on top of the liquid phase when separating the precipitate by centrifugation, and they were thus excluded from the P-AEF.

5.1.4 Insoluble lignin-carbohydrate residues

The third hydrolysis step was similar to the first one with the aim to further enrich the lignin content of the insoluble residue after the protein removal. The outcome was somewhat different to the first carbohydrate hydrolysis as this time not only carbohydrates were solubilised. It seemed that the previous alkaline treatment had opened up cell wall structures and enabled the release of some of the remaining lignin and a small amount of lipids as well. The residue (INS) contained 40 % of lignin (Table 5), which was twice the content of the starting material. The amount of lignin in the residue corresponded to 39 % of the initial lignin in BSG. The rest of the material was composed of the most resistant cell wall polysaccharides that remained unhydrolysed even after the extensive hydrolytic treatments. As INS still contained some ferulic acids (Figure 17), it can be assumed that lignin in INS was cross-linked to carbohydrates making it insoluble in the alkaline conditions of the proteolytic treatment.

In addition to the enzymatic fractionation, a separate alkaline extraction was carried out for BSG. Its purpose was to remove the ester-bound ferulic acids, and thus reduce the presence of ferulic acid metabolites in the further *in vitro* colon model study, as they may be confused with lignin metabolites. Ferulic acid and its dimers, although minor components in BSG (ca. 0.3 %) (Hernanz *et al.* 2001), are among the most abundant of phenolic compounds of BSG. Strong alkali dissolved a significant amount (62 %) of the whole BSG leaving an insoluble residue rich in cell wall polysaccharides and lignin (denoted as deferuloylated BSG or DEFE) (Table 5). It should be noted that the alkaline conditions of the reaction (pH 14) were more severe than in the proteolytic treatment (pH 10).

As BSG carbohydrates turned out to be difficult to be removed enzymatically when using only a mechanical milling pre-treatment, and the protein-lignin interactions further complicated the enriching of lignin, it was apparent that samples with very high lignin content could not be obtained from BSG with the applied methods. However, lignin content of die-

tary fibre in common fibre-rich foods, such as kale and whole grain wheat, is around 5–7 % (Bunzel *et al.* 2011). This converts to approximately 1–2 % of lignin in the whole food material. Therefore, the lignin contents of BSG and its fractions could be considered lignin-rich compared to common foods. Three different lignin-rich preparations were obtained from BSG by the enzymatic and chemical methods. In the protease-alkaline extracted fraction, lignin was together with proteinaceous material but almost free of carbohydrates. The insoluble residue after the three hydrolyses contained the most recalcitrant lignin-carbohydrate matrix. Finally, the fraction deferuloylated by alkali, which also consisted of lignin-carbohydrate matrix, had a low content of ferulic acid, which ideally would facilitate the distinguishing between ferulate and lignin metabolites in further metabolic study.

5.2   Metabolism of lignin-rich fractions in a colon model

Lignin is the major phenolic component in BSG (Rencoret *et al.* 2015). The nature of lignin of the BSG fractions produced in the present study has been characterised by Py-GC/MS (Ohra-aho *et al.* 2016) and the results were in agreement with the previous findings (Rencoret *et al.* 2015). The interactions between lignin and human faecal microbiota were studied in order to assess if faecal microbiota is able to degrade and convert lignin in the colon. The main focus was on detecting phenolic metabolites, which could originate from lignin. The analysis was divided to quantitative, targeted analysis using authentic standards and non-targeted, semiquantitative analysis, in which metabolites were identified using compound libraries and comparison of mass spectra with relevant literature.

   A variety of mono- and dimeric phenolic metabolites were detected and identified. In Figure 20 the chemical structures are presented for the most potential lignin metabolites, which could be fully identified. BSG and DEFE, which had the lowest lignin contents, also gave rise to the least amount of phenolic metabolites, although it is unlikely that the lignin content alone affected the metabolite profile, but lignin accessibility and presence of other compounds contributed as well. The lower amount of phenolic metabolites formed from BSG was apparently due to the abundance of more easily digestible material and poor accessibility of the microbiota to lignin, as no treatments to open up the cell wall matrix had been carried out. In DEFE, the content of ferulic acid was very low (Figure 17) due to the strong alkaline extraction. The alkaline conditions most likely released most of the easily fermentable material, but on the other hand the cleavage of ferulate cross-links could have facilitated the decomposing of the remaining cell walls. The amounts of phenolic metabolites originating from P-AEF and INS were notably higher compared to BSG and DEFE. This was probably affected by the increased amount and availability of lignin as a result of cell wall digestions and the dissolving effects of alkaline conditions. In INS lignin remained together with carbohydrates, whereas in P-AEF it appeared to be covered by proteinaceous material (Figure 15).

5.2.1   Potential lignin metabolites

Catechol compounds were formed from all of the studied fractions. 4-methylcatechol was the only one of the quantified metabolites (Figure 17), which was not a known ferulic acid metabolite (Russell *et al.* 2008, Braune *et al.* 2009). Its amount increased more rapidly in the beginning of the fermentation but slowed down towards the end, suggesting that it was a preliminary metabolite. Moreover, there was no apparent connection between the con-

centrations of 4-methylcatechol and ferulic acid supporting the hypothesis that 4-methylcatechol originated from something else than ferulic acid. Thus lignin seems like the most likely source. The origin of catechols could be guaiacyl type compounds from which the methoxyl group on the phenolic ring was converted to a hydroxyl group.

Among the most interesting compounds was 3-syringylpropanoic acid, which was formed from INS and P-AEF. As syringyl compounds have two methoxyl groups, they cannot be formed from ferulic acid, so it is probable that they originate from lignin. Sinapic acid, which could be an alternative source for syringyl compounds, has not been detected in barley or BSG (Hernanz *et al.* 2001, Holtekjølen *et al.* 2006), and it was neither detected in the BSG used this study. Another compound closely resembling lignin in structure was coniferyl aldehyde formed from P-AEF. In addition, unidentified guaiacyl compounds were formed from BSG and P-AEF, and two of them from P-AEF had especially high fold change values of 46 and 48 implicating that their concentrations were markedly higher than the faecal background. Guaiacyl structures are abundant in lignin and therefore it can be assumed that these compounds were lignin related.
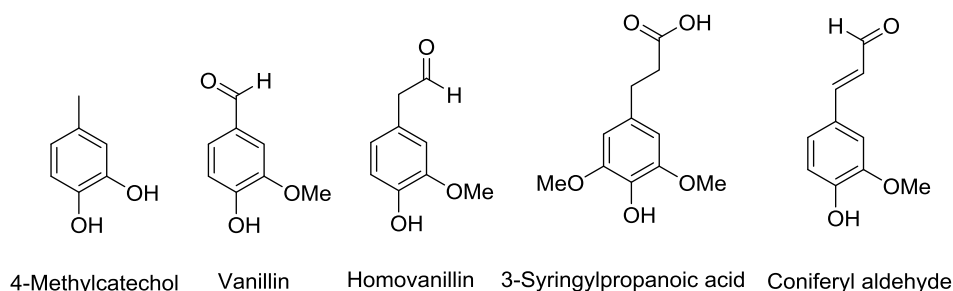
Figure 20. Structures of identified potential lignin metabolites.

Vanillin was another metabolite having structural similarity to lignin with fold changes 5 and 10 (Figures 16b and c). Vanillin is a well-known degradation product of lignin, and produced at industrial scale from sulphite pulping side streams (Borregaard 2015). Therefore vanillin as well could be lignin-related. On the other hand, vanillic acid seemed to be present in the sample already in the beginning of the fermentation (Figure 16c), which would suggest that it originated from BSG. The increase in the response of vanillic acid at the end of the fermentation could have come from oxidation of vanillin. Homovanillic acid (Figure 16b and c) is an intermediate in the conversion of 8-*O*-4 linked ferulic acid dimer (Braune *et al.* 2009), but it could also have come from oxidation of homovanillin (Figure 16b). Alternatively, homovanillin could be, in theory, formed from homovanillic acid by reduction of the carboxylic acid to an aldehyde. This is not, however, a common reaction in the metabolism of 8-*O*-4 dehydrodiferulic acid (Braune *et al.* 2009) and therefore homovanillin can also be considered a potential lignin metabolite.

Several dilignols were produced by the microbiota (Figure 16). Dilignols consist of two monolignols and they are also potential lignin metabolites. Plant lignans, which are dilignols in structure, are present in BSG (Paper III, Table 2) and theoretically the detected dilignols could be lignans released from BSG instead of being cleaved from lignin polymer. However, as in most cases the dilignol concentrations increased towards the end of the fermentation, it would indicate that they were formed relatively slowly. Furthermore, it has

been demonstrated that lignans (or dilignols) can be obtained from lignin through the activities of gut microbiota (Begum *et al.* 2004).

It has been claimed that lignin is not at all degraded in the human gastrointestinal tract (Holloway *et al.* 1978). However, in the *in vivo* study conducted by Holloway *et al.* (1978) the amount of lignin excreted was markedly higher than the amount of lignin consumed, which should not be theoretically possible, suggesting significant inaccuracies in the analysis method. On the contrary, Williams and Olmsted (1936) proposed that notable lignin degradation occurs in humans. However, the analytics involved quantitation of lignin as an acid-insoluble residue, which unlikely yielded reliable results from faecal mass. Instead of the gravimetric lignin quantitation, some studies based on analysis of aromatic metabolites have also been carried out. Silanikove and Brosh (1989) measured lignin degradation in goats based on urinary hippuric and benzoic acids concentrations. Notable increases in the acid concentrations were detected indicating lignin degradation. Csonka *et al.* (1929) measured the amount of lignin methoxyl groups and urinary aromatic acids to determine lignin digestion in cows and dogs. They concluded that based on the loss of methoxyl groups and increase in aromatic acids in urine, lignin was degraded in both animals. However, in the view of current knowledge (Russell *et al.* 2008, Braune *et al.* 2009) and the abundance of catechols as metabolites in the present study, it should be reconsidered whether or not the disappearance of methoxyls can be regarded as evidence of lignin degradation, as demethylation of the phenolic ring substituents appears to be a common reaction by faecal microbiota resulting in the loss of a methoxyl group by conversion to a hydroxyl group. It does not seem impossible that such a reaction could also occur on polymeric lignin, without necessarily involving a further fragmentation of the polymer. On the other hand, the increased amounts of aromatic metabolites support their conclusions.

Although the studies discussed above date back more than several decades, standardised or generally accepted methods for quantitatively detecting lignin degradation by faecal microbiota do not exist. This is likely to be one of the main factors limiting the research of this particular topic. A more reliable way of demonstrating lignin degradation, could be, for instance, using isotope labelled lignin. This has been performed by Begum *et al.* (2004), who prepared deuterated synthetic lignin and followed its conversion in rats. Deuterated enterolactone was detected from the urine of rats unambiguously confirming the degradation of lignin by gut microbiota *in vivo*. However, with the methods applied in the present study, the origin of any metabolite cannot be verified with 100 % certainty, although links to lignin seem very likely with several metabolites. With further identification of the metabolites, their conversion reactions and particularly the converting enzymes the metabolite pathways could also be studied. Knowing the pathways would confirm the origin of the metabolites and provide new information on the activities of gut microbiota.

5.2.2 Extent of lignin degradation by gut microbiota

Regarding the extent of lignin degradation by human faecal microbiota no definite conclusions can be drawn, as the analysis of metabolites was only semiquantitative (except for 4-methylcatechol), and instead of absolute concentrations it provides information on a) how much higher the concentration of a given compound is relative to the faecal background (fold change) and b) whether the concentration of the compound is increasing or decreasing over time. As the faecal background most likely contained some lignin as well, the con-

centrations of metabolites originating from the background affected the fold change. The higher the background the lower the amount of a given metabolite is relative to it. Alternatively, if the concentration of a certain metabolite is very low in the background, even a smaller amount of it produced from the studied sample may result in a relatively large fold change. Therefore, the fold changes should only be considered as indicative.

Some measure of lignin degradation can be drawn from the amount of 4-methylcatechol, which was quantitated. The amount of 4-methylcatechol produced during the fermentations corresponded to approximately 40–80 ppm of the lignin present in the samples in the beginning of the fermentation. As the amounts of other metabolites were not quantitated, the extent of lignin degradation cannot be measured from one metabolite, but it provides some idea of the level of degradation. Also the rat study by Begum *et al.* (2004) gives some indication. The amount of deuterated enterolactone produced was 655 nmol/g of synthetic lignin, which corresponds to approximately 0.2 mg of enterolactone per g of lignin also indicating a low degree of lignin conversion. However, the amount of only two metabolites, enterolactone and enterodiol (not detected), were measured, and there is a possibility that other deuterated conversion products were also formed, which would suggest a more extensive conversion. Regarding the rat study, it should be noted though that rat and human microbiota are different from each other and may have different capabilities to degrade lignin.

The degradation of lignin could be limited also by inability of microbiota to cleave carbon-carbon bonds between phenolic units. As seen with diferulates, ether-linked dimers are cleaved to monomers, whereas carbon-carbon linked dimers are not (Braune *et al.* 2009, Russell *et al.* 2008, Schendel *et al.* 2015). However, the β-*O*-4 aryl ethers are the most abundant linkages in BSG lignin (Rencoret *et al.* 2015) suggesting that despite the possible limitations sites for cleavage are still numerous. Nevertheless, based on the research showing more limited fermentation of carbohydrates in lignin-containing dietary fibre (Grabber *et al.* 2009) it can be assumed that lignin is not an easy substrate for microbiota. Thus, it would seem reasonable that human microbiota is able to convert only a small part of it, but according to the present study it seems more than likely that lignin is not completely inert.

5.2.3 Phenolic metabolites derived from non-lignin sources

In addition to lignin, ferulic acid and its dimers are present in BSG (Hernanz *et al.* 2001), and thus they were another source of phenolic metabolites. The amount of ferulic acid in the colon model was quantitated. Its content was highest in P-AEF and it was metabolised for the most part during the fermentation (Figure 17). BSG, INS and DEFE contained markedly less ferulic acid, and in INS and DEFE its concentration did not change notably over time. Moderate changes were observed for BSG as ferulic acid was rapidly metabolised upon release from the matrix. For INS and DEFE the formation of 3,4-dihydroxyphenylacetic acid was on a similar level compared to ferulic acid, suggesting that it was a major metabolite of ferulic acid, but for P-AEF the concentrations of these two compounds did not correlate similarly.

It should be pointed out that there are some differences in the naming of diferulates and dilignols (Figure 21). In both the phenolic ring carbons are denoted as carbons 1–6 starting from the carbon containing the side chain. However, in monolignols the propenyl side chain carbons are denoted as α, β and γ, whereas in ferulic acid (and other hydroxycinnam-

ic acids) the equivalent carbons are simply numbered 7, 8 and 9. Thus, for instance β-*O*-4 and 8-*O*-4 are similar aryl ether bonds but the first one links two monolignols and the latter ferulic acids.

R¹=R²=H: *p*-coumaryl alcohol
R¹=H, R²=OMe: coniferyl alcohol
R¹=R²=OMe: sinapyl alcohol

R=H: *p*-coumaric acid
R=OMe: ferulic acid
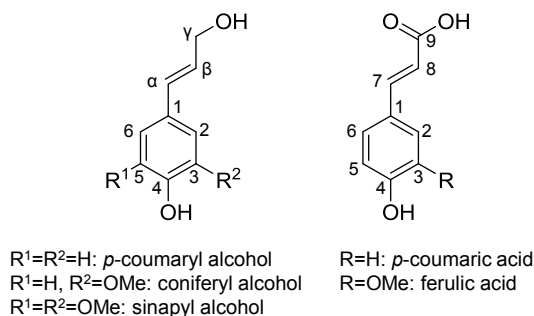
Figure 21. Naming of carbon atoms in monolignols and hydroxycinnamic acids.

Ferulic acid is the component, which in this study interferes most with the interpretation of the lignin metabolites. The conversions of ferulic and diferulic acids by faecal microbiota have been studied and their metabolites characterised, and thus the obvious ferulic acid metabolites could be excluded from the potential lignin metabolites. The metabolites originating from ferulic acid include *p*- and *m*-coumaric acid, cinnamic acid and phenylpropanoic acid (mono-, di- and non-hydroxylated) (Russell *et al.* 2008). In addition to these 3,4-dihydroxyphenylacetic acid, 3-(3,4-dihydroxyphenyl)lactic acid and homovanillic acid are formed from 8-*O*-4 linked dehydrodiferulic acid (Braune *et al.* 2009). The ether bond in 8-*O*-4 dehydrodiferulic acid can be cleaved by faecal microbiota, whereas in 8-5 coupled dehydrodiferulic acid the carbon-carbon bond between the two ferulic acid units is not cleaved resulting in only dimeric metabolites (Schendel *et al.* 2015). For 5-5 linked diferulic acid only one metabolite has been identified, a dimeric compound with hydrogenated aliphatic side chains but no alterations in the methoxyl groups (Russell *et al.* 2008). Many of the ferulic acid and 8-*O*-4 dehydrodiferulic acid metabolites were produced from the BSG fractions, and although they have structural similarities with lignin as well, due to the presence of ferulic acid in the studied fractions, the origin of these metabolites was probably not lignin.

Metabolites that probably originated from sources other than lignin and ferulic acid include aminobenzoic acids and 4-hydroxyphenylpentanoic acid. 4-Aminobenzoic acid is formed in the synthesis of aromatic amino acids in certain bacteria including *Escherichia coli* (Herrmann 1995). In 4-hydroxyphenylpentanoic acid (5 carbons) the aliphatic chain is too long to be formed from lignin structure, as the aliphatic chains in lignin contain 3 carbons.

5.2.4 Potential bioactivities of lignin and phenolic metabolites

Lignin and other polyphenolics may induce beneficial properties in humans upon ingestion. These include acting as an antioxidant and radical scavenger (Lu *et al.* 1998, Dizhbite *et al.* 2004) due to phenolic hydroxyl groups (Barclay *et al.* 1997), and controlling post-prandial glycemic response (Hanhineva *et al.* 2010). Lignin has been shown to adsorb carcinogenic compounds in conditions mimicking the small intestine and colon (Funk *et al.* 2006, Funk *et al.* 2007), which might help protect from cancer. Lignin may also exert an-

timicrobial activity towards certain intestinal pathogens, such as *E. coli* (Baurhoo *et al.* 2007a). Moreover, the part of lignin that remains in polymeric form may further contribute to bulking effect and bowel movement (Raninen *et al.* 2011) improving gut health. In addition to lignin, the effects of low molecular weight phenolic metabolites on human health should also be further elucidated.

Phenolic metabolites may induce bioactive effects upon release and conversion by colon microbiota, including antioxidative (Castelluccio *et al.* 1995, Vinson *et al.* 2003), antimicrobial (Barber *et al.* 2000), anti-inflammatory (Karlsson *et al.* 2005) and anticarcinogenic (Stich and Rosin 1984) properties. For example, ferulic acid alone possesses all the aforementioned characteristics (reviewed by Ou and Kwok 2004). Other phenolic metabolites, such as 4-methylcatechol and 3,4-dihydroxyphenylacetic acid have also been found good antioxidants *in vitro* (Gläßer *et al.* 2002). Plant lignans, are known to be bioactive in many ways; they are antioxidant (Eklund *et al.* 2005, Willför *et al.* 2003) and antitumorigenic (Thompson *et al.* 1996, Hausott *et al.* 2003), and when converted to mammalian lignans enterodiol and enterolactone may protect from hormonal cancers, such as breast cancer (Adlercreutz 2002). The exact structures of the dilignol metabolites detected in the present study could not be confirmed, but characteristics such as dimeric form with guaiacyl structures were identified. Due to their structural similarities with lignans, it can be hypothesized that these dilignols as well could possess similar properties. Thus it appears that lignin-related metabolites may potentially induce bioactive effects in humans, but their impact is not known and needs to be characterised before any conclusions can be drawn. It seems likely that lignin is an undervalued compound of dietary fibre, and its role in diet and digestion by intestinal microbiota requires more attention in the future.

5.3 Effects of lignin on microbial carbohydrate fermentation and growth

5.3.1 Formation of SCFA from the lignin-rich fractions

Carbohydrates that are resistant to digestion in the stomach and small intestine are transferred to the colon where they are partially fermented to short chain fatty acids (SCFA) (Cummings and MacFarlene 1997). The most important SCFA include acetic, propionic and butyric acids, which have several physiological functionalities, such as being the major source of energy of colon epithelial cells (Comalada *et al.* 2006). In the present study the concentrations of SCFA were measured in the colon model to see if their formation was suppressed, which could indicate that lignin inhibits enzymatic activities of the microbiota. No significant suppression was detected (Figure 18). Although in the beginning of the fermentation SCFA levels were slightly lower for BSG compared to the faecal background, they exceeded them at the end, and thus it would seem that carbohydrate fermentation was slowed down but not completely suppressed. Previously suppression of SCFA formation and phenolic metabolism have been noticed to occur in the presence of condensed tannins, or proanthocyanidins isolated from apples and Syrah grapes (Bazzocco *et al.* 2008, Aura *et al.* 2013). These types of tannins are mostly composed of epicatechin units and although they differ in their chemical structure compared to lignin, it was important to assess if lignin has similar effects towards microbial enzymes.

The untreated BSG was probably a slowly fermentable substrate, as no enzymatic breakdown of the cell walls had been carried out prior to the *in vitro* fermentation. Thus more time for the microbiota to release sugars and ferment them to SCFA was needed. In the

case of INS the more easily digestible carbohydrates had been removed by enzymatic treatments, and only the most resistant carbohydrates most tightly bound to each other and lignin remained. In DEFE the cleavage of (di)ferulate cross-links should have facilitated the degradation of cell wall polysaccharides, but the amount of SCFA was not higher compared to the other fractions. It is possible that the strong alkaline treatment also dissolved the easily digestible material and thus DEFE was not notably more fermentable despite the loss of cross-links. P-AEF was very low in carbohydrates (4 %), but it was still fermented at significantly higher rate and extent compared to the faecal background suggesting that these few carbohydrates were accessible to the microbiota. Therefore, it would seem probable that slow SCFA production was not as strongly affected by the presence of lignin in the material but was rather dependent on the resilient nature of the carbohydrate matrix.

The lignin contents of the samples were not very high, in the range of 20–40 %, and the original idea was to obtain BSG fractions with higher lignin purity. However, if lignin isolation had been more comprehensive, most likely the results would have been completely different. As lignin also has antimicrobial characteristics (Baurhoo *et al.* 2007a), purer lignin could have had detrimental effects on the faecal microbiota, and therefore the metabolism of the samples and SCFA formation might have been significantly more limited. As lignin is ingested as part of dietary fibre i.e. inside the plant cell wall matrix, it is not present as a pure component in the colon, and therefore the impurities present in this study may actually have given a more realistic result. Furthermore, the carbohydrates and proteins in the studied fractions provided nutrients and energy to the microbiota and likely enabled them to survive longer in the colon model experiment.

5.3.2   Lignin-rich fraction as a growth substrate for lactobacilli and bifidobacteria

The capability of beneficial colonic bacteria to grow on the protease-alkaline extracted fraction (P-AEF) was studied in order to detect potential inhibition of growth by a lignin-rich substrate. For this purpose lipids were extracted from P-AEF increasing its lignin content to 41 %. The bacteria were grown on P-AEF, and positive and negative controls were also prepared. Glucose was used as growth substrate in the positive control, and the negative control contained only the basal medium without added glucose or P-AEF. Four lactobacilli and three bifidobacterial strains, which can be commonly found in the human intestinal microbiota, were selected for the study, with the exception of *L. salivarius*, which is more often present in saliva than colon (Goldstein *et al.* 2015). Lactobacilli and bifidobacteria are considered beneficial for human health as they regulate the colonisation of pathogenic bacteria in the colon, help recover from diarrhoea and relief irritable bowel syndrome among many other things (Gibson and Wang 1994, Ouwehand *et al.* 2002, Gomes and Malcata 1999).

Using a lignin-rich substrate (10 g/L) did not inhibit the growth of lactobacilli and bifidobacteria, but instead the fraction enabled a longer growth of bifidobacteria than glucose (Figure 19). The colony counts of both strains of *L. rhamnosus* were almost equal in the presence of P-AEF and in the controls. The MRS medium of lactobacilli did not contain carbohydrates, but apparently peptides and amino acids in the medium (15 g/L) enabled growth also in the negative control. In the absence of glucose the growth was somewhat lower for *L. paracasei* and *L. salivarius*, indicating that they were more sensitive to the lower amount (in P-AEF sample) or complete lack (in the negative control) of fermentable

sugars than *L. rhamnosus*. However, the colony counts of bacteria grown on P-AEF were always above the negative control demonstrating that no inhibitory effects were induced by the lignin-rich growth substrate.

In the positive controls of *B. adolescentis* and *B. longum* the amount of viable cells was surprisingly low already after 3 days, but in the presence of P-AEF there was remarkable growth (10^7–10^8 CFU/mL). Also in the negative controls bifidobacteria grew almost equally well (10^7 CFU/mL). After 7 days of incubation, there was still significant growth (10^6–10^7 CFU/mL) in bifidobacterial cultures with P-AEF, whereas in the positive controls culturable cell counts were in the level of 10^2 CFU/mL. A similar protective effect on bifidobacteria has been previously observed with fermented oat bran preparations, but no single growth factor was identified in that study (Kontula *et al.* 1998). In the negative control the growth was in the level of 10^5 CFU/mL.

After lipid removal, P-AEF was low in carbohydrates (7 %), but especially rich in proteinaceous material (45 %) as the result of the partial precipitation of peptides after the proteolytic step. Peptide preparations, such as dairy protein hydrolysates and yeast extract, are known to stimulate growth of bifidobacteria (Gomes and Malcata 1999). Although the effects of cereal proteins on bifidobacteria are not as well known, presumably the peptides in P-AEF were the factor, which enabled the prolonged growth. The *Bifidobacterium* medium was free of carbohydrates but contained 25 g/L of peptides from different sources, which explains the growth in the negative control. However, the growth was very limited in the positive controls, except for *B. breve* at 3 d. This can be explained by a very rapid growth during the first incubation days induced by the easily available glucose in the medium. The high numbers of cells resulting from this exponential growth quickly depleted the nutrients from the medium, and therefore after 3 days there were no nutrients left and the culturable cell counts were thus very low. The bacteria growing on P-AEF or the basal medium without carbohydrates (negative control) grew more slowly due to the low amount or lack of easily available sugars, and thus the amount of nutrients originating from peptones in the medium was sufficient to maintain growth even after 7 days.

When dosed at low levels (1.25 %), lignin has been shown to increase the numbers of lactobacilli and bifidobacteria in broiler gut and to reduce the amount of *E. coli* in the faeces (Baurhoo *et al.* 2007b). A higher lignin dose (2.5 %) was highly efficient in preventing populating of *E. coli* in the cecum of *E. coli* challenged broilers, but on the other hand the numbers of lactobacilli and bifidobacteria were also reduced (Baurhoo *et al.* 2007a). It seems that lignin can have both growth-inducing and inhibitory effects on lactobacilli and bifidobacteria depending on the dosage. It is possible that at a higher dosage BSG lignin could have exerted antimicrobial properties as well. Another factor affecting the reactivity of lignin is the isolation method. In the case of the broilers lignin was isolated using the Alcell process, which is an organosolv process producing hydrophobic low-molecular weight lignin with higher purity and most probably with a higher content of free phenolic hydroxyl groups compared to the BSG fractions of the present study (Lora and Glasser 2002). Similarly, condensed tannins when isolated from fruits were inhibitory to the conversions of faecal microbiota but did not exert such effects in their natural state inside the fruits (Bazzocco *et al.* 2008, Aura *et al.* 2013). It is possible that the proteinaceous material in P-AEF, which seemed to be associated with lignin, could have acted as a barrier reducing the antimicrobial effects of lignin. Therefore, it seems likely that not only the lignin content but also the isolation method and association of lignin with cell wall polysaccharides or

proteinaceous material influence the potentially antimicrobial and inhibitory properties of lignin.

5.4 Methodological considerations

5.4.1 Analysis of lignin content in biomass

Accurately measuring the lignin content of non-wood biomass is not straightforward, and therefore BSG lignin contents reported in the literature (measured as Klason lignin) are likely to be exaggerated. Klason lignin is the insoluble residue of a biomass after acid hydrolysis, and its amount is measured gravimetrically. As demonstrated by Bunzel *et al.* (2011), Klason lignin from non-wood biomasses may contain protein, ash, cutin, suberin, fats and waxes, which all interfere with the analysis. However, measuring the contents of all the aforementioned components to correct the lignin content, would require much more work, and therefore it is not routinely performed. The Klason method is better suited for wood material as the contents of protein, ash and extractives in wood are sufficiently low so they do not induce a significant error in the Klason lignin determination

Hatfield and Fukushima (2005) have discussed the issue of accurately measuring lignin. They concluded that "there is no single method that is rapid, noninvasive, handles large sample numbers, and provides accurate measure of cell wall lignin contents". Instead, they recommend that one should always use the same method enabling comparison between samples rather than using different methods for different samples. In the present study, the Klason method (including acid-soluble lignin) was found to give exaggerated values for some samples (data not shown). In order to counteract this, lignin content was estimated by subtracting other major components (carbohydrates, protein, extractives and ash) from 100 %, as the analytical methods for those were believed to be more accurate than the Klason method. Furthermore, in addition to carbohydrates and lignin, faeces contain also other types of insoluble components, such as waxes, cutin and a considerable amount of bacterial mass, and thus the Klason lignin analysis would most likely yield unreliable results. Therefore, the amount of lignin degraded during the fermentation was not quantitated.

5.4.2 Use of the *in vitro* colon model

The *in vitro* colon metabolic model is a batch model, in which microbial conversions occur in anaerobic conditions for defined periods of time. Fresh human faeces are used as the inoculum to obtain a diversified microbial population. The transit time in colon is approximately 28 h (Arhan *et al.* 1981) and the fermentation time in the present study was set accordingly (24 h). Although the conditions of the model, such as temperature, pH, anaerobic atmosphere and the microbiota simulate the conditions of the proximal part of the colon, incubation in a batch model still provides different dynamics as compared to the continuous system of the colon.

In humans, before entering the colon food has been digested in the stomach and the small intestine leaving only the dietary fibre and other indigestible components to be fermented by the colonic microbiota. Therefore to obtain the most realistic results, samples should be similarly pre-digested with alimentary enzymes to remove the digestible food components before introducing the residue to the colon model (Aura *et al.* 1999). However, this was not performed in the present study. BSG has been exposed to hydrolytic enzymes

in mashing and contains only a residual amount of starch (ca. 1 %). The rest is mostly dietary fibre and protein (Table 1). A major part of the protein in BSG is encapsulated in aleurone cells (Figure 14a) and is thus not available to peptidases without decomposition of the cell walls. Moreover, the protein content of cereal materials after a pre-digestion with an upper intestine model can still be as high as 25 %, as demonstrated with wholemeal rye bread (Aura *et al.* 1999), suggesting that BSG protein level was not especially high for the fermentation. The protein (peptide) content was highest and most accessible in P-AEF. However, aromatic protein metabolites, such as indoles, could be distinguished from those of phenolic origin, and thus protein was not considered to interfere with the analysis of lignin metabolites. P-AEF was also rich in lipids but they were easily excluded from the phenolic metabolic profile.

Statistical significance of the responses of the metabolites compared to the faecal background was estimated using two-way ANOVA and denoted with asterisks. It is worth pointing out, that many metabolites with high FC values, such as guaiacyl compounds derived from P-AEF (Figure 16b) had only one time point statistically different from the faecal background, whereas some metabolites with low FCs had several of these points (e.g. 4-methylcatechol from BSG (Figure 16a)). The statistical significance is partially affected by the repeatability of the parallel measurements. As lignin is presumed to be a resilient substrate for the microbiota, it is not surprising that the repeatability between replicates was not very high. Another factor affecting the lack of statistical difference for the metabolites is most likely their low levels and origination of the same compounds also from the faecal background. If the background levels are high, the difference between the sample and the background is relatively smaller than if the same amount of a given metabolite was produced and compared against a low level in the background.

6. Conclusions and future prospects

The objectives of this thesis were to produce lignin-rich fractions from BSG and to study the interactions of the lignin in these fractions with colon microbiota *in vitro*. As BSG contains the outer parts of the grain, which are the protective layers, it is resistant to hydrolysis by cell wall degrading enzymes, if no pre-treatment is applied. The first specific aim was to evaluate the effectiveness of milling as a pre-treatment to enhance the enzymatic hydrolysis of carbohydrates. Of the different types of milling pre-treatments ball-milling was the most efficient increasing carbohydrate solubilisation from 23 to 45 % and thus notably improving the enzymatic carbohydrate digestibility. However, it was not effective enough to enable total solubilisation of BSG carbohydrates. Masuko milling was somewhat less effective than ball milling enabling 35 % enzymatic carbohydrate solubilisation, but on the other it was more convenient, as a larger amount of material could be processed and no drying was required. Enzymatic hydrolysis of carbohydrates was most probably restricted by several factors: steric hindrance, presence of lignin, cross-linking of arabinoxylans and lignin by (di)ferulates and possibly the absence of feruloyl esterases or other enzymatic activities. It is evident that a more efficient pre-treatment is required to obtain high sugar yields from BSG resulting in purer lignin in the residue.

The second specific aim regarding the fractionation was to produce different types of lignin-enriched fractions from BSG. Despite the limited extent of carbohydrate solubilisation and complications arisen from the co-solubilisation and co-precipitation of lignin and protein, two lignin-rich fractions were obtained by a three-step enzymatic hydrolysis. In addition, a separate alkaline extraction provided BSG-derived material with low ferulic acid content. Lignin contents in the fractions were 20–40 %, which can be considered high compared to common foods and thus sufficient for the colon model studies, although in only one of the fractions the lignin content was notably enriched (from 19 % in BSG to 40 %).

The third and fourth specific aims were to study the interactions of colon microbiota with lignin *in vitro*. More specifically, the aims were to assess if lignin is degraded and metabolised by colon microbiota and if lignin suppresses microbial conversions in the colon. A number of phenolic metabolites structurally similar to lignin were formed from BSG and the fractions by microbial conversions. Although the origin of the metabolites could not be determined exactly, it seemed very likely that several of the metabolites, including 3-syringylpropanoic acid, 4-methylcatechol, coniferyl aldehyde and dilignols originated from lignin. Based on these results it seems that lignin is not inert but is degraded and metabolised to a limited extent in the colon. Most likely microbiota is able to cleave the edges of lignin but a major part of the macromolecule remains intact. No notable suppression was detected based on the formation of short chain fatty acids from carbohydrates. Also, the presence of lignin did not inhibit the growth of lactobacilli and bifidobacteria, but instead the bacteria seemed to be capable of using the carbohydrates and peptides of the fraction

as carbon and energy sources. Thus it appears that lignin does not suppress microbial conversions in the colon and it seems not to be inhibitory to bacterial growth. However, this is likely to depend on the state of lignin as an isolated compound or bound in a matrix. Association of lignin with carbohydrates or proteinaceous material may reduce the possible antimicrobial effects of lignin, as free phenolic hydroxyl groups are believed to have a key role in the antimicrobial activities of lignin.

The hypotheses of this thesis were that lignin-rich fractions can be produced from BSG using enzymatic methods, and that lignin is at least partially metabolised by human intestinal microbiota. It can be stated that three different types of lignin-rich fractions were obtained from BSG (although one with chemical and not enzymatic methods) and that lignin-related metabolites were shown to be formed from all fractions and from the original BSG upon digestion by human gut microbiota. Although the analytical methods used did not allow quantitation and in some cases full identification of the metabolites, and the results provide qualitative rather than quantitative information on lignin degradation, it can be considered that both hypotheses were correct.

The results of the present study provide new information on the significance of lignin as part of dietary fibre. Based on the findings of the present study it appears that instead of being completely inert and resistant to digestion lignin would be a source of phenolic metabolites and thus an undervalued component of dietary fibre. As in foods lignin is part of the dietary fibre complex and most likely degraded only to a limited extent, it does not seem probable that consuming lignin as part of plant matrix even at high dosages would result in harmful effects.

In order to find applications for BSG outside of feed industry in the future, its fractionation should be further developed and optimised. Pre-treatment methods such as steam-explosion could improve the hydrolysis of carbohydrates e.g. for fermentable sugars. Furthermore, understanding the interactions observed between lignin and proteins could help find more efficient ways to recover these two components separately. Utilisation of dietary fibre and (undegraded) protein from BSG as food additives is another alternative to the hydrolytic fractionation approach.

Lignin is generally considered to be resistant to bioconversion by human colon microbiota, and thus its digestibility has been the attention of only a limited amount of studies, many of which date back several decades and have been carried out with methods that may not be the most accurate. The results of the present study suggest that more research is needed to further elucidate the role of lignin as a source of low molecular weight phenolic compounds, which potentially possess bioactive properties upon being absorbed in the colon. For instance, lignans have been shown to be released from lignin *in vivo*, and a number of studies provide information on their physiological functionalities.

Expensive materials, such as labelled synthetic lignin may be required to obtain indisputable results. In addition, as in gastrointestinal tract lignin remains as part of the cell wall matrix, a synthetic lignin material not bound to polysaccharides may not be the most representative sample. More information on how the surrounding cell wall polysaccharides affect the antimicrobial properties and the accessibility of lignin to intestinal microbiota is needed. The key metabolites derived from lignin should be identified in order to quantitate them, which would enable the determination of the extent of lignin degradation. Determining the bioactivities of the metabolites is also crucial to confirm the safety and potential beneficial impacts of lignin as part of food. An accurate method for measuring lignin con-

tent in food and faecal materials is an essential tool and requires adaptation of current lignin analytics to suit versatile biomaterials, such as BSG, which consist of a variety of compounds, many of which may interfere with the traditional Klason lignin analysis.

In conclusion, potential evidence of lignin metabolism by colon microbiota was provided in the present study. Furthermore, no suppression of carbohydrate fermentation or inhibitory effects on bacterial growth was observed. However, due to limitations in analyses and presence of interfering phenolic compounds in the fermentation, these results are preliminary and need to be confirmed with optimised methods. Further research to develop quantitative analysis methods for microbial lignin degradation and identification of the key metabolites would enable a better understanding of the effects of lignin as a component of dietary fibre.

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Effect of a milling pre-treatment on the enzymatic hydrolysis of carbohydrates in brewer's spent grain

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ABSTRACT

Millions of tonnes of brewer's spent grain (BSG) are annually produced worldwide as a by-product of the brewing industry. BSG has the potential to be a valuable source of food, chemicals and energy if cost-efficient fractionation methods can be developed. A 2-fold improvement in carbohydrate solubilisation could be achieved through the introduction of a milling step prior to enzymatic hydrolysis. Course and fine milled fractions were characterized by particle size distribution and light microscopy. Fine milling decreased particle size down to the micron level and this in turn improved the carbohydrate solubility yield by a multi-enzyme mixture from 23% up to 45%. Carbohydrate solubilisation could be further increased through the supplementation of this enzyme preparation with additional cellulases. The physical degradation caused by the milling also liberated soluble carbohydrates without the requirement of any enzymatic treatment.

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1. Introduction

Brewer's spent grain (BSG) is the most abundant side stream generated by the brewing industry. BSG is the insoluble residue that is separated from the mash before fermentation. In 2010, 1.69 billion hl of beer from barley was produced worldwide (FAO-STAT, 2010). Based on current technologies, approximately 15–20 kg of BSG is produced per every hectolitre of beer, resulting in annual production of 30 million tonnes of BSG worldwide. Thus BSG is a universally abundant, low-cost material and available throughout the year. Current use has been limited mainly to low-cost ruminant feed. Nevertheless, as BSG is rich in arabinoxylan, cellulose, lignin and protein, it could provide important ingredients and precursors for the food, chemical and energy industries (Mussatto et al., 2006), if cost-efficient fractionation methods are developed.

There have been many reports over the last 10 years about the potential utilization of BSG. As almost all components of BSG could be used in more valuable ways than just animal feed, methods to isolate the individual components of BSG while retaining their functionality are required. Several studies on solely enzymatic solubilisation of BSG using carbohydrate and protein degrading enzymes have been already reported (Faulds et al., 2008; Forssell et al., 2008; Treimo et al., 2009). The protein in BSG is fairly easily

degradable: up to 80% of protein can be removed from the insoluble biomass by a simple proteolytic treatment (Treimo et al., 2009). However, the carbohydrates are more resistant to enzymatic hydrolysis; the degree of solubilisation with one-step hydrolysis is only 28–30% (Forssell et al., 2008; Treimo et al., 2009). The resistance of BSG carbohydrates to hydrolytic enzymes is probably due to several factors, including inaccessibility of the carbohydrates to the enzymes caused by the cross-linking and substitution of the polymers in a complex, lignin-rich matrix. The xylan backbone in cereals is substituted with arabinose, xylose, galactose, glucuronyl and acetyl residues (Collins et al., 2010), which restrict the action of xylanase. Some of the arabinose residues are further substituted with ferulic acid either in mono or dimeric, and higher oligomeric forms (Bunzel, 2010). Ferulic acids form diferulate cross links between arabinoxylan molecules and also between arabinoxylan and lignin (Ralph et al., 1995), and such cross linking hinders the access of enzymes to the cell wall polysaccharides (Grabber et al., 1998). In addition, cellulase activity is inhibited by the presence of lignin through the non-specific binding of the enzyme to the polymer (Palonen et al., 2004), and this has been shown to negatively affect the hydrolysis of BSG (Mussatto et al., 2008).

Some attention has already been paid to the effects of possible pre-treatments of BSG prior to the enzymatic hydrolysis. Pre-treatments that decrease the particle size, open up the cell wall structures and reduce cellulose crystallinity make the biomass more accessible to enzymes and thus improve the digestibility (Hendriks and Zeeman, 2009). Previously, BSG has been pre-treated, for example, by coarse milling, using a 0.5 mm (Forssell et al., 2008; Beldman et al., 1987) or 1 mm (Treimo et al., 2009) sieve, but that

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is not sufficient to affect micrometer scale cell wall structures or crystallinity (Beldman et al., 1987). Other types of mechanical pre-treatments that have been attempted include extrusion, homogenisation with an Ultra Turrax (Beldman et al., 1987; Macheiner et al., 2003), autoclaving, microwave radiation (Macheiner et al., 2003) and thermo-mechanical pre-treatment using high pressure and temperature (Pierre et al., 2011). Nevertheless, only minor improvements were detected except for the thermo-mechanical pre-treatment, which significantly enhanced the enzymatic hydrolysis of BSG cellulose. In addition, a hot water treatment or autohydrolysis has been applied for the removal of hemicelluloses from BSG (Carvalheiro et al., 2004). However, the conditions of autohydrolysis cause conversion of some of pentoses to furfural (Carvalheiro et al., 2004), which can be undesirable in certain processes. Thus, the aim of this study was to improve the enzymatic solubilisation of BSG carbohydrates through the use of mechanical milling pre-treatments and to compare different carbohydrate degrading enzymes in carbohydrate solubilization.

2. Methods

2.1. BSG

BSG was obtained from the Sinebrychoff brewery in Kerava, Finland. A portion of it was hot air dried for dry milling experiments and the rest was stored undried at −20 °C.

2.2. Composition analysis

Klason lignin content was determined gravimetrically after removing extractives by heptane extraction and carbohydrates by acidic hydrolysis (Puls et al., 1985). The absorbance of acid soluble lignin was measured from the hydrolysate at 203 nm and calculated using absorptivity of 128 l/g. Carbohydrate composition was determined from the hydrolysate by HPAEC-PAD using a Dionex CarboPac PA-1 column in a Dionex ICS-3000 system (Dionex Corp., Sunnyvale, CA) with electrochemical detection. Analysis was run as described previously (Tenkanen and Siika-aho, 2000) with minor modifications; equilibration with 15 mM NaOH and isocratic elution with water. Starch and β-glucan contents were determined with the Megazyme Total Starch (amyloglucosidase/α-amylase method) and Mixed-linked β-glucan (McCleary method) kits, respectively, according to the manufacturer's instructions. Lipid content was determined by extraction of lipids with boiling petroleum ether in Soxhlet and quantifying the lipids gravimetrically after evaporating the solvent. Nitrogen content was analyzed using the Kjeldahl method (Bernard, 1992). Ash content was determined gravimetrically after burning the samples in a muffle furnace at 550 °C overnight. Phenolic acids were extracted from BSG by adding 2 M NaOH to dry sample and incubating for 16 h at room temperature. The samples were then acidified with 5 M HCl and extracted with ethyl acetate three times. The organic phase was collected and the solvent was evaporated. The dried residue was dissolved in 50:50 mixture of methanol and water. HPLC analysis was performed using Hypersil BDS C18 column and with UV detection at 324 nm. All analyses were carried out in duplicate.

2.3. Milling pre-treatments

Three types of dry milling techniques: pin disc milling, TurboRotor and ball milling, were studied, in addition to two types of wet milling techniques: a Masuko grinder and a microfluidizer. In the pin disc milling the dried material was milled at a rotor speed of 17,800 rpm (tip speed 180 m/s) and a feed rate of 10 kg/h, using a Hosokawa Alpine 100 UPZ-lb Fine impact mill with stain-

less steel pin disc grinders (Hosokawa Alpine AG, Augsburg, Germany). TurboRotor treated material was provided by Mahltechnik Görgens GmbH (Dormagen, Germany) and the milling was carried out using TurboRotor type G-55 with a rotor speed of 113 m/s and feed rate of 28 kg/h. The air flow was set to 1200 m³/h and the gap between the rotor and the inner liner was 3 mm. For ball milling, the dried BSG was first coarse milled with the pin disc mill as above. Ball milling was carried out with 500 g of stainless steel balls and rotation speed of 300 rpm, using a Fritsch Pulverisette 5 planetary ball mill (Fritsch GmbH, Idar-Oberstein, Germany) for 1 and 24 h under argon atmosphere. The mills used in the wet milling experiments were Masuko Supermasscolloider MKZA10–15 J, (Masuko Sangyo Co. Ltd., Kawaguchi-city, Japan) and Microfluidizer Processor M-110Y (Microfluidics, Newton, MA, USA). For wet milling experiments, BSG was suspended in tap water with 0.02% sodium azide to ensure microbiological stability. In the Masuko experiments, a 6% BSG suspension was passed through MKGA10-80 grinding stones seven times with a grinding speed of 1500 rpm. For microfluidizer experiments, the BSG suspension was first pre-treated with the Masuko grinder. A 5% suspension was passed through MKE10-46 grinding stones eight times and twice using MKGA10-80 grinding stones. The pre-ground suspension was passed through the microfluidizer six times using 1000 bar pressure and chamber sizes 100 and 200 µm. Particle size distributions after millings were measured using Coulter LS230 (Beckman Coulter, Miami, FL, USA) taking the average of two measurements. Both suspension and dry particles can be analysed with Coulter LS230.

2.4. Enzymatic hydrolyses

The enzymes used were Depol740L from *Humicola* sp. (Biocatalysts Ltd., Cefn Coed, Wales, UK), Celluclast 1.5 L from *Trichoderma reesei* (Novozymes, Bagsvaerd, Denmark), Novozym188 from *Aspergillus niger* (Novozymes, Bagsvaerd, Denmark) and *T. reesei* endoglucanase I and cellobiohydrolase I (purified according to (Suurnäkki et al., 2000)). The activities of the enzymes (Table 1) were determined as described previously (endoglucanase and β-glucosidase: (Bailey and Nevalainen, 1981), xylanase: (Bailey et al., 1992), β-xylosidase: (Poutanen and Puls, 1988), α-arabinosidase: (Poutanen et al., 1987), feruloyl esterase: (Forssell et al., 2008) and cellulase (filter paper assay): (Xiao et al., 2004)). Enzymatic hydrolyses were performed on a laboratory scale using 3% solids (w/w) in 50 mM ammonium acetate buffer (pH 5.0) containing 0.02% sodium azide at 50 °C for 5 h. Measurements were performed in triplicate. Depol740 (dosage of 5000 nkat of xylanase activity per 1 g of substrate) was used to study the effects of milling on enzyme activity. For cellulase addition experiments, the activities were 50 FPU/g substrate for Celluclast and 500 nkat of β-glucosidase activity per 1 g of substrate for Novozym188. For the pure EGI the dosage was 1000 nkat/g and CBH dosage was four

Table 1
Different hydrolytic activities in the commercial enzyme preparations. Nd = not detected, below detection limit. The left column indicates activity per ml of preparation and the right column the activity dosed for hydrolyses per g of BSG. Cellulase activity is given as FPU (filter paper units) per ml or per g.

Activity	Depol740L		Celluclast 1.5L		Novozym188	
	nkat/ml	nkat/g	nkat/ml	nkat/g	nkat/ml	nkat/g
Endoglucanase	1124	500	17,500	17,857	736	62
β-Glucosidase	386	172	430	439	5908	500
Xylanase	11,230	5000	14,900	15,204	2968	251
β-Xylosidase	95	39	959	979	103	9
α-Arabinosidase	35	16	230	235	478	40
Feruloyl esterase	25	11	Nd	Nd	Nd	Nd
Cellulase (FPU)	0.3	0.1	49	50	Nd	Nd

times the amount of EG based on protein content. The amount of solubilised sugars in the supernatant was determined with HPAEC-PAD after acidic hydrolysis as described in the composition analysis section above. Acid hydrolysis of the supernatants was carried out by adding 200 µl of 70% sulphuric acid into 4 ml of diluted (sample/water 1:3) sample solution and autoclaving the samples for 50 min at 121 °C. The sample volume was filled up to 10 ml with distilled water and the samples were filtered (pore size 0.45 µm) before analyzing them by HPAEC-PAD. The amount of reducing groups was determined by the DNS method (Bernfeld, 1955) scaled down to a 96 well microtiter plate.

3. Results and discussion

3.1. BSG composition

BSG is a heterogeneous material, which contains the insoluble outer layers of the grain, the husks and also residues of the starchy endosperm. Approximately half of the material (46.7%) was composed of carbohydrates, mainly arabinoxylan (22.9%) and glucans (20.9%), most of which was cellulose. Residual amounts of starch (2.8%) and mixed linked β-glucan (0.32%) were also present. Other major components were protein (23.3%), lignin (19.4%), lipids (7.8%) and ash (4.9%). The amounts of the main phenolic acids were 2.7 mg/g for ferulic acid and 1.3 mg/g for p-coumaric acid.

3.2. Effects of milling on structural characteristics of BSG

The original BSG contained up to 5 mm size particles (Fig. S1a, Supplementary information) and by different milling techniques the particles size could be decreased down to the micron scale (Fig. 1). Pin disc milling, TurboRotor milling and ball milling were carried out with dry BSG, whereas Masuko grinding and microfluidization were performed at high water content (>90%). The smallest particles were obtained by ball milling, and the pin disc mill was the least effective, mainly producing particles of 100–1000 µm particle size. With the ball milling, 50% of the particles were <5 µm and 95% had a diameter <33 µm. It has been noted previously (Van Craeyveld et al., 2009) that ball milling of cereal brans produced particles from low micrometer to high nanometer range, but the particles tended to aggregate and therefore the measured particle size values should be considered as apparent values. TurboRotor milling gave variable sized particles between 2 and 200 µm, with 80% of the particles between 10 and 100 µm in size. Wet milling produced more uniform particle size distributions than dry milling. With the microfluidizer 95% of the particles were

Fig. 1. Particle size distributions of BSG samples milled with different types of mills.

<30 µm, and with Masuko 95% of the particles were <40 µm in size (Fig. 1).

The original, undried BSG when diluted further with water prior to the milling sedimented rather quickly. After one pass through the Masuko grinder a notable increase in the swelling properties was observed. The wet-milled samples produced a homogenous slurry in the course of the milling process, whereas the dry milled samples sedimented rapidly when resuspended in water. Drying is known to cause fibrils of cellulosic fibers to aggregate and to decrease the pore size of the fibers (Häggkvist et al., 1998). The mechanism of this phenomenon, called hornification, is proposed to be an increased cross-linking between microfibrils due to additional hydrogen bonds, which are formed during drying but not broken when the fibers are resuspended in water (Minor, 1994). It was observed also for BSG that this phenomenon was not reversed by reintroducing water to the fibers, and the fibers in the dried BSG samples remained more aggregated than in the wet milled samples.

The reduction in particle size and changes in microstructure were also evident in the light microscopy images taken from the differently milled samples (Fig. S1, Supplementary information). In the light microscopy images aleurone cell walls and the lignified cell walls seemed to be more resistant towards milling as compared to the proteinaceous material. In the unmilled and pin disc milled BSG cellular structures were clearly visible. In the TurboRotor milled BSG, the aleurone cell particles had decreased in size but intact cells were still present. On the other hand, there were also very fine particles present. In the wet milled samples the particles looked quite different from dry milled having a long and thin shape and hardly any cellular structures visible. Both wet-milled samples had similar appearance in the microscopy images as well as in the particle size measurement. The uniformity of their size distributions seen in Fig. 1 correlated well with the particle sizes seen in the microscopy images. However, there was a difference in the overall color of the images. The Masuko ground sample had overall more red color than the microfluidized one, although otherwise they looked similar. In the microfluidizer treated sample the proteins may have been degraded to such small particles that they were not as well visible. Alternatively, the physicochemical properties of the BSG components could have been changed by the size reduction, if, for example, the treatment has caused the sample to heat up. Ball-milled BSG still contained cellular structures after 1 h, but after 24 h it had become a very fine powder. In the 24 h ball-milled sample separate particles could not be distinguished anymore, but colors blended together upon staining forming an overall brown color. Larger aggregates could be seen among the fine particles after 24 h, which could be due to the charges caused by the frictional electricity of the milling. In addition, the lipids in BSG could have been mixed with carbohydrates and protein forming a dough-like material. A similar aggregation phenomenon has been observed also with ball-milled cereal bran particles (Van Craeyveld et al., 2009). It is likely that these aggregates represented the larger particles (15–50 µm) seen in the size distribution (Fig. 1).

3.3. Effect of particle size on the solubilization of BSG carbohydrates

Milling had a notable impact on the particle size and carbohydrate solubility even without any enzymatic treatment (Fig. 2). This effect is especially pronounced with ball milled BSG where about 20% of the carbohydrates were soluble, compared to 1.7% in unmilled BSG. Total yield of soluble carbohydrates was further increased in the presence of Depol740. With a milling pre-treatment, the total carbohydrate solubilization in the presence of enzymes could be increased 2-fold, from 23% (unmilled) up to 45% (ball-milled 24 h). It is worth pointing out that the difference be-
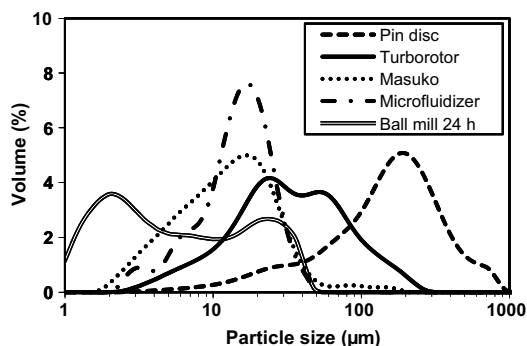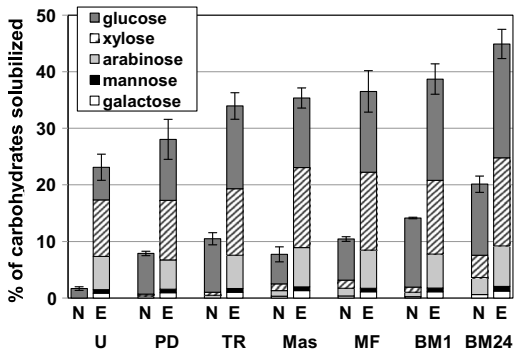
Fig. 2. Solubilization of carbohydrates by Depol740 from BSG samples milled with different types of mills. The solubilized carbohydrates also include other soluble carbohydrates and not only monosaccharides. All solubilized carbohydrates were hydrolyzed to monosaccharides by acid after the enzymatic hydrolysis to enable their feasible quantitative detection by HPLC. Abbreviations: U, unmilled BSG; PD, pin disc mill; TR, TurboRotor; Mas, Masuko; MF, microfluidizer; BM1, ball mill (1 h); BM24, ball mill (24 h); N, reference (no enzyme); E, with enzyme.

tween the enzymatic and non-enzymatic solubilization did not vary greatly between the milling techniques; the difference was 21.5% units for the unmilled sample and the largest difference, 26.1% units, was obtained with the microfluidizer treated sample. It appeared that milling had the most significant effect on carbohydrate solubility and that the hydrolytic efficiency of the enzymes was mostly unaltered. It is clear that the mechanical degradation of cell walls enabled the enzymes to access locations that were impenetrable in unmilled BSG.

From the milled samples, ball milling gave the highest total yield of solubilized sugars (45%) after the enzymatic treatment and pin disc milling the lowest (28%), as could be expected based on size distribution (Fig. 1) and microstructure (Fig. S1, Supplementary information). TurboRotor, Masuko and microfluidizer all produced yields in a similar range, between 34% and 37% carbohydrate solubilization. The latter methods are more practical and economical than ball milling, which requires a lot of time and can process only small amounts of sample at one time. In addition, Masuko and microfluidizer can process wet material, which saves time and energy when drying of BSG is not required. Previously, the solubilization of BSG carbohydrates with Depol740 using coarse milling as a pre-treatment has been reported to be 28–30% (Forssell et al., 2008; Treimo et al., 2009). The reference sample of unmilled BSG without any enzyme contained only small amounts of glucose and solubilized glucose-based oligosaccharides, which most likely originated from the hydrolysis of starch during mashing.

The monosaccharide profile of the solubilized sugars (Fig. 2) showed that in the unmilled BSG samples, Depol740 was able to degrade mainly arabinoxylan and much less of cellulose was hydrolyzed, which can be expected based on the high xylanase and relatively low cellulase (FPU) activities in Depol740 (Table 1). However, the improved yield in the milled samples was mostly caused by an increased cellulose hydrolysis, which was partially caused by solubilization through mechanical degradation, as seen in samples with no enzyme. The increase in xylose and arabinose solubilization due to milling was not as great as with cellulose, although enhancement was seen there as well. Without milling, Depol740 was able to solubilize only 13% of all glucans but with a milling pre-treatment this could be increased more than 3-fold, up to 45%. Arabinoxylan (AX) solubilization was higher already without any milling treatment, 32%, and it was increased to 46%

by milling. Thus, milling had a stronger effect on cellulose than xylan degradability. In fact, fine milling is known to reduce the crystallinity of cellulose increasing the portion of amorphous regions (Zhao et al., 2006). Furthermore, crystallinity has a negative impact on the initial rate of enzymatic hydrolysis of cellulose (Hall et al., 2010). Therefore, the decrease in cellulose crystallinity caused by the milling treatments most likely explains the enhancement of cellulose hydrolysis also with BSG. In addition, it has been reported that ball milling increases the amount of water-extractable AX in cereal brans by breaking covalent bonds inside arabinoxylan molecules (Van Craeyveld et al., 2009), and this could enhance AX solubilization. The ratio of solubilized arabinose to solubilized xylose (A/X) after enzymatic treatment decreased from 0.59 in the unmilled sample to 0.46 in the 24 h ball-milled sample, indicating that milling is more likely to degrade covalent bonds in the less substituted parts of xylan. This observation was supported by the decrease in A/X also in the reference samples, in which the A/X of solubilized sugars decreased from 1.0 (pin disc milled sample) to 0.76 (ball milled sample) with a more powerful milling. The low-substituted xylan may be able to form crystalline structures on the surfaces of cellulose fibrils and in that way it could be more susceptible to the impact of grinding, whereas the highly substituted xylan is more flexible avoiding the degradation caused by the grinding energy.

The effect of treatment time on the hydrolysis was also investigated. The reaction rate of the carbohydrate digestion slowed down depending on the pre-treatment already after 3–5 h reaching at that time approximately 80% of the yield obtainable in 24 h (Fig. 3). BSG is not microbiologically stable in the conditions of the hydrolysis reaction (Robertson et al., 2010), and a limited hydrolysis time is required, unless antimicrobial agents are used. Therefore, the short incubation (5 h) time is adequate as no biocide addition is needed. In addition, Fig. 3 shows that not only does the milling improve the final yield but also the initial reaction rate is higher due to the increased amount of water-soluble carbohydrates. Most likely, the cell walls of inner parts of the grain, especially the aleurone cells, are the easiest for the enzymes to digest and they are therefore first hydrolyzed, whereas the lignin-containing outer layers and the husk are more difficult to degrade. It has been shown on wheat bran that xylanase cannot diffuse into testa and pericarp cell walls due to their narrow pore size, but cell wall disassembly is first required for xylanase to penetrate into these tissues (Beaugrand et al., 2005). In addition, the enzymatic digestion of wheat bran is initiated from the aleurone cell walls and proceeds from there towards the inner parts of the bran. The
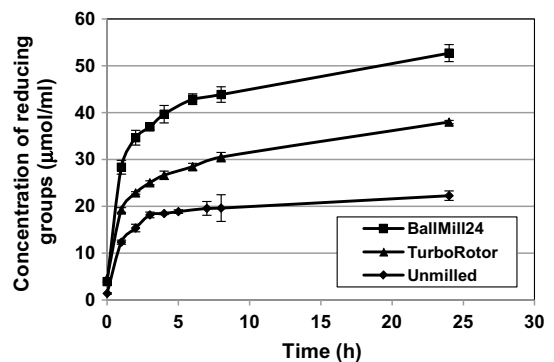
Fig. 3. The concentration of reducing groups produced in the enzymatic hydrolysis reaction as a function of time.

lipophilic layers present in inner bran also create a barrier for enzymes (Beaugrand et al., 2005). These observations are in line with the results obtained in this study; fine milling enhances the enzymatic solubilization due to the physical breakage of cell walls, which enables enzymes to more easily access the cell wall polymers.

3.4. BSG solubilization by different enzyme mixtures

The impact of different enzyme combinations on the hydrolysis of Masuko ground BSG was further studied. Celluclast 1.5 L, containing various cellulase and hemicellulase activities, and Novozym188, containing mainly β-glucosidase activity, were added to the hydrolysis reactions with Depol740. This was compared to Depol740 supplemented with purified *T. reesei* EG I and CBH I. Saturating cellulase levels were used (Table 1), so that the maximum possible solubility could be obtained.

The solubilization of different sugars is shown in Fig. 4. After grinding with the Masuko mill, 8% of carbohydrates were solubilized in the absence of any enzyme. The highest yield 48% was obtained with the combination of Depol740 and Celluclast. The additional cellulase (FPU) activity clearly boosted the ability of Depol740 to solubilize more carbohydrates. Adding Novozym188 in addition to these did not further improve the yield. Celluclast alone was found to be fairly efficient solubilizing 42% of all carbohydrates. Celluclast has also been previously found to work effectively on BSG cellulose after a thermo-mechanical pre-treatment (Pierre et al., 2011). Compared to Depol740, Celluclast was almost equally efficient in the release of xylose and arabinose and more efficient in cellulose hydrolysis. As seen in Table 1, Celluclast lacks the detectable feruloyl esterase activity that Depol740 has. Because of the ferulate substitution on arabinoses and the diferulic cross-linking between hemicelluloses and between hemicelluloses and lignin, it could be assumed that Depol740 would be more efficient towards arabinoxylan hydrolysis but such result was not seen in this study. Certain feruloyl esterases also display deacetylating activity (Faulds et al., 2011) and Depol740 contain acetylesterase activity (Faulds et al., 2008). Thus, these enzymes could act on the acetyl substitutions on xylan and thus provide more suitable hydrolysis sites for the xylanases present in the enzyme cocktails, but nevertheless, only minor difference in the xylan hydrolysis was observed between Celluclast and Depol740. The feruloyl esterase in Depol740 is known to be more efficient towards monomeric ferulic acid than diferulates (Faulds et al., 2002), and so apparently the diferulates, which form the crosslinks, were not as effectively cleaved as would have been needed for more complete cell wall degradation.
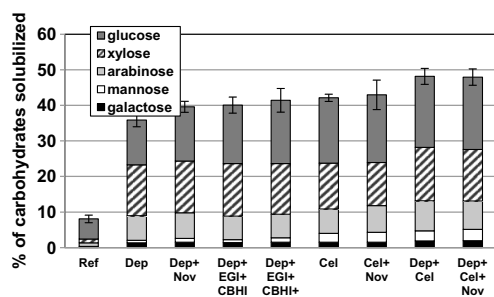
The addition of monocomponent cellulases (EG I and CBH I) resulted in a lower yield of sugar release than with Celluclast, indicating that additional enzymes present in this cellulase preparation are required to improve the extent of BSG solubilization. The enzyme dosages used were so high that increasing them would not necessarily increase the yield. The overall differences in efficiencies between the enzyme cocktails in this study were not very large. As only half of the carbohydrates were solubilized, it indicates that the enzymes used in this study cannot easily access the remaining polysaccharides in the recalcitrant residue.

4. Conclusions

In conclusion, a milling pre-treatment significantly decreased BSG particle size leading to a 2-fold improved carbohydrate solubilization. Furthermore, the yield was influenced by the choice of the mill. Wet milling can process larger amounts of BSG without the energy consuming step of drying, and would thus have potential for further scaling-up of the process, as cost-efficiency must also be considered. Cellulose solubility especially was improved by milling, possibly by decreasing the crystallinity. However, the high amount of insoluble carbohydrates remaining indicated that BSG cell walls are a complex structure, which the hydrolytic enzymes have difficulties penetrating.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2012.04.043.

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Fig. 4. Solubilization of carbohydrates from Masuko milled BSG by different enzyme cocktails. Abbreviations: Ref, no enzyme; Dep, Depol740; EGI, endoglucanase I; CBHI, cellobiohydrolase I; Nov, Novozym188; Cel, Celluclast 1.5.

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Pre-hydrolysis with carbohydrases facilitates the release of protein from brewer's spent grain

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HIGHLIGHTS

- BSG has the potential to be a valuable source of food, chemicals and energy.
- Entrapped protein was released by the combination of mild alkali and protease.
- Initial carbohydrase treatment breaks down cellular structures around the protein.
- 53% of the protein was solubilised after carbohydrase treatment by buffer alone.

ARTICLE INFO

ABSTRACT

Brewer's spent grain (BSG) is the most abundant side-stream from brewing. It is food-grade being rich in dietary fibre and protein and thus having potential as their source for both food and non-food applications. Initial treatment of milled BSG with a carbohydrase cocktail from *Humicola insolens* significantly enhanced the subsequent solubilisation of protein from the residual biomass. When treated with an alkaline protease, 76% of BSG protein was solubilized, whereas the yields were significantly lower with neutral or acidic proteases. In alkaline conditions significant amount of protein (53%) as predominantly low molecular weight protein was solubilized even without any protease addition. The degree of protein solubilisation was influenced by the time of exposure of modified BSG to the alkaline environment. The non-enzymatic protein solubilisation was, however, only observed when BSG had been initially treated with the carbohydrase, suggesting the protein is surrounded by cell wall polysaccharides restricting its initial release.

1. Introduction

Brewer's spent grain (BSG), the most abundant by-product generated by the brewing industry, is the insoluble residue of the malts that is separated from the mash before fermentation. In 2011 1.8 billion hectoliters of beer from barley was produced worldwide (FAOSTAT, 2011) With current technologies approximately 15–20 kg of BSG is produced per every hectolitre of beer, which results in an annual production of over 30 million tonnes of BSG worldwide. BSG is an abundant, low-cost biomass, rich in carbohydrates (arabinoxylan and cellulose), lignin and protein, and so could provide important ingredients and precursors for the food, chemical and energy industries (Mussatto et al., 2006), if cost-efficient fractionation and separation methods are developed. The current use of BSG is mainly ruminant feed. It has been demonstrated that BSG can be enzymatically separated into carbohydrate-rich and protein-rich soluble fractions (Forssell et al., 2011; Robertson et al., 2011) and enzyme-aided solubilisation is enhanced upon particle size reduction (Niemi et al., 2012a).

BSG contains between 10% and 24% of protein on a dry weight basis (Robertson et al., 2010). During malting, the barley proteins are partially degraded to amino acids and small peptides by the endogenous peptidases, but there is very little detail about the residual proteins present in BSG. Barley proteins have previously been classified by their sequential extractabilities by the procedure developed by Osborne (1924). Albumins, which are mainly en-

zymes, are water-soluble. Globulins may be enzymes or storage proteins and are extractable by salt-solutions. Hordeins, which are the main storage proteins in barley, are alcohol-soluble, and glutelins, which are structural proteins, may be extracted with dilute acid or alkali or with detergents (Celus et al., 2006). Mechanical protein separation from biomass (milling, pressing, etc.,) combined with an alkaline extraction has been studied to separate proteins from different plant biomasses (Chiesa and Gnansounou, 2011). However, if the biomass had been previously dried and not used fresh, the extraction usually requires very high pH (10–12) and elevated temperatures (40–90 °C). This can lead to lower protein quality in the extract and impaired digestibility. In addition, despite the harsh extraction conditions, the protein yields may be as low as 20–30% (Chiesa and Gnansounou, 2011).

The protein component in BSG has potential to be used as a source of human and animal dietary protein, if it can be extracted without too much deterioration in its techno-functional properties. Direct enzymatic treatment of BSG with commercial bacterial proteases has been used to extract up to 77% of the total protein at pH 8 as low molecular weight peptides (Treimo et al., 2008). A two-stage hydrolysis protocol with carbohydrases and proteases also resulted in the almost complete removal of protein from BSG (Forssell et al., 2011). Enzymatic protein extraction yields of 90% from distiller's grain (Cookman and Glatz, 2009), 90% from soybean meal and 50–80% from microalgae and rapeseed meals (Sari et al., 2013) have also been recently reported under alkaline conditions. In this paper, the effect of pH and the type of protease used in the extraction of protein and peptides from milled BSG have been explored.

2. Methods

2.1. Materials

Brewer's spent grain (BSG) was obtained from Sinebrychoff brewery (Kerava, Finland) directly from the mashing process and stored at −20 °C. Before enzymatic hydrolyses, the BSG was thawed, dried using hot air and milled to a fine powder using an Alpine mill with a 0.3 mm sieve (Hosokawa Alpine AG, Augsburg, Germany). The composition of dried BSG was 42.2% of carbohydrates (arabinoxylan 23.1%; glucans 17.1%), 19.4% of lignin, 22.8% of protein, 11.0% of lipids and 4.7% of ash (Niemi et al., 2012b). *Bacillus licheniformis* Alcalase 2.4L was obtained from Novozymes (Bagsvaerd, Denmark), *Humicola insolens* Depol 740L and *Carica papaya* Promod 144GL from Biocatalysts Limited (Cefn Coed, Wales, UK), and *Aspergillus niger* Acid Protease A from Amano Enzyme USA Co. Ltd. (Elgin, Illinois, USA).

2.2. Composition analysis

Extractives, which mainly include lipids in BSG (Niemi et al., 2012b), were removed by heptane extraction in a Soxhlet apparatus and quantitated gravimetrically. After the heptane extraction, carbohydrates were hydrolyzed with acid. 3 ml of 70% H_2SO_4 was added to 300 mg of extracted material and incubated at 30 °C for 1 h. After adding 84 ml of water the material was autoclaved at 121 °C for 50 min. After filtration, carbohydrate composition was determined from the hydrolysate by HPAEC-PAD using a Dionex CarboPac PA-1 column in a Dionex ICS-3000 system (Dionex Corp., Sunnyvale, CA) with electrochemical detection. Analysis was run as described previously (Tenkanen and Siika-aho, 2000) with minor modifications; equilibration with 15 mM NaOH and isocratic elution with water. Klason lignin content was determined from the acid-insoluble residue. The absorbance of acid-soluble lignin was measured from the hydrolysate at 215 and 280 nm and calculated according to Goldschmid (1971). Nitrogen content of solid samples

was analysed using the Kjeldahl method. To degrade the proteins in the sample, 11 ml of 98% sulphuric acid and 8 ml of 30% hydrogen peroxide were added to 1 g of sample in the presence of a catalyst containing 5.0 g K_2SO_4, 0.15 g $CuSO_4 \cdot 5H_2O$ and 0.15 g TiO_2. The reaction mixtures were burned at 420 °C for 35 min. The formed ammonium sulphate was converted to ammonium hydroxide by adding 32% NaOH and water in a Kjeltec 2300 system (Foss Tecator, Höganäs, Sweden). The ammonium hydroxide was distilled and reacted with boric acid to form ammonia, which was titrated using 0.1 M HCl. The nitrogen content was calculated from the amount of HCl consumed, and the nitrogen content was converted to protein by multiplying with a factor of 6.25. Soluble protein in the hydrolysates was determined with Lowry method using the Bio-Rad DC Protein Assay. Bovine serum albumin (BSA) was used as the standard protein.

2.3. Enzymatic digestions

2.3.1. Depol 740L digestion

Milled BSG was treated with Depol 740L (100 U of xylanase-equivalent activity per g of dry matter) in distilled water as a 10% (w/v) slurry, for 5 h at 50 °C with continuous stirring. The liquid phase was separated from the residue by centrifugation at 4000 rpm for 30 min, and the supernatant was frozen. The insoluble residue was washed with distilled water to remove any residual enzyme and solubilized carbohydrates. The resultant material was termed rBSG-D.

2.3.2. Protease digestion

The Depol 740 treated BSG residue (rBSG-D) was treated with different proteases, Alcalase 2.4L, Promod 144GL and Acid Protease A (10 U of protease activity/g). The hydrolysis was carried out at 10% (w/v) solids content, at 40 °C for 4 h with continuous stirring. The pH of the reaction was adjusted to the optimum of each enzyme preparation: pH 9.5 (50 mM sodium carbonate buffer) for Alcalase, pH 6.5 (50 mM McIlvaine's buffer) for Promod 144 and pH 3.5 (50 mM sodium citrate buffer) for Acid Protease A. Controls with no enzyme addition were also prepared. For the Alcalase reaction pH had to be readjusted to 9.5 with NaOH after mixing all the reagents, as the addition of BSG notably lowered the pH. The respective control sample was treated similarly. Reactions were stopped by centrifugation at 4000 rpm for 30 min in a pre-cooled (4 °C) centrifuge, and the supernatants were collected and stored at −20 °C. The residues were washed with distilled water, and the solid residues were collected and lyophilised. After lyophilisation the amount of remaining solids were measured to determine to total solubilisation of each reaction.

2.3.3. Effect of pH on non-enzymatic protein solubilization

The effect of pH on BSG and rBSG-D solubilisation was measured by incubating the samples at different pHs from 3.5 to 10 for 4 h at 50 °C. The buffers used were sodium citrate (pH 3.5 and 4.0), McIlvaine's buffer (pH 5.0, 5.5, 6.0 and 6.5), sodium phosphate (pH 7.0, 7.5 and 8.0), Tris–HCl (8.5), and sodium carbonate (pH 9.0, 9.5 and 10.0). After incubation solids and liquid were separated by centrifugation. The total solubilisation was determined from the amount of recovered residue, and the amount of solubilized protein was measured from the liquid phase with the Lowry method described above.

2.3.4. Protein solubilization as a function of time

rBSG-D (900 mg) was suspended in pH 9.5 sodium carbonate buffer with a solids 10% (w/v) solids content and Alcalase (10 U/g of solid material) was added. Controls consisted of rBSG-D suspended in buffer only. The reactants were mixed and incubated at 40 °C with continuous stirring. Aliquots (450 μL) were removed

every 30 min and the amount of protein solubilised determined using the Lowry method described above.

2.3.5. Effect of temperature on non-enzymatic protein solubilization

rBSG-D (900 mg) was suspended in pH 9.5 sodium carbonate buffer with a 10% (w/v) solids content. The obtained slurries were mixed and incubated at 30, 40, 50, 60 and 70 °C with continuous stirring. Aliquots (450 µL) were removed every 30 min, centrifuged for 5 min at 14,000 rpm, and the amount of solubilised protein was determined from the supernatants using the Lowry method described above.

2.4. SDS–PAGE

Protein electrophoresis was done in denaturing conditions by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli, 1970). Samples (0.5 mg/ml estimated based on the amount of soluble protein) were run on a Bio-Rad DC Criterion TGX Stain-Free Precast 4–20% gradient gel (Bio-Rad Laboratories, Inc., California, USA) together with protein standards (Precision Plus Protein Standard, Bio-Rad Laboratories, Inc., California, USA). The gel was visualized with a Criterion stain-free imaging system (Bio-Rad Laboratories Inc., Hercules, CA) where protein visualization is based on UV-light driven reaction of tryptophan residues in the presence of trichlorocompounds (Kazmin et al., 2002).

2.5. Microscopy imaging

Light microscopy was carried out as described previously (Van Craeyveld et al., 2009). In brief, samples were embedded in hydroxyethyl methylacrylate matrix from which 2 µm thick sections were cut. Prior to imaging with the microscope, the sections were treated with chemical dyes Calcofluor and Acid Fuchsin to enable visualization of different components of BSG. Acid Fuchsin stains protein red, and Calcofluor stains β-glucan bright blue. The autofluorescence of lignin and other phenolics are seen as yellow and green.

3. Results and discussion

3.1. Solubilization of BSG with Depol 740

Depol 740 solubilised 17% of the Alpine-milled BSG (Table 1). Most of the solubilized material was carbohydrates but small amounts of protein, lignin and extractives were also released. Depol 740 is a mixture containing several cell wall degrading activities, such as xylanase, arabinosidase and cellulases, and but also some feruloyl esterase and protease activity (Niemi et al., 2012a; Forssell et al., 2008). A dosage of 5000 nkat of xylanase activity per g of BSG has been previously applied in BSG solubilisation with Depol 740 (Niemi et al., 2012a; Forssell et al., 2008), as arabinoxylan is the most abundant polysaccharide in BSG. However, the

addition of xylanase activity above 100 U (or 1700 nkat) per g of BSG resulted in no significant increase in the solubilisation yield (results not shown). Therefore, the amount of Depol 740 equivalent to 100 U of xylanase per g of BSG was applied in this study. BSG is also rich in cellulose and Depol 740 contains some endoglucanase activity, although the total cellulase activity (measured as filter paper units) is quite low (Niemi et al., 2012a; Forssell et al., 2008).

The recalcitrant nature of BSG carbohydrates has been previously addressed, and the maximal carbohydrate solubilisation with current enzymatic methods accounts for approximately 50% of BSG carbohydrates (Niemi et al., 2012a). As BSG contains the hulls and the outermost layers of the grains, which are meant to protect the nutrients inside the grain, it is not surprising that these structures are not easily degraded by fungal enzymes. In addition, the hydrophobic cuticular layers of the grains may create a physical barrier for the hydrolytic enzymes.

3.2. Effect of pH on biomass and protein solubilization

3.2.1. Solubilities of different components

Three proteases with different pH optima and a broad substrate specificity were selected to determine the influence of the type of protease in the solubilisation and digestion of proteins from rBSG-D; Alcalase (subtilisin), an alkaline protease from *B. licheniformis*, Promod144 (papain), a neutral protease from the papaya fruit, and Acid Protease A, an acidic protease from *A. niger*. Alcalase treatment on intact BSG or after carbohydrate treatment has previously been examined in several studies (Forssell et al., 2011; Robertson et al., 2011; Treimo et al., 2008, 2009; Faulds et al., 2008, 2009). Alcalase proved to be the most efficient protease solubilising 76% of proteins from rBSG-D within 4 h, whereas the solubilization obtained with Promod144 and Acid Protease was only 21% and 30%, respectively. Together with the Depol 740 treatment, the amount of protein solubilization after Alcalase hydrolysis was 86%. From previous studies, it is known that Alcalase effectively dissolves BSG at high pHs (9–10) (Faulds et al., 2008). However, the previous focus has mainly been on the total biomass solubilization instead of analyzing the individual components, and therefore a more detailed composition analysis was carried out in this study.

The total solubilization of the biomass was also more efficient at high pH (Table 1). Alcalase treatment at pH 9.5 solubilized 35% of BSG, whereas with the other two proteases the degree of solubilization was only 14%. In addition to protein, the high pH affects the release of lipids and lignin (Niemi et al., 2012b). The extractive content of the residue after Alcalase treatment (rBSG-D/Alc) was 5% accounting for 80% solubilization of all extractives that were present in the initial BSG. With Acid Protease and Promod the solubilizations were 11% and 23%, respectively. A similar behaviour was observed with lignin as well; after the Alcalase treatment, 40% of lignin had been removed compared to the 14% and 16% removal after the Acid Protease A and Promod treatments, respectively. Carbohydrates were mostly not affected by the proteases or

Table 1
Solubilization of BSG by different treatments, composition of the residues and extraction of protein from BSG.

Material	Solubilization of BSG (%)	Recovered residue (g)	Components in the residue (g)						% Of initial BSG protein
			Xylose	Arabinose	Glucose	Lignin	Extractives	Protein	
BSG		100	16.5	6.6	17.1	19.4	11.0	22.8	100
rBSG-D	17.1	82.9	12.2	4.3	16.8	18.8	9.6	20.5	89.9
rBSG-D/AcP	13.7	69.3	11.1	4.0	13.9	16.6	9.8	13.7	60.2
rBSG-D/P144	13.5	69.4	10.5	4.6	13.6	16.3	8.5	15.7	68.8
rBSG-D/Alc	34.8	48.2	10.5	3.5	12.9	11.7	2.2	3.3	14.4
rBSG-D/pH3.5	8.2	74.8	11.8	4.1	14.7	18.3	8.7	20.8	91.2
rBSG-D/pH6.5	8.7	74.3	10.6	3.8	13.6	18.7	8.5	17.5	76.9
rBSG-D/pH9.5	30.7	52.3	10.3	3.7	12.8	13.0	0.8	8.4	36.8

buffers, but a small decrease in the carbohydrate content was seen with all samples. This could have been caused by solubilization of other components, which had carbohydrates attached to them, for example proteins or lignin.

To verify if the protein solubilization was due to the protease alone or if it was enhanced by pH, control samples without proteases were prepared. The protein solubilization in buffer was highest, 53%, at pH 9.5, 13% at pH 6.5 and no protein solubilization occurred at pH 3.5 (Table 1). It was clear that pH was a significant factor in BSG protein solubilisation, and solubility was increased at higher pH. However, the maximal solubilization required the additional action of a protease. To confirm that the solubilisation in the control samples was not due to residual protease activity from Depol 740, the experiment was repeated with boiling rBSG-D prior to the addition of Alcalase, and the same results were obtained, thus excluding the possibility of residual Depol 740 protease activity.

3.2.2. Solubility of different protein classes and effect of pI

The protein content in barley varies from 8% up to 15% of the dry weight (Jadhav et al., 1998). During malting the amount of peptides and free amino acids increases as the endosperm proteins are partially degraded by endogenous proteases to generate nutrients for the germinating embryo (Jones and Budde, 2005). Although the malting process results in approximately 10% material losses, the protein content of malts does not significantly differ from that of unmalted barley (Celus et al., 2006). In mashing approximately 22–26% of malt proteins are dissolved in the wort (Jones and Budde, 2005). 90% of proteins that remain in BSG are water-insoluble hordeins and glutelins (Celus et al., 2006). Hordeins are the main storage proteins in barley, and glutelins act as structural components of the cells. Mashing causes disulphide bridge formation in hordeins, and their extraction requires a reducing agent in addition to a high alcohol content in the extraction solvent (Celus et al., 2006). Therefore it seems unlikely that hordeins would be solubilized by alkaline conditions without protease activity. As glutelins are alkali-soluble (Linko et al., 1989), these are the proteins most likely to be affected by the high pH. The remaining 10% of BSG proteins consist of water- and salt-soluble albumins and globulins (Celus et al., 2006). Part of them may have been already solubilized during the Depol 740 treatment, but possibly some were also solubilized in the second step.

The improved solubility in alkaline pH was most likely affected by the pI of the proteins, which is the pH at which the net charge of the protein is zero. As the charged functional groups improve water-solubility, the further away from the pI the reaction pH is, the better the solubility is due to the increasing amount of charges. It has been shown that water-solubility of barley proteins is lowest from pH 4 to 6 suggesting that the pI of the proteins is in that range, and the solubility is highest from pH 10 to 11 (Yalçın and Çelik, 2007). Although mashing can induce some changes in the proteins, a similar pH effect was observed for BSG proteins too. Addition of a protease further improved the solubility by decreasing the molecular weight (MW) of the proteins.

3.2.3. Molecular weights of solubilized proteins

The MW of enzymatically solubilized proteins were mostly lower than 15 kDa (Fig. 1, lanes 1–3), but also some very light bands were seen at above 250 kDa. The results were very similar for Promod and Alcalase, but for Acid Protease fewer bands were observed and the intensity of the bands was somewhat lower except for the band above 250 kDa. The MW of alkali-soluble proteins (pH 9.5, lane 6) were somewhat higher than with Alcalase, but mostly under 20 kDa and some thin bands at approximately 25 kDa. It appeared that alkaline conditions were able to solubilize only low MW proteins. With pH 3.5 and pH 6.5 controls (lanes 4
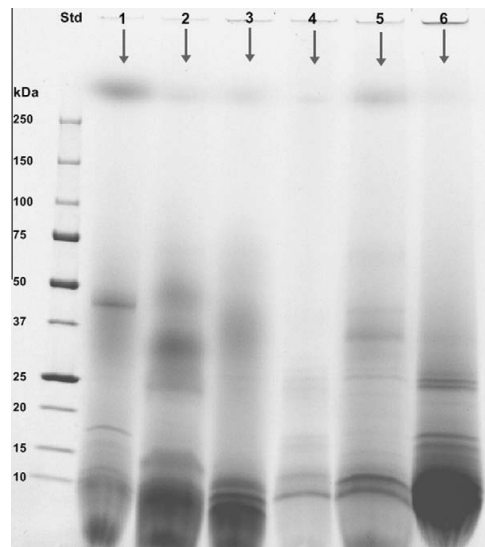
Fig. 1. Molecular weights of proteinaceous material solubilized from rBSG-D by different proteolytic treatments (1–3) and the respective controls (4–6). (1) Acid Protease A, (2) Promod144, (3) Alcalase, (4) pH 3.5 control, (5) pH 6.5 control, (6) pH 9.5 control.

and 5) only two bands between 10 and 15 kDa were observed. Especially for pH 3.5 control (lane 4) the band intensities were low as could be expected from the low solubilization results. The observed bands on the gel were generated by the action of the proteases on rBSG-D and not by interference by the protein in the enzymes used, as this amount was too low to be visible on these gels (results not shown).

3.2.4. Effect of pH on non-enzymatic solubilization

Depol 740 treatment significantly increased non-enzymatic solubilization of protein as well as solubilisation of other components from BSG (Fig. 2). Total biomass solubilization was improved over the whole pH range (3.5–10.0) after the carbohydrate digestion (Fig. 2a). At lower pHs the total solubilization increased from 2–3% to 5–7% and at higher pHs from 3–5% to 10–15% (Fig. 2a). The lipids were released due to de-esterification and saponification caused by the high pH, and the water-solubility of lignin was also improved by the alkalinity (Niemi et al., 2012b). Polysaccharides, such as arabinoxylan, are not extractable from BSG with sodium carbonate but require a stronger base such as KOH (Mandalari et al., 2005). Carbohydrates may also be susceptible to acidic environments, but in this study the conditions were not harsh enough to cause any notable change in these polymers. A significant amount of protein was released under alkaline conditions alone without the addition of any protease (Fig. 2b). While there was no significant difference at acidic pH, at pH 8 the difference in protein solubilization between initial BSG and rBSG-D was already five-fold and at pH 10 it had increased to seven-fold. This demonstrated that an initial carbohydrate digestion is necessary for enhanced protein solubilization from BSG. The high pH may also loosen the cell wall matrix making it more accessible for enzymes. It has been previously shown that over 60% of BSG is extractable with a strong base (Mandalari et al., 2005). The notable difference in protein solubility between acidic and alkaline conditions may also explain why Alcalase was so much superior to Promod and Acid Protease.
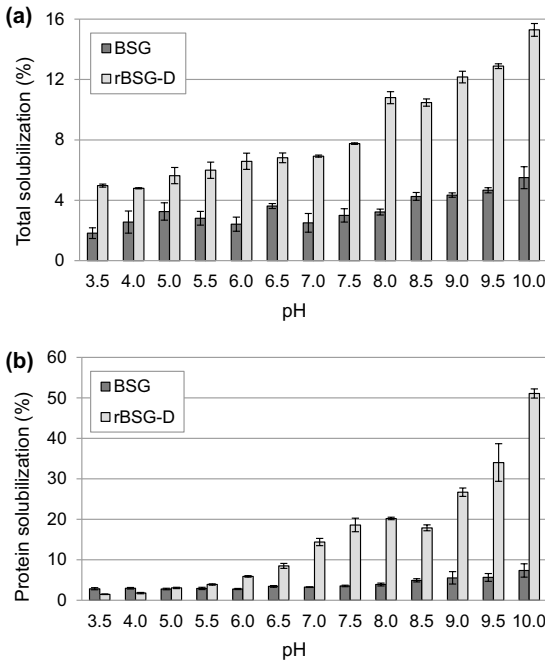
Fig. 2. Non-enzymatic biomass solubilization; (a) and solubilization of protein; (b) from BSG and rBSG-D at different pHs.

Fig. 4. Effect of temperature on non-enzymatic protein solubilization from rBSG-D at pH 9.5.

amount of protein was solubilized in the pH 9.5 control sample, 37% of all protein remained insoluble, as seen by the level of red color in Fig. S1c. It should also be pointed out that where there were undigested aleurone cells left after the Depol 740 treatment, it seemed that the protein inside of them had remained intact despite the alkalinity and the protease (Fig. S1c and d), suggesting that the carbohydrates truly are a barrier for both enzymatic and mild chemical extraction of protein.

3.3. Effect of time and temperature on protein solubilization from BSG

Protein solubilization was almost linear starting from the first 0.5 h and up to 7 h (Fig. 3). At 0.5 h two thirds of the solubilized protein resulted from enzymatic digestion, whereas at 7 h the enzymatic solubilization accounted for only 50%. The enzymatic cleavage of peptide bonds produces carboxylic acid groups, and this quickly decreased the pH of the reaction. Most likely this negatively affected the protease, which has an alkaline pH optimum (Faulds et al., 2008), as pH was not readjusted during these reactions. The reaction rate could possibly be increased, if pH was maintained constant during the hydrolysis. In the control, pH did not change significantly, as proteins were dissolved without the hydrolysis of peptide bonds. The amount of non-enzymatically solubilized proteins increased almost linearly over time suggesting that the alkaline conditions solubilized the protein gradually rather than all at once. It was evident that both the protease and the alkaline conditions had an impact on protein solubilization from BSG.

The optimal temperature for non-enzymatic protein solubilization over the range of 30–70 °C was also determined (Fig. 4). The highest solubility was obtained at 50 °C. Generally the solubilization is improved by elevated temperatures, but on the other hand too high heat also increases the risk of protein denaturation. For rBSG-D, heating to 60 and 70 °C in alkaline conditions may have caused the proteins to undergo some solubility decreasing changes. Some of these reactions could have begun already in mashing, as the mashing temperature may rise close to 80 °C. On the other hand, in mashing most of the storage proteins were protected inside cells, whereas after Depol 740 treatment they were more exposed and may have reacted with other components. On the contrary, Alcalase has been previously found to work more efficiently at 60 °C than at 50 °C on both untreated and carbohydrase-treated BSG (Treimo et al., 2008).

4. Conclusions

Carbohydrate digestion prior to a proteolytic treatment significantly enhanced the solubilization of protein from BSG, as without this pre-hydrolysis, low yields were obtained. In addition, a notable

3.2.5. Effect of aleurone cell walls on protein solubility

To examine the changes in BSG caused by Depol 740 and Alcalase more closely, the initial material and the residues were imaged by light microscopy with different cell wall components visualized by differential staining (Fig. S1, Supplementary information): aleurone and residual endosperm cell walls appear blue and protein red, lignified cell walls appear yellow and light green due to their autofluorescence and protein appears red. In BSG, a great deal of the protein is located in aleurone cells, which for the most part remain intact after mashing, storing the protein inside (Fig. S1a, white circles point out examples of protein-rich aleurone cells). The carbohydrate digestion with Depol 740 treatment degraded most of the aleurone cell walls remaining after the milling step (Fig. S1b) exposing the protein for solubilization and digestion at different pHs. As seen in Fig. S1c, nearly all protein was removed by the Alcalase treatment, and only the most recalcitrant lignified cell walls of husk and pericarp remained. Although a notable
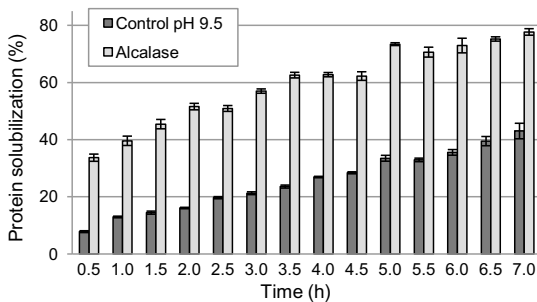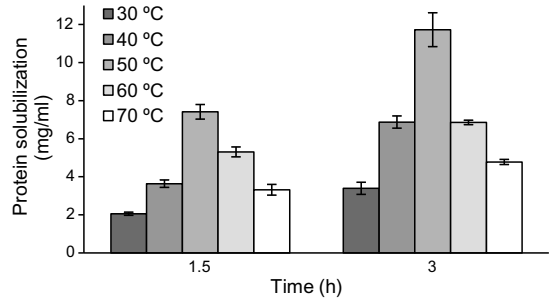

Fig. 3. Enzymatic and non-enzymatic solubilization of protein from rBSG-D.

amount of protein was solubilized under alkaline conditions in the absence of protease. Alkalinity was an important factor, as much higher yields were obtained with an alkaline protease compared to neutral and acidic proteases. At high pH, in the absence of a protease, protein was released over time, suggesting that the alkaline conditions gradually loosened the cell walls and enhanced protein solubility. Both pH and temperature were important parameters affecting the protein solubilization.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2013.03.076.

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III

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Characterization of Lipids and Lignans in Brewer's Spent Grain and Its Enzymatically Extracted Fraction

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ABSTRACT: Brewer's spent grain (BSG), the major side stream of brewing, consists of the husks and the residual parts of malts after the mashing process. BSG was enzymatically fractionated by a two-step treatment with carbohydrate- and protein-degrading enzymes, which solubilized 66% of BSG. BSG contained 11% lipids, which were mostly triglycerides, but also a notable amount of free fatty acids was present. Lipids were mostly solubilized due to the alkaline pH applied in the protease treatment. The main fatty acids were linoleic, palmitic, and oleic acids. Several lignans were identified in BSG, syringaresinol and secoisolariciresinol being the most abundant, many associated with the cell wall matrix and released by the alkaline-protease treatment.

KEYWORDS: *brewer's spent grain, enzymatic solubilization, lipid, lignan*

■ INTRODUCTION

Brewer's spent grain (BSG) is the most abundant side stream from brewing with an annual production of 30 million tons worldwide. Traditionally, this material rich in carbohydrates, protein, and lignin goes mainly to cattle feed, with little or no profit to the breweries. BSG contains several potentially valuable components suitable for utilization as food ingredients or raw materials for microbial or chemical conversions. Thus, advanced technologies for deconstruction and fractionation of BSG in the development of new value chains for breweries are still required. Both chemical and enzymatic fractionations of BSG have been previously studied in order to separate the carbohydrate, protein, and lignin moieties for further uses.[1−3] For instance, the carbohydrates have been studied as fermentable sugars,[4] arabinoxylan for functional oligosaccharides,[5] and xylitol,[6] and the solubilized peptides from BSG could have potential as foaming agents and emulsifiers.[7] Nevertheless, large-scale studies, not to mention practical applications, have been rarely reported.

In mashing, almost all of the starch and β-glucan and some proteins within the malts are enzymatically solubilized. BSG thus contains the husk and the outermost layers of the barley kernel and is a heterogeneous material rich in arabinoxylan (22−28%), cellulose (17−25%), lignin (12−28%), and protein (15−24%).[8] As BSG originates from the brewing process, it is a food-grade material and could be an interesting ingredient in food production, provided that cost-efficient processing methods to separate the different components can be developed.

In food processing, enzymatic treatments are preferable compared to chemical treatments, and enzymatic fractionation methods for protein and carbohydrates from BSG have been especially studied over the last 10 years.[1,2,7,9,10] However, BSG contains bacterial and fungal spores that are not destroyed by the high temperature in mashing;[11] to avoid their extensive growth and hence spoilage of the material, the hydrolysis time

has to be limited. Incubations of 4−5 h have been often reported,[1,10] but with current enzymatic methods, this time period can only release 50% of the total carbohydrates in a solubilized form.[12] However, the proteins are more easily removed and can be almost completely released by protease activity during the short time under optimal conditions.[10] Due to the different pH and temperature optima of carbohydrate- and protein-degrading enzymes, the hydrolysis treatments are more efficient when carried out in a sequential manner rather than combined in one step. A sequential carbohydrase−protease treatment can solubilize up to 40−60% of the total material, depending on the enzymes and conditions applied.[10,13,14]

In the previous studies, the focus has been mostly on the carbohydrates and proteins, whereas the lipids and phenolic compounds other than hydroxycinnamic acids have not been well characterized. If BSG is aimed to be utilized as a food component, the compositions of these aforementioned compounds are also of interest. Lignans are low molecular weight, polyphenolic compounds composed of similar structural units as lignin. They are known to have several health-promoting effects, such as antioxidant and anticarcinogenic activity,[15−19] but their content in BSG has not been previously determined. Furthermore, lipids are also an important constituent in BSG, but they have not been previously characterized. The aim of this study therefore was to characterize the lipids and lignans in BSG and their extractability in a hydrolysis process.

■ MATERIALS AND METHODS

Materials. BSG was obtained from Sinebrychoff brewery (Kerava, Finland) and stored as is at −20 °C. The composition of BSG is shown in Table 1. The main components were arabinoxylan, glucan

Table 1. Composition of BSG and the Residual Fractions Obtained after the Hydrolysis Steps[a]

|		hydrolysis residue			
component	initial BSG	1st	2nd	P-AEF	extr P-AEF
carbohydrate	42.2	22.2	49.4	4.0	6.7
arabinoxylan	22.2	15.9	31.2	2.3	2.7
glucan	17.1	4.5	15.9	0.7	1.1
lignin	19.3	29.1	33.5	24.4	40.7
protein	22.8	28.6	4.8	27.2	45.2
lipid	11.0	15.5	4.1	39.9	−
ash	4.7	6.4	8.1	4.5	7.4

[a]The values are given in mass %. Extr. P-AEF: P-AEF from which lipids were removed with heptane extraction. The amount of lignin is determined by subtracting other components from 100%.

(mainly cellulose), protein, lignin, lipids, and ash. Small amounts of starch (1.3%) and mixed link β-glucan (0.36%) were also present. The amounts of ferulic and p-coumaric acids, which are mostly associated with lignin, were 3.0 and 1.1 mg/g, respectively.

BSG Composition Analysis. Total lipids were analyzed by extracting a dry sample with heptane for 5 h in a Soxhlet apparatus. After extraction, the solvent was evaporated at room temperature, and the lipids were quantitated gravimetrically. The Klason lignin content was determined gravimetrically after removing extractives by heptane extraction and carbohydrates by acidic hydrolysis.[20] The acid-soluble lignin was measured from the hydrolysate by UV absorbance.[21] The carbohydrate composition was determined by analyzing the monosaccharides from the hydrolysate with HPAEC-PAD using a Dionex CarboPac PA-1 column (guard column 4×50 mm^2 and analytical column 4×250 mm^2) in a Dionex ICS-3000 system (Dionex Corp., Sunnyvale, CA) with electrochemical detection. The analysis was performed as described previously[22] with minor modifications, that is, equilibration with 15 mM NaOH and isocratic elution with water. The starch and β-glucan contents were determined with Megazyme kits total starch (amyloglucosidase/α-amylase method) and mixed-linked β-glucan, respectively, according to the manufacturer's (Megazyme, Bray, Ireland) instructions. The nitrogen content was analyzed by total nitrogen analysis at Analytische Laboratorien Prof. Dr. H. Malissa and G. Reuter GmbH (Lindlar, Germany) with a standard method ASTM D-5291. The nitrogen content was converted to protein content by multiplying by a factor of 6.25. The ash content was determined gravimetrically after burning the samples in a muffle furnace at 550 °C overnight. Phenolic acids were extracted from BSG by adding 2 M NaOH to the dry sample and incubating for 16 h at room temperature. The samples were then acidified with HCl and extracted with ethyl acetate three times. The organic phase was collected, and the solvent was evaporated. The dried residue was dissolved in a 50:50 mixture of methanol and water. High-performance liquid chromatography (HPLC) analysis of the phenolic acids was performed with a Waters 600S system controller equipped with a Waters 616 pump (Waters, Milford, MA) using a 4.6×150 mm^2, 5 μm, Hypersil BDS-C18 column (Agilent) with UV detection at 324 nm. A gradient solvent system using 5% formic acid in water (solvent A) and 100% acetonitrile (solvent B) was used. The volumetric proportions of solvents were 95%/5% A/B (5 min), 90%/10% A/B (10 min), 60%/40% A/B (5 min), and 10%/90% A/B (5 min). The flow rate was 0.7 mL/min. All analyses were carried out in duplicate.

Enzymatic Hydrolyses. BSG was defrosted at 4 °C overnight and suspended in tap water; a 6% BSG suspension was milled with a Masuko supermasscolloider MKZA10-15J, (Masuko Sangyo Co. Ltd., Kawaguchi-city, Japan) with a 7 times passing through MKGA10−80

grinding stones with a grinding speed of 1500 rpm. After grinding, a portion of the water was removed by centrifugation, and the dewatered suspension was stored overnight at 4 °C. Enzymatic hydrolysis consisted of two steps (Figure 1) and was carried out in a 10 L reactor
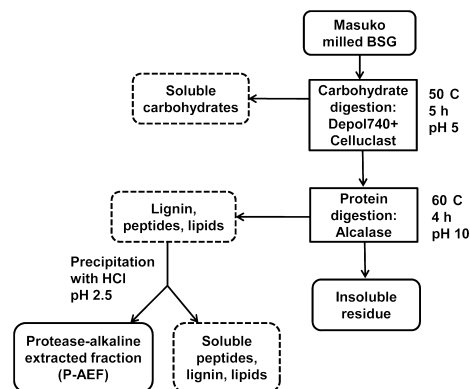

Figure 1. Scheme of BSG hydrolysis steps.

using a 10% (w/w) solids content. The quantity of BSG in the first hydrolysis step was 1000 g, and the enzymes used were Depol740L (Biocatalysts Ltd., Cefn Coed, Wales, U.K.) (5000 nkat of xylanase activity per g of BSG) and Celluclast 1.5 L (Novozymes, Bagsvaerd, Denmark) (50 FPU per g of BSG). The BSG suspension was preheated to 50 °C before adding the enzymes. The pH of the reaction mixture after adding the enzymes was 5.4 and was not further adjusted. The mixture was continuously stirred at 48 rpm. After 5 h, the suspension was cooled down by centrifuging in a precooled SorvallRC12BP centrifuge with a H12000 rotor (Sorvall Products L.P., Newton, CT, USA) at 4 °C and 4000 rpm for 30 min. The supernatant was collected, and the solid residue was washed twice with tap water to remove solubilized compounds and enzymes. The residue was weighed, and small aliquots were withdrawn to determine the dry matter content of the centrifuged material and for composition analysis of the insoluble material. The rest of the insoluble material was used in the second step of the hydrolysis. The second proteolytic step was carried out with Alcalase 2.4 L (Novozymes, Bagsvaerd, Denmark) (20 μL of enzyme preparation per g of BSG). The hydrolysis was carried out as before but this time at 60 °C for 4 h and in 100 mM, pH 10 sodium carbonate buffer. The decrease in pH during the hydrolysis was adjusted back to pH 10 with 10 M NaOH. The hydrolysis was terminated as done previously by cooling the suspension while separating the solids from liquids and by washing the solids.

The soluble fraction of the second hydrolytic step was precipitated by lowering the pH to 2.5 with 5 M hydrochloric acid. The solids were separated by centrifugation and washed twice with acidic water (pH 2.5). The precipitate is referred as the protease-alkaline extracted fraction (P-AEF). For lignan analysis, P-AEF was extracted with heptane as described above. All the insoluble fractions and samples taken between different hydrolytic steps were freeze-dried for further analyses.

Microscopy Analyses. Light microscopy was carried out as described previously.[23] In brief, samples were embedded in hydroxyethyl methylacrylate matrix from which 2 μm thick sections were cut. Prior to imaging with the microscope, the sections were treated with chemical dyes Calcofluor and Acid Fuchsin to enable visualization of different components of BSG. Acid Fuchsin stains protein red, and Calcofluor stains β-glucan bright blue. The autofluorescence of lignin and other phenolics are seen as yellow and green. Cutin appears as orange due to its autofluorescence. For the autofluorescence images, the samples were not stained, and the
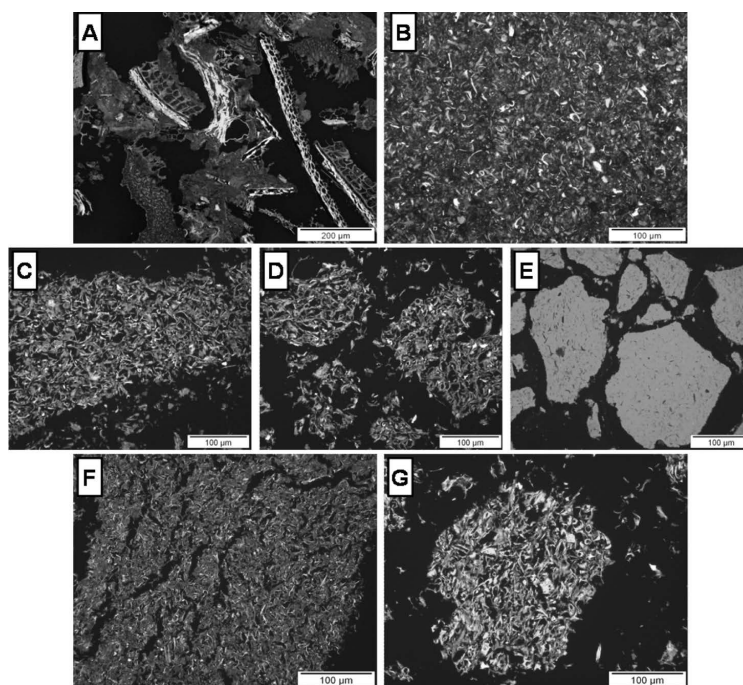
Figure 2. Microscopy images of BSG and the enzyme-treated fractions: light microscopy images of (A) unmilled BSG, (B) Masuko-milled BSG, (C) residue after the first carbohydrase treatment, (D) residue after the protease treatment, and (E) protease-alkaline extracted fraction; autofluorescence images of (F) the residue after the first carbohydrase treatment and (G) the residue after the protease treatment.

excitation and emission wavelengths used were 330–385 nm and >420 nm, respectively.

Lipid Analysis with Pyrolysis–GC/MS. Methylation pyrolysis in the presence of tetramethylammonium hydroxide (TMAH) was performed using a platinum foil pulse pyrolyzer Pyrolab2000 (Pyrolab, Lund, Sweden) connected to a gas chromatograph–mass spectrometry (GC/MS) Varian 3800 GC-Varian 2000 MS. The column used was a 30 m × 0.25 mm, i.d., 1 μm, fused silica capillary column DB-1701 (Agilent). About 40 μg of the sample was weighed accurately and transferred to the foil. A 10% TMAH aqueous solution (4 μL) was added and mixed with the sample. The sample mixture was inserted in the pyrolyzer maintained at 175 °C and heated at 400 °C for 4 s. The degradation products were led into the capillary column for separation using helium as the carrier gas, with a flow rate of 1.0 mL/min. The column temperature was programmed from 80 °C (2 min) to 160 °C at 8 °C/min and extended from 160 to 280 °C at 5 °C/min. The final temperature was held for 15 min. The mass spectrometer was operated in EI mode (70 eV). Fatty acid composition was calculated after normalization of peak areas to 100%.

Lipid Classification with TLC–GC. The separation of major lipid classes (phospolipids, free fatty acids, di- and triglycerides) was carried out with thin layer chromatography (TLC).[24] The heptane-extracted lipids were redissolved in a small amount of chloroform/methanol (100:1) and supplemented with the internal standards. The mixture was applied on silica plates, and the plates were developed with petroleum ether/diethyl ether/acetic acid (80:30:1). Lipid classes were visualized under UV light after spraying 0.01% Rhodamine 6B GO, scraped off, and used for quantitation.

Fatty acids were quantitated as their methyl esters.[25] The samples were first suspended in an excess (1 mL) of saponification reagent (3.7 M NaOH in 49% methanol) at 100 °C for 30 min. Samples were cooled down to room temperature, and 4 mL of methylation reagent (3.3 M HCl in 48% methanol) was added. After mixing, samples were held at 80 °C for 10 min. Fatty acid methyl esters were extracted in 1.5 mL hexane/methyl-*tert*-butyl ether solution (1:1). The methyl esters were analyzed by GC with a flame ionization detector (GC-FID) with an HP-88 column (30 m × 0.25 mm, phase 0.20 μm; Agilent). The amount of fatty acid methyl esters were calculated with corresponding standards. Three replicate samples were analyzed.

Analysis of Lignan Contents. BSG and the heptane-extracted P-AEF sample were extracted using accelerated solvent extraction (ASE). Approximately 0.5–0.7 g of the samples was ASE extracted as described by Smeds et al.[26] with some modifications, that is, 3 × 5 min static cycles were applied, and the heptane-extracted P-AEF was extracted with acetone and acetone/water (70:30, v/v), only. The acetone and acetone/water fractions were combined and diluted to 100 mL with acetone. A portion (10 mL) of this extract was withdrawn, and the solvent was evaporated to dryness in a water bath at 40 °C using a stream of nitrogen gas. The extracts were then enzymatically hydrolyzed by adding 490 units of purified β-glucuronidase (from *Helix pomatia*) (Sigma–Aldrich) dissolved in 1 mL of 10 mM sodium acetate buffer (pH 5.0) and kept at 37 °C for 19 h. The internal standards matairesinol-d_6, enterolactone-d_6, and dimethylated pinoresinol-d_6 (dissolved in methanol) were added in amounts of 484, 285, and 570 ng, respectively. The solutions were liquid–liquid extracted with 2 × 0.75 mL of ethyl acetate, and the ethyl acetate phase was evaporated to dryness using a stream of nitrogen gas. Methanol/0.1% acetic acid (500 μL, 20:80, v/v) was added, the samples were sonicated for approximately 30 s, and the solutions were filtered using PTFE 0.45 μm syringe filters. A portion (10 μL) of the solutions were injected into a HPLC-MS/MS system, and the lignans were determined as described previously.[26,27]

■ RESULTS AND DISCUSSION

Enzyme-Aided Solubilization of BSG. A two-step hydrolysis of BSG was carried out with carbohydrate- and protein-degrading enzymes. Prior to the hydrolyses, BSG was pretreated with a Masuko grinder. Reducing the particle size of BSG by grinding has been shown to improve the efficiency of enzymatic hydrolysis of BSG carbohydrates releasing some soluble material by the mechanical impact alone.[12] The changes in the microstructure of BSG were followed with light microscopy starting from unmilled BSG to Masuko-ground BSG and through the enzymatic treatments (Figure 2). In Figure 2A–E, the bright blue stain represents cell walls of residual endosperm cells, the red stain indicates protein, and lignin or phenolic acids contained in the aleurone, pericarp, and husk cells are seen because of their autofluorescence (green and yellowish colors).

The initial BSG contained several millimeter long particles with intact cellular structures (Figure 2A). Due to grinding of BSG with the Masuko mill, the particle size was significantly decreased, and the cellular structures were disrupted (Figure 2B). The hydrolysis was initiated with a mixture of two carbohydrase preparations, Depol740 and Celluclast 1.5, previously found to be effective in the solubilization of BSG carbohydrates.[12] Celluclast is high in cellulolytic and xylanolytic activities whereas Depol740 contains feruloyl esterase activity in addition to several hemicellulolytic and cellulolytic activities.[12] In the first hydrolysis step, the enzymes solubilized 26% of the milled BSG. After the first treatment, the carbohydrate content had decreased from 42% to 22%, whereas the contents of protein, lignin, and lipids had increased accordingly (Table 1). It should be noted that the analysis of lignin content with nonwood materials is challenging and due to several factors the Klason lignin method often exaggerates the results.[28] The same phenomenon was observed also with the BSG fractions, and therefore, the lignin contents (Table 1) were calculated by subtracting the amounts of other components from 100%.

When observed by microscopy, the blue color had disappeared almost completely after the carbohydrase treatment (Figure 2C). Presumably, the nonlignified endosperm and aleurone cell walls were more accessible for enzymes than the more resistant, protective outer layers. It has been shown with an endoxylanase on wheat bran[29] that the enzyme preferentially acted on the aleurone cells and progressed into the bran only after degradation of the aleurone layer. This was due to lower arabinose substitution of the aleurone layer and because the xylanase was too large to diffuse into the cells without further arabinoxylan degradation. A small amount of protein was also solubilized in the first hydrolysis as Depol740 contains some protease activity.[2] However, protein content of the residue increased due to the carbohydrate removal, which was confirmed by an increase in red-colored components (Figure 2C).

The aim of the second hydrolysis treatment was to digest and hence release the proteins from the residual matrix. Alcalase has previously been shown to work effectively on BSG proteins, especially at alkaline pH (9.5–10).[1,30] While the first hydrolysis step with Depol740/Celluclast removed 6% of the protein, the Alcalase treatment resulted in 93% of all BSG protein being solubilized by the two steps. The removal of protein was confirmed by microscopy as the amount of red stain decreased significantly (Figure 2D). Most of the water-soluble barley protein is removed already in mashing, and the proteins left in

BSG are mainly the water-insoluble storage proteins, hordeins, and structural proteins, glutelins.[31] In addition, BSG often contains some precipitated protein from wort boiling. The insoluble residue after the protease treatment contained thin and long orange particles, which should not be confused with protein (Figure 2D). Most likely, they were parts of the cutin, which is a polyester in the hydrophobic cuticle layer covering the outer surface of the husk. Cutin has also aromatic components and can therefore be seen due to its autofluorescence. In samples rich in protein, the red color was so dominant that it was difficult to distinguish the cutin, but as the protein was removed, the cutin fragments became more visible.

The Alcalase treatment solubilized 40% of BSG, and therefore, after the two-step treatment, a total of 66% of BSG had been solubilized. A portion (58%) of the calculated BSG lignin was present in the insoluble residue after the protease treatment, whereas practically no solubilization of lignin occurred during the carbohydrate digestion. Previously, it has been speculated that, when BSG is treated with carbohydrate- and protein-degrading enzymes, lignin is partly cosolubilized during an alkaline protease treatment[13] due to its linkages to other components or because of the high pH in the proteolytic treatment. Therefore, in the current work, the supernatant collected after the Alcalase treatment was acidified to precipitate the solubilized lignin (protease-alkaline extracted fraction, P-AEF). The amount of the obtained precipitate was 113 g (after removal of lipids 68 g). Nearly half of the lignin was released by the action of Alcalase (Table 1), but only a small part of it, approximately a third, precipitated when the pH was lowered to 2.5. It is known that lignin is covalently linked to carbohydrates,[32] and possibly, some carbohydrates were associated with the lignin present in the soluble phase making it more soluble in water. The protease treatment also released 87% of the lipids present in BSG (Table 1), with almost half of them associated with the precipitate after the subsequent acidification. The main components of P-AEF were lipids, protein (or peptides), and lignin (Table 1). In Figure 2E, precipitated proteinaceous material presumably covered lignin and thus hid some of the autofluorescence as well. The degree of solubilization in the second hydrolytic step was 40% of the initial BSG, and as the amount of the subsequent precipitate was 11%, it demonstrated that most of the material solubilized in the proteolytic treatment remained in the soluble form despite the lowering of the pH.

Figure 2F, G represents the autofluorescence of the hydrolysis residues when excited with UV light. The major autofluorescent component in BSG is lignin, but several other aromatic compounds such as phenolic acids and lignans contribute as well.[33] Although some of the aromatic compounds are released by the enzymatic treatments, the autofluorescence of the residues increased in the processing. This is consistent with the composition data (Table 1) showing also that the lignin content of the solid residue was enriched by the hydrolytic treatments. In addition to the enrichment of lignin, its autofluorescence may become more visible as the protein and carbohydrates covering it are removed.

Lignin is one of the major constituents of BSG, but so far, nothing is really known about the characteristics of BSG lignin. Recently, there have been studies on the delignification of BSG lignin[34] and the further use of the obtained lignin fraction,[35] but in these studies, no characteristics of the processed lignin were determined. In the literature, the lignin content of BSG is generally expressed simply as "Klason lignin", without further

considerations of what this acid-insoluble residue actually consists.[28] The autofluorescence of the insoluble residue combined with the lignin content data strongly supports the assumption that this material truly is lignin for the most part.

BSG Lipids. Figure 3A presents the results of fatty acid analyses with pyrolysis−GC/MS. The fatty acid content of BSG
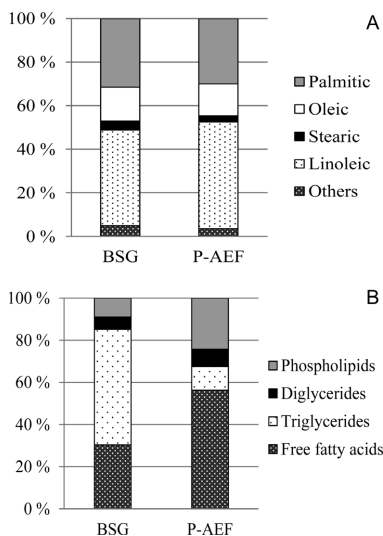
Figure 3. (A) Proportions of different fatty acids in BSG and protease-alkaline extracted fraction (P-AEF). (B) Proportions of different lipid classes in BSG and P-AEF.

was 11%, and the most abundant lipids were linoleic (18:2), palmitic (16:0), and oleic acids (18:1). Small amounts of other fatty acids, such as stearic (18:0) and linolenic (18:3) acids, were also present. The benefit of pyrolysis−GC/MS is that it is able to detect all fatty acids in the material. More often solvent-extractable lipids are analyzed, but all lipids are not necessarily extractable as they may be bound in the matrix to structures such as cutin. The lipid content of unmalted barley varies between 1.0−2.6%,[36,37] but in mashing, the lipids are enriched, and their concentration in BSG is from 5.8% up to 11%.[6,8,12] Only minor changes in fatty acid composition occur during malting and mashing,[38] and therefore, the fatty acid composition of BSG is similar to that of barley, although in barley, the linoleic acid content has been reported to be slightly higher and palmitic acid content consequently somewhat lower than in BSG. However, this is most likely due to differences in environmental conditions in which the barley plants were cultivated.[39]

Lipid classification (Figure 3B) showed that most lipids in BSG are triglycerides (55%), although the amount of free fatty acids was also notable (30%). Phospholipids and diglycerides accounted for 9.1% and 5.7%, respectively. Compared to unmalted barley, BSG has less triglycerides and phospholipids and more free fatty acids, whose proportions in barley are 69%, 27%, and 2%, respectively.[38] The increased amount of free fatty acids in BSG is probably a result of endogenous lipase activity that released free fatty acids from triglycerides and phospholipids during malting and mashing. Triglycerides are the main form of storing lipids for energy in barley, whereas

phospholipids constitute biological membranes. The lipids in barley are located in the endosperm and embryo, as their role is to provide nutrients and energy for the new, germinating barley plant. Although the endosperm is almost completely solubilized in mashing, most of the lipids remain with spent grains and are not transferred to wort.[38,40] During the protease treatment, the high pH caused hydrolysis of alkali-labile ester bonds of the di- and triglycerides and saponifying of the fatty acids, as in P-AEF, the proportion of triglycerides was only 11%.
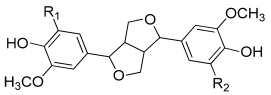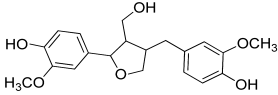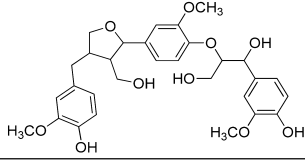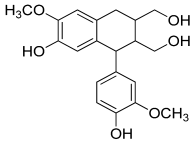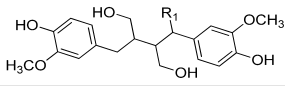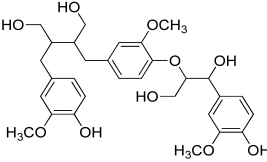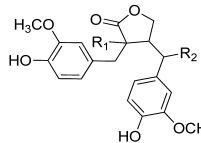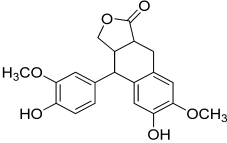
Lignans in BSG and the Protease-Alkaline Extracted Fraction. Several lignans were detected in BSG (Table 2). The total content of the analyzed lignans amounted up to 1300 μg/ 100 g, which is similar to the lignan content of unmalted barley.[26] Compared to other cereals, such as wheat and rye, barley has much lower lignan content.[26] The most abundant lignans in BSG were syringaresinol **3** and secoisolariciresinol **7**, which had significantly higher concentrations than any other lignan. Lignan concentrations in P-AEF were notably higher than in BSG indicating that most lignans were released from the matrix in the conditions of the second hydrolysis step and were then concentrated in the precipitate. Syringaresinol **3** was the most abundant lignan in both BSG and P-AEF, but the amounts of medioresinol **2**, nortrachelogenin **13**, and α-conidendrin **14** were considerably increased as well in P-AEF. For lariciresinol **4**, nortrachelogenin **13**, α-conidendrin **14**, and secoisolariciresinol-sesquilignan **9**, the concentrations in P-AEF were higher than what seemed theoretically possible to obtain from BSG. In addition, medioresinol **2** and 7-hydroxymatair-esinol **11** were not detected at all in BSG but were found in P-AEF in significant amounts.

Lignans are mostly polyphenolic compounds, excluding a few lignans that lack the phenolic hydroxyl group such as sesamin and hinokinin. Lignans are commonly found in plants, in which their function is to act as defensive substances, and they have been reported to have several disease-preventing and health-promoting properties.[15−19] The amounts of lignans in several cereal grains, including barley, have been quantified[26] and show that a number of these lignans are a part of our everyday diet. In addition, the conversion of plant lignans to the mammalian lignans enterodiol and enterolactone by gut microbiota has been demonstrated.[41,42]

When comparing barley[26] and BSG, it can be noticed that lignans behave differently in the mashing process. The concentrations of certain lignans, such as 7-hydroxymatairesinol **11** and lariciresinol **4**, which are among the most abundant lignans in barley, were much lower in BSG indicating that they are solubilized in mashing and appear in the wort. Many lignans in cereals exist as glycosides,[26] which may improve their solubility. On the other hand, some lignans, such as syringaresinol **3** and secoisolariciresinol **7**, appeared more enriched in BSG than barley indicating that they are not soluble in aqueous environment. However, as lignan concentrations vary between barley varieties and the harvesting year,[43] comparison between barley and BSG may not be completely accurate but should be considered only as indicative.

During the enzymatic processing, most lignans seemed to be solubilized by the alkaline-protease treatment and subsequently precipitated by the acidification, as the concentrations of lignans in P-AEF were systematically higher than in BSG. The increase in the concentration could be due to some lignans being bound in the cell walls with ester linkages and released by the alkaline pH effect.[26] It has been shown for certain lignans, including medioresinol **2** and lariciresinol **4**, that alkaline and

Table 2. Molecular Structures and Concentrations of Lignans in BSG and the Delipidated Protease-Alkaline Extracted Fraction (P-AEF)[a]

Molecular structure	Name	μg/100g in BSG	μg/100g in P-AEF
	$R_1=R_2=H$: Pinoresinol **1**	29	203
	$R_1=OCH_3$, $R_2=H$: Medioresinol **2**	nd	1249
	$R_1=R_2=OCH_3$: Syringaresinol **3**	982	2357
	Lariciresinol **4**	4.8	229
	Lariciresinol-sesquilignan **5**	13	82
	Cyclolariciresinol **6**	22	26
	$R_1=H$: Secoisolariciresinol **7**	233	595
	$R_1=OH$: 7-Hydroxy-secoisolariciresinol **8**	nd	nd
	Secoisolariciresinol-sesquilignan **9**	6.2	112
	$R_1=R_2=H$: Matairesinol **10**	18	142
	$R_1=H$, $R_2=OH$: 7-Hydroxymatairesinol **11**	nd	477
	$R_1=H$, $R_2=O$: 7-Oxomatairesinol **12**	1.0	nd
	$R_1=OH$, $R_2=H$: Nortrachelogenin **13**	24	1791
	α-Conidendrin **14**	12	1239

[a] Nd: not detected.

acidic treatments are required to achieve the best possible extraction yield from barley.[26] This could well explain why these lignans were detected in higher concentrations in P-AEF than BSG. In addition, structures of some lignans, for example, pinoresinol **1** and lariciresinol **4**, are incorporated in lignin,[44] and it has been suggested that they are released by acidic conditions.[26] Interestingly, 7-hydroxymatairesinol **11** and medioresinol **2**, which were below the detection levels in the initial BSG, were identified in P-AEF. 7-Hydroxymatairesinol

11 is the most abundant lignan in barley[26] but seems to be released in mashing, as it was not detected in BSG. 7-Hydroxymatairesinol **11** is not stable in alkaline or acidic conditions,[26] and therefore, its reappearance in P-AEF was unexpected.

The quantitation of lignans is complicated by several factors. Acidic and alkaline pHs are known to cause structural changes in several lignans and conversion of certain lignans to other lignans.[26] Furthermore, finding the optimal extraction method

for lignans is not always straightforward and strongly depends on the properties of the starting material. For example, for barley, the ASE extraction gives the highest overall lignan yield, but there are some lignans that may not be entirely extractable with this method.[26]

A two-step enzymatic treatment solubilized 66% of BSG. In the first step, carbohydrates were partially resistant to the action of the carbohydrate-degrading enzymes, whereas the proteins and lipids were almost completely solubilized by the alkaline-proteolytic treatment. The main form of lipids in BSG was triglyceride, but a notable amount of free fatty acids were also detected. The main fatty acids were linoleic, palmitic, and oleic acids. Most of the lipids were solubilized by the proteolytic treatment, most likely due to the high pH. Several lignans were detected in BSG, and many of them were concentrated in the precipitated fraction. Lignans seemed to be partially bound in the cell wall matrix and were probably released by the effects of alkaline and acidic pH. Also, some chemical conversion may have occurred due to the changes in the pH during the enzymatic processing. The results of this study showed that, in addition to dietary fiber and protein, which have been previously characterized, BSG is rich in unsaturated fats and lignans with beneficial properties for health. Therefore, BSG or its enzymatically extracted fractions have great potential to be used in applications such as added-value food components.

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Notes
The authors declare no competing financial interest.

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IV

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Interactions of a Lignin-Rich Fraction from Brewer's Spent Grain with Gut Microbiota in Vitro

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ABSTRACT: Lignin is a constituent of plant cell walls and thus is classified as part of dietary fiber. However, little is known about the role of lignin in gastrointestinal fermentation. In this work, a lignin-rich fraction was prepared from brewer's spent grain and subjected to an in vitro colon model to study its potential bioconversions and interactions with fecal microbiota. No suppression of microbial conversion by the fraction was observed in the colon model, as measured as short-chain fatty acid production. Furthermore, no inhibition on the growth was observed when the fraction was incubated with strains of lactobacilli and bifidobacteria. In fact, the lignin-rich fraction enabled bifidobacteria to survive longer than with glucose. Several transiently appearing phenolic compounds, very likely originating from lignin, were observed during the fermentation. This would indicate that the gut microbiota was able to partially degrade lignin and metabolize the released compounds.

KEYWORDS: *brewer's spent grain, dietary fiber, lignin, lignan, in vitro fermentation, colon microbiota, lactobacilli, bifidobacteria*

■ INTRODUCTION

Cereal dietary fiber (DF) is commonly known to provide several benefits for human health, including reduced risk of obesity, cardiovascular disease, and type 2 diabetes.[1] DF consists mainly of the carbohydrates resistant to human digestive enzymes, such as arabinoxylan and β-glucan, but other noncarbohydrate compounds, for example, lignin, are also included in DF. According to the European Union (EU) definition, lignin is included as a component of DF when it remains closely associated with the original plant polysaccharides.[2] Lignin is a polymeric and phenolic compound acting as the glue between the cellulose–hemicellulose matrix in plant cell walls. It is formed from three monomers: *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Figure 1), which are linked together in a branched network structure by radical-induced condensation reactions. Depending on the plant origin, lignins vary in monomer ratios and in the types of linkages between the monomers. The most typical linkage between the monolignol units is the β-arylether (β-O-4) linkage (Figure 1D). Other types of linkages include phenylcoumaran (β-5), biphenyl (5-5′), diarylpropane (β-1), α-arylether (α-O-4), diarylether (4-O-5), and pinoresinol (β-β) linkages.[3] Wood lignin has been widely studied, but very little is known about cereal lignin. However, the presence of lignin in cereal grains has been demonstrated.[4] Cereal lignin differs from wood lignin in the monomer composition and by having ferulate cross-links to carbohydrates.

Lignin is generally considered to be an inert compound in the human gastrointestinal tract and resistant to the metabolic activities of gut microbiota.[5] In in vitro studies, fiber fractions high in lignin have been shown to be less extensively fermented by gut microbiota than low lignin fibers.[6] As lignin in cereals is covalently linked to carbohydrates,[4] it causes steric hindrance for carbohydrate-degrading enzymes and could hinder the fermentation of lignin-rich DF. Lignin can also adsorb enzymes, such as cellulases, and thus inhibit their action.[7] Nevertheless, some wood-feeding insects including termites have the ability to digest lignin.[8] There is also some evidence of lignin degradation in animals such as goats, cows, and dogs.[9,10] A few studies demonstrating lignin digestion in humans have been reported,[11,12] which contradicts the common conception of the inertness of lignin. Biodegradation of lignin by white rot fungi is better known,[13] and there is also evidence of lignin degradation by soil bacteria.[14] However, the interactions between lignin and
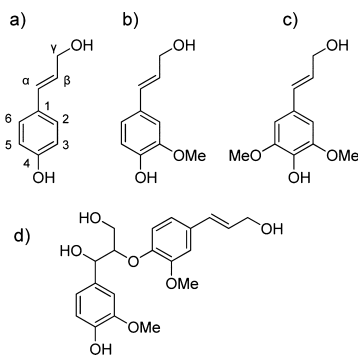
Figure 1. Lignin monomers: (a) *p*-coumaryl alcohol; (b) coniferyl alcohol; (c) sinapyl alcohol; (d) β-O-4 linked dilignol structure.

human colon microbiota have hardly been studied. Although lignin has been consumed in the human diet since ancient times, its possible degradation in the human gastrointestinal tract and its effects on the gastrointestinal microbiota have not been adequately elucidated.

Because lignin is a polymer, it is not absorbed but remains in the gut lumen and could interact with dietary components or have effects on gut microbiota or its conversion activities as has been demonstrated for isolated apple and grape tannins.[15,16] Isolated condensed tannins inhibit DF fermentation, which can be monitored as a formation of short-chain fatty acids (SCFA).[15] Tannins have been shown to bind proteins and thus inhibit enzymes,[17] which may explain the suppression of SCFA formation.

The aim of this study was to investigate if a lignin-rich fraction from brewer's spent grain (BSG) is degraded in an in vitro human colon metabolic model with concurrent release and conversion of low molecular weight lignin-like components. The fraction was extracted from BSG by enzymatic methods as described previously[18] and contained a low amount of carbohydrates in relation to lignin, which in all likelihood makes the lignin more accessible for the microbiota than as part of an intact cell wall. The second aim was to investigate if the lignin-rich fraction is inhibitory to carbohydrate degradation in the in vitro colon model and if the fraction has an effect on the growth of lactobacilli and bifidobacteria.

■ MATERIALS AND METHODS

Material. The lignin-rich fraction was prepared from BSG as described previously.[18] In brief, BSG was first treated with carbohydrases and then with an alkaline protease. The hydrolysate of the proteolytic treatment was acidified to produce a precipitate, which was called protease-alkaline extracted fraction (P-AE fraction). The composition of the fraction was 4.0% carbohydrates, 24% lignin, 27% protein, 40% lipids, and 4.5% ash.[18] For the studies with lactobacilli and bifidobacteria, lipids were removed by extracting the material with heptane for 5 h in a Soxhlet apparatus.

Chemicals. In the targeted analysis *trans*-2-hydroxycinnamic acid (Aldrich, St. Louis, MO, USA) was used as the internal standard. In addition, the following compounds were quantitated using the same compounds for external standards: 3-hydroxybenzoic acid, 3-(4′-hydroxyphenyl)propionic acid, 3-(3′,4′-dihydroxyphenyl)propionic acid (Aldrich, Steinheim, Germany); 4-hydroxybenzoic acid, 2-(3′-hydroxyphenyl)acetic acid, 2-(3′,4′-dihydroxyphenyl)acetic (Sigma, St. Louis, MO, USA); 3-phenylpropionic acid, 3,4-dihydroxybenzoic acid (Fluka, Buchs, Switzerland); 3-(3′-hydroxyphenyl)propionic acid (Alfa Aesar, Karlsruhe, Germany); 4-methylcatechol (Aldrich, Steinheim, Germany); vanillic acid (Fluka); *p*-coumaric acid (Sigma); gallic acid (Extrasynthèse, Genay, France); and ferulic acid (Sigma-Aldrich, St. Louis, MO, USA). *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide (Sigma) and methoxyamine (Thermo Scientific, Bellefonte, PA, USA) were used as the derivatization reagents. The standards used in SCFA analysis were acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, and heptanoic acid (internal standard) (Fluka Analytical, Buchs, Switzerland). Deuterated lignans (matairesinol-d_6, secoisolariciresinol-d_6, enterolactone-d_6, and dimethylated pinoresinol-d_6) used as internal standards in the lignan analysis were prepared at the Laboratory of Organic Chemistry at Åbo Akademi University as described previously.[19,20]

Fermentation of the P-AE Fraction in the Colon Model. In vitro fermentation was performed as described previously,[21] with minor modifications. Human feces were collected from five healthy volunteers, who had not received antibiotics within the previous 3 months. The donors of the feces were not given any limitations regarding their diets prior to the feces collection. Freshly passed feces were immediately placed in an anaerobic chamber, pooled, and homogenized (2 min) with the culture medium using a Waring

blender (18000 rev/min). The slurry was diluted to 20.8% (w/v) by adding the culture medium, filtered through a 1 mm sieve, placed on ice, and used immediately as an inoculum in the experiment. Two hundred milligrams of the P-AE fraction was weighed into bottles (50 mL) and suspended with 2 mL of the culture medium 1 day before incubation to reduce the lag in fermentation rate due to the hydration of the fraction. Prehydrated P-AE fractions were inoculated with 8 mL of fecal suspension, and a 16.7% (w/v) final concentration of fresh fecal matter was obtained. Bottles were tightly closed and incubated in a water bath at 37 °C under agitation (250 rev/min) for 0, 2, 4, 6, 8, and 24 h. Fecal controls were similarly produced except that they did not contain any P-AE fraction. Fermentations were carried out in triplicate. After the incubation, the fermentation samples were rapidly cooled using an ice water bath, and the pH was measured. They were then rapidly frozen using liquid nitrogen and stored at −20 °C for further analyses. Averages and standard deviations were calculated from triplicate measurements at each time point.

Extraction of Fermentation Samples. Extraction of SCFA to diethyl ether was carried out as described previously.[22] For phenolic compound, lignan, and metabolome analyses 1 mL of the fermented sample was thawed. One milliliter of 2% NaCl solution was added to salt out the lipids contained within the P-AE fraction. Fifty microliters of 6 M HCl was added to lower the pH to nearly 1. For the analysis of phenolic compounds and the metabolome 15 μL of internal standard (123 mg/L in MeOH) was added to the fermented samples, which were then extracted twice with 3 mL of ethyl acetate. The organic phases were collected, combined, and evaporated under nitrogen. For lignan extraction, 20 μL of an ethyl acetate solution containing the internal standards matairesinol-d_6 (430 ng), secoisolariciresinol-d_6 (524 ng), enterolactone-d_6 (316 ng), and dimethylated pinoresinol-d_6 (756 ng) was added to 1 mL of the fermented samples, which were then extracted twice with methanol (3 mL), and the liquid phases were combined. The methanol−water mixture was evaporated under nitrogen stream. The dried samples were stored under a nitrogen atmosphere at −20 °C until analyzed.

SCFA Analysis. Diethyl ether extracts (2 μL, splitless injection) were analyzed with gas chromatography with a flame ionization detector (GC/FID) (Agilent 6890 Series, Palo Alto, CA, USA). Analytes were separated on a DP-FFAP capillary column (30 m × 0.32 mm) with a phase thickness of 0.25 μm (Agilent). Helium was used as the carrier gas at 2.7 mL/min. Both the injector and FID were kept at 250 °C. The temperature program started at 50 °C with 3 min of holding time, then increased at 25 °C/min to 100 °C, and finally increased at 10 °C/min to the final temperature of 240 °C, which was maintained for 10 min. Compounds were quantitated with corresponding standards.

Metabolomics. The analysis was performed as described previously[15] using a two-dimensional gas chromatograph coupled with a time-of-flight mass detector (GC×GC-TOFMS). Sample derivatization was done automatically by Gerstel MPS autosampler and Maestro software. The data processing of GC×GC-TOFMS responses has been described earlier.[15] Briefly, the peaks were identified by ChromaTOF software, which matches deconvoluted spectra against the NIST05 mass spectral library. The compounds in different data sets were aligned and normalized using an in-house developed software, Guineu,[23] for further analyses. Alignment of the data was performed on the basis of retention indices, second-dimension retention times, and spectra.

The GOLM Metabolome Database (GMD)[24] and the Guineau program[23] were utilized for second-stage identification of those compounds that lacked spectral matches from the NIST05 or in-house collected libraries. GMD allows searching of the database on the basis of submitted GC-MS spectra, retention indices, and mass intensity ratios. In addition, the database allowed a functional group prediction, which helped characterize the unknown metabolites without available reference mass spectra in the GMD.

The visualization was performed by calculating 2-based logarithmic fold-changes of the relative peak areas from GC×GC-TOFMS analysis against the fecal control. The profile of the individual metabolite was visualized as color intensities (red as overexpression and blue as

underexpression) and the time point specific significances (t test p-values) as asterisks against the corresponding control. The nontargeted metabolite profiling was semiquantitative. The names of the overexpressed metabolites were verified by comparing the mass spectra with those found in GMD, and the unknowns were named according to the group specifications and displayed in the final heat maps. Clustering of the metabolites was performed according to the similarity of the time profiles.

Lignans. To the dry fermentation methanol extracts was added 1.5 mL of 10 mM sodium acetate buffer (pH 5.0), and the solutions were sonicated for 1–2 min or until the material was completely dissolved. The solutions were then centrifuged for 15 min, and the supernatant was carefully removed and then centrifuged again for 15 min. The clear supernatant was solid-phase extracted using Oasis HLB 30 mg cartridges (Waters Corp., Milford, MA, USA) according to a previously described method.[20] After evaporation of the solvent to dryness, 200 μL of methanol/0.1% acetic acid in Milli-Q water (20:80, v/v) was added, the solution was sonicated for 1–2 min, and 10–20 μL was injected into the HPLC-MS/MS. The HPLC-MS/MS method and conditions were the same as described previously.[25] Quantitation was carried out using standard solutions containing the internal standards and six concentration levels of the analyzed lignans, as described previously.[25] The standard solutions were solid-phase extracted and redissolved as the real samples.

Studies with Lactobacilli and Bifidobacteria. To evaluate possible microbiological impurities within the P-AE fraction after removal of lipids, 2% heptane extracted P-AE fraction was anaerobically incubated for 3 days at 37 °C in de Man–Rogosa–Sharpe (MRS) medium (Oxoid, Basingstoke, UK), Reinforced Clostridial Medium (RCM) (Difco, Franklin Lakes, NJ, USA), and *Bifidobacterium* medium (DSMZ medium 58; www.dsmz.de). After incubation, the optical density (OD) and pH of each medium were measured. The media containing the fraction were serially diluted in prereduced peptone saline containing 0.5 g of L-cysteine/HCl (pH 6.3; Merck, Darmstadt, Germany) and plated on corresponding solid culture medium (i.e., MRS medium to MRS agar) in an anaerobic workstation.

Four lactobacilli strains (*Lactobacillus rhamnosus* VTT E-97800, *L. rhamnosus* VTT E-97948, *Lactobacillus paracasei* VTT E-97949, and *Lactobacillus salivarius* VTT E-981006) and three bifidobacteria strains (*Bifidobacterium adolescentis* VTT E-981074, *Bifidobacterium breve* VTT E-981075, and *Bifidobacterium longum* VTT E-96664) were used to evaluate the potential of the lignin-rich fraction as a bacterial growth substrate. The following culture media were utilized (incubation at 37 °C): (1) unmodified MRS medium as a positive control medium for lactobacilli; (2) MRS medium without carbohydrates and the fraction as a negative control medium for lactobacilli; (3) MRS medium without carbohydrates as a basal medium with 0.5, 1.0, or 2.0% (w/w) addition of the fraction as test media for lactobacilli; (4) unmodified MRS medium as a basal medium with 0.5, 1.0, or 2.0% (w/w) addition of the fraction as extra media for lactobacilli; (5) unmodified *Bifidobacterium* medium as a positive control medium for bifidobacteria; (6) *Bifidobacterium* medium without carbohydrates and the fraction as a negative control medium for bifidobacteria; (7) *Bifidobacterium* medium without carbohydrates as a basal medium with 0.5, 1.0, or 2.0% (w/w) addition of the fraction as test media for bifidobacteria. Bacterial growth was monitored using OD and pH. In addition, the bacterial suspensions containing 1% of the P-AE fraction were serially diluted as described above and plated on MRS agar (lactobacilli) or RCM agar (bifidobacteria). The plates were incubated in Anoxomat WS8000 anaerobic jars (Mart Microbiology, Lichtenvoorde, The Netherlands) containing 10:5:85 $H_2/CO_2/N_2$ for 3 days (lactobacilli and bifidobacteria) and 7 days (bifidobacteria) at 37 °C.

Statistical Analyses. For statistical analyses of the quantitated phenolic compounds and SCFA, the responses were measured in triplicates, and two-way ANOVA with repeated measures using a Bonferroni adjustment was used to test significance ($p < 0.05$) between the fraction and the control. The statistics were performed with using MatLab Version R2008b. Two-way ANOVA was also performed for nontargeted GC×GC-TOFMS data as described previously.[15] Significantly different response levels between lignin

fraction and the fecal control within a time point are indicated with asterisks (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

RESULTS

Interactions of the Lignin-Rich Fraction with Fecal Microbiota. *SCFA Formation.* The conversion of carbohydrates to SCFA was measured to determine if the lignin-rich P-AE fraction suppressed the fermentation of remaining carbohydrates from the fraction and fecal suspension, expressed as SCFA formation (Figure 2). No suppression of SCFA
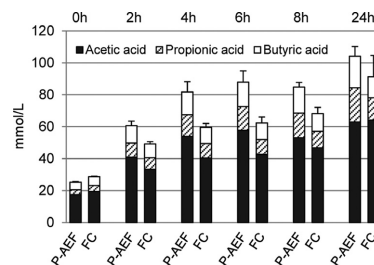

Figure 2. Concentrations of linear short-chain fatty acids formed during in vitro fermentation. FC, fecal control; P-AEF, protease-alkaline extracted fraction. The error bar is the combined standard deviation of the three fatty acids.

formation was observed in the presence of the fraction. In the beginning of the fermentation, acetic acid, which was the most abundant SCFA, was formed more rapidly from the P-AE fraction than from the fecal suspension, but toward the end of the fermentation the formation rate decreased, and at the end the concentration of acetic acid in the fecal control reached the same level as in the P-AE fraction. Propionic and butyric acids were constantly produced in higher concentrations in the P-AE fraction than in the fecal control. All data points were found to be significantly different from the fecal control ($p < 0.05$) with the t test, except for the 24 h point for acetic acid.

Effects of the Lignin-Rich Fraction on Lactobacilli and Bifidobacteria. No bacterial growth was observed after incubation of 2% (delipidated) P-AE fraction in MRS, RCM, or *Bifidobacterium* medium. The effect of the fraction on growth medium pH was first evaluated to assess the possibility of using pH as a measurement of growth. All of the concentrations of the fraction decreased the pH of the medium, independent of bacteria, during the 2 day incubation period. The pH in MRS medium with 0.5% fraction inoculated with lactobacilli was 6.1–6.2, depending on the lactobacilli strain used, whereas the pH in MRS medium with 0.5% fraction and no bacterial inoculum was also 6.1. In addition, the P-AE fraction concentration affected the pH; the higher the concentration of the fraction, the lower the pH. With 0.5% concentration of the P-AE fraction, the pH of MRS medium was 6.1, whereas with 2% concentration the pH was 5.5. Therefore, pH was not further used for growth measurements.

The effect of the P-AE fraction in the OD values was also evaluated. The addition of the fraction increased the OD, independently of bacteria. The ODs of 0.5, 1, and 2% PA-E fraction in MRS medium without the addition of other carbohydrates were 0.110, 0.140, and 0.180, respectively. The ODs of the same medium with the fraction inoculated with *L. salivarius* did not differ from the OD of the P-AE fraction,
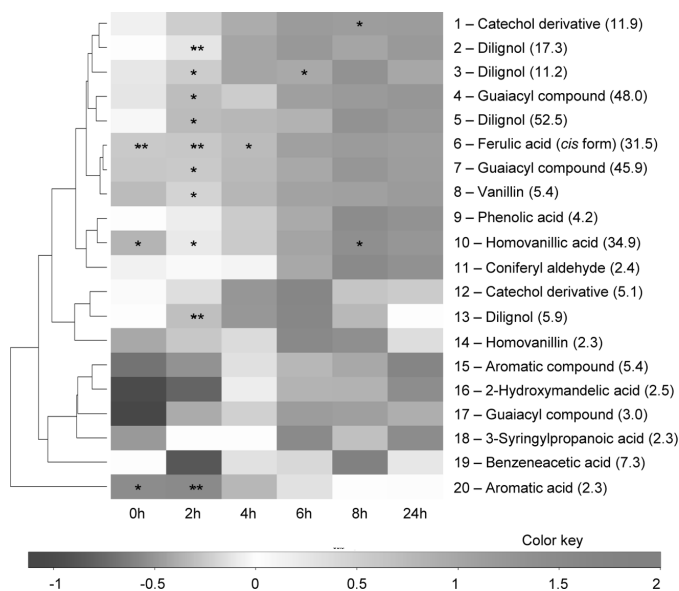
Figure 3. Heat map of the 20 aromatic metabolites from the metabolome. The number after the name in parentheses is the FC of the compound. The color key is the 2-log value of the FC. A blue color indicates an underexpression and red color represents an overexpression of the metabolite compared to the fecal control.

suggesting that *L. salivarius* did not grow on the P-AE fraction as the only substrate. Better growth was observed with the other lactobacilli, and the average ODs of the other lactobacilli with 0.5, 1, and 2% addition of the fraction were 0.220, 0.270, and 0.340, respectively. When basal MRS (including carbohydrates) enriched with the P-AE fraction was used for the incubation of lactobacilli, the OD was significantly higher (1.250−1.500) in the lactobacilli-inoculated media than in the medium without lactobacilli (OD = 0.180−0.300).

The culture-based studies indicated that the growth of *L. rhamnosus* strains in medium supplemented with 1% P-AE fraction (8.0−8.1 log units) was similar to the growth in the glucose-supplemented medium (8.2−8.3 log units). The colony counts of *L. paracasei* were slightly lower in 1% P-AE fraction supplemented medium (7.8 log units) than in the glucose-supplemented medium (8.2 log units). The colony counts of *L. salivarius* were 1.5 log units lower in the medium supplemented with 1% P-AE fraction than in the glucose-supplemented medium. The bifidobacterial colony counts after 3 days of incubation in the medium supplemented with 1% P-AE fraction were similar to the ones in the glucose-supplemented medium (7.5−8.2 log units, depending on the strain). However, after 7 days of incubation, the colony counts of *B. adolescentis*, *B. breve*, and *B. longum* in the medium supplemented with 1% P-AE fraction were 6.3, 7.1, and 6.5 log units, respectively, whereas the colony counts in the glucose-supplemented medium were below 2 log units after 7 days.

Metabolite Formation in the in Vitro Colon Model.
Metabolomics. The formation of metabolites from the P-AE fraction in the in vitro colon model fermentation was followed over 24 h, and approximately 1800 metabolites were discovered. The metabolites were filtered according to the difference in responses between the fraction and the fecal control, that is, the fold-change (FC). FC was calculated as

average from all six time points. Metabolites with FC < 2 were excluded. There were 699 metabolites with FC > 2, and from those the compounds with structural similarity to lignin-derived components were selected. With these criteria, the total number of compounds was narrowed down to 26. On the basis of the mass spectra, as many as 20 of them turned out to be aromatic in nature; they are presented in the heat map with their FC values (Figure 3). Several of the identified metabolites showed low response in the beginning of the fermentation. However, toward the end of the fermentation the response of most compounds was intensified, displayed as a deeper red color in the heat map.

Several monomeric compounds could be fully identified using a commercial library and extensive collection of literature data.[26,27] These included several typical lignin-type compounds with guaiacyl (4-hydroxy-3-methoxyphenyl) structure (compounds **8**, **10**, **11**, and **14**) and one with syringyl (4-hydroxy-3,5-dimethoxyphenyl) structure (compound **18**). In addition, the mass spectra of several other compounds (**4**, **7**, and **17**) showed certain features characteristic of guaiacyl derivatives. Two of them (**17** and **7**) are clearly homologous compounds, with molar masses of 414 and 428 (as TMS derivatives). Despite that and the presence of intense m/z 209 ions, their final structures are still unknown. Compound **4** represents a 4-hydroxy-3-methoxybenzyl alcohol derivative (as shown by the base peak at m/z 297), but the molar mass could not be reliably determined. In addition to the above lignin-type monomeric compounds, several other aromatic monomers (**1**, **9**, **12**, **15**, **16**, **19**, and **20**) were also found. Some of them could be characterized only as catechol derivatives. Compound **15** represents 3,4-dihydroxycinnamyl alcohol with high probability.

Several late-eluting (retention indices >2700) aromatic compounds, dilignols or lignans, were also found. Without more detailed discussions, it can be said that in most cases their
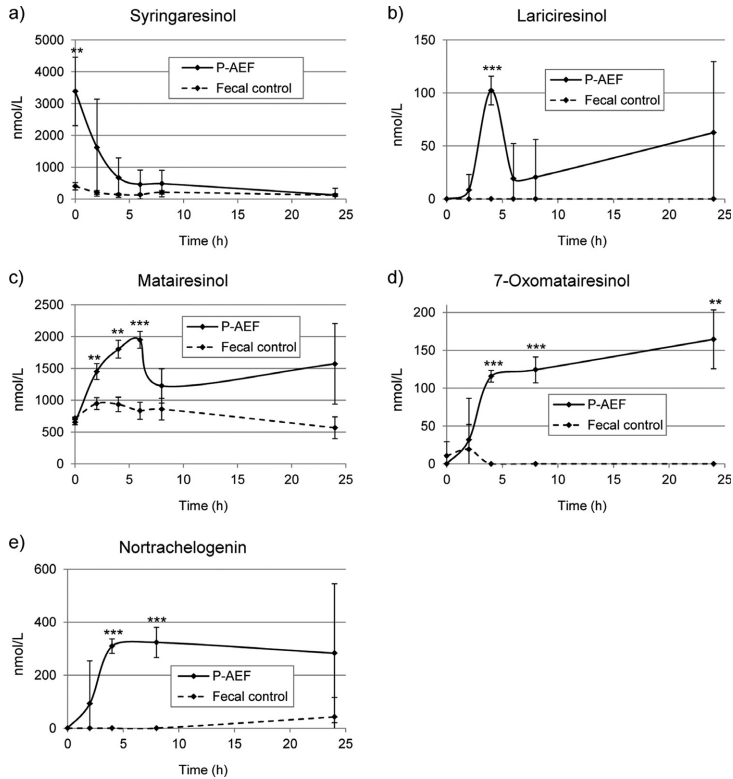
Figure 4. Concentrations of lignans during in vitro fermentation: (a) syringaresinol; (b) lariciresinol; (c) matairesinol; (d) 7-oxomatairesinol; (e) nortrachelogenin. P-AEF, protease-alkaline extracted fraction.
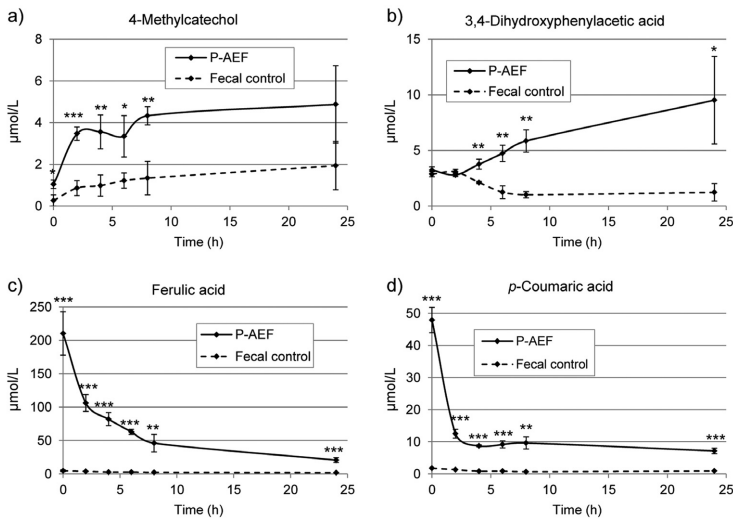

Figure 5. Concentrations of phenolic compounds during the in vitro fermentation: (a) 4-methylcatechol; (b) 3,4-dihydroxyphenylacetic acid; (c) ferulic acid; (d) p-coumaric acid. P-AEF, protease-alkaline extracted fraction.

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mass spectra exhibited intense m/z 179, 209, 267, or 297 ions, indicating the presence of guaiacyl or catechol structures. Their molar masses (as the TMS derivatives) could not always be reliably determined, but several peaks in the m/z range from 500 to 650 were usually found.

Lignans. The concentrations of several lignans, analyzed by HPLC-MS/MS, increased in the course of the fermentation of the lignin-rich fraction (Figure 4). Nortrachelogenin was formed in quantities many times higher than in the fecal control. Matairesinol and its derivative 7-oxomatairesinol also showed significant increase compared to the control. Lariciresinol was above the control, but there were large variations between the replicates. However, as lariciresinol was always below the detection limit in the control, it was considered a metabolite and not originating from the fecal matrix. Enterolactone and its derivative 7-hydroxyenterolactone levels were below the control, and enterodiol formation was somewhat above the control, but there were large variations between the replicates (data not shown). Cyclolariciresinol, pinoresinol, syringaresinol, and α-conidendrin seemed to be present already in the P-AE fraction, because their concentrations were significantly higher in the beginning of the fermentation compared to fecal control, so they were not considered metabolites, and secoisolariciresinol seemed to originate from the fecal background (data not shown except for syringaresinol).

Phenolic Compounds. A number of different phenolic compounds were identified and quantitated, from which 4-methylcatechol and 3,4-dihydroxyphenylacetic acid differed significantly from control (Figure 5). The concentrations of both ferulic acid and p-coumaric acid declined rapidly during the fermentation. Other detected phenolic acids did not differ significantly between the P-AE fraction and the fecal control and thus were not considered metabolites from the P-AE fraction.

■ DISCUSSION

BSG is the most abundant side-stream generated by the brewing industry. It contains the hulls and outer layers of the barley kernels, and it is thus rich in cellulose, arabinoxylan, lignin, and protein. A lignin-rich fraction was prepared from BSG by enzymatic methods as described previously,[18] by precipitation of the liquid phase after an alkaline protease treatment carried out on carbohydrase-pretreated BSG. The fraction, named the protease-alkaline extracted fraction, was considered lignin-rich compared to commonly consumed foods, even though its lignin content was no higher than 24%. In addition, the insoluble residue after the protease treatment contained more lignin (34%)[18] than the P-AE fraction; nevertheless, the P-AE fraction was used for the in vitro studies, because the lignin in it was considered more accessible for the microbiota and enzymes as it was not trapped inside a carbohydrate network. It can be assumed that lignin in the P-AE fraction is not as much cross-linked to carbohydrates as lignin in the insoluble residue, which contains the most recalcitrant carbohydrates. For the experiments with lactobacilli and bifidobacteria, lipids were removed from the fraction to avoid giving the bacteria an alternative carbon source, which increased the lignin content of the fraction to 41%.

The carbohydrates that are resistant to human digestive enzymes are fermented by colon microbiota to SCFA, which are major metabolites in the colon. It is possible to use the SCFA formation as an indicator in determining whether or not

the fermented substrate suppresses the conversion activity of the microbiota, as shown earlier.[15] As seen in Figure 2, the concentrations of linear SCFA were higher in the P-AE fraction than in the fecal control, suggesting that the lignin in the P-AE fraction did not suppress microbial metabolism. The small increase in SCFA production was most likely due to the residual carbohydrates present in the fraction. The production of SCFA in the colon is desirable, as, for example, butyric acid is the major energy source for colon epithelial cells and may help to protect against colon cancer.[28] Propionic acid is also associated with beneficial health effects, such as lower lipogenesis and serum cholesterol.[29] Therefore, a diet high in slowly fermentable DF is necessary for the health and well-being of humans and especially of the gut. It is also worth pointing out that according to these results, even the very high ratio of lignin to carbohydrates (6:1) did not prevent microbes from fermenting the carbohydrates present in the P-AE fraction to SCFA.

The lignin-rich fraction as a growth substrate did not inhibit the growth of lactic acid bacteria and bifidobacteria, which are regarded as beneficial bacteria in the human colon. Moreover, it seemed that some component of the fraction enabled a longer growth of bifidobacteria, because after 7 days of incubation, there was still growth in bifidobacterial cultures with 1% lignin preparation as the sole carbohydrate source (10^7–10^8 cfu/mL), whereas with glucose the numbers of bifidobacteria were below 10^2 cfu/mL. A similar protective effect on bifidobacteria has been previously observed with oat fiber preparations.[30] The carbohydrate content of solvent-extracted P-AE fraction was only 6.7%, but there was also 45% of proteinaceous material. There is not much information in the literature regarding proteolytic abilities of bifidobacteria. Most of the studies are related to dairy products and report only limited proteolytic activity of bifidobacteria toward dairy proteins.[31] The effect of cereal proteins on bifidobacterial growth is not well studied, and therefore it is difficult to estimate if bifidobacteria could survive on the peptides extracted from BSG. Nevertheless, some component in the P-AE fraction kept the bifidobacterial cells alive longer than an equal amount of glucose.

The in vitro colon model used in this investigation is a metabolic model designating biochemical changes in introduced components due to their metabolism by the fecal microbiota, which is pooled and collected from several donors and used as an inoculum. This model has been used in biochemical conversions of carbohydrates, phenolic components, and pharmaceuticals detected by targeted GC-MS and nontargeted GC×GC-TOFMS methods as described in previous studies.[15,32] The batch model is not suitable for studies concerning changes in microbial composition, because the incubation time and batch design are not sufficient, but changes in microbial metabolites depend only on the components in the studied substrates and are not due to changes of the microbiota. Therefore, the model is well suited for the detection of the release of low molecular weight aromatic compounds from lignin.

The major phenolic compounds in BSG are p-coumaric acid, ferulic acid and its dimers,[33] and lignin. As the possible degradation of lignin was studied, low molecular weight phenolic compounds were the most searched metabolites. Lignin is generally considered to be an inert material in the colon. However, in this study several metabolites, which very likely originated from lignin, were identified (Figure 3). For some of the metabolites the exact structure could not be

determined, and therefore they were indicated with a group-specific name only. The possibility of some of the metabolites originating from phenolic precursors other than lignin, such as ferulic acid, cannot be excluded. However, the metabolism of ferulic acid in the gut is well-known; the main metabolite from monomeric ferulic acid is 3-(3′,4′-dihydroxyphenyl)propionic, and in addition 3′,4′-dihydroxyphenylacetic acid is formed from 8-O-4-linked diferulate with homovanillic and dihydroferulic acids as intermediates.[34] Approximately two-thirds of the ferulic acid in BSG occurs in monomeric form and one-third as different types of dimers.[33]

Different types of guaiacyl compounds and dilignols with FC as high as around 50 were detected, but, on the other hand, many of the possible lignin-derived compounds also had lower FC, such as vanillin (5.4), coniferyl aldehyde (2.4), and 3-syringylpropanoic acid (2.3) (Figure 3). The latter is especially interesting, as it contains two methoxyl groups and thus cannot be formed from ferulic acid, which has only one methoxyl group. The release of these low molecular weight phenolic compounds would indicate that partial degradation of lignin could have occurred due to microbial action. As the metabolite analysis is semiquantitative, it is difficult to estimate the amount of the released phenolic metabolites, but it was assumed to be very limited as indicated by the low FCs. The late appearance of many of the lignin-related metabolites also suggests that the release of these compounds occurs slowly. This would seem reasonable and in line with the idea that lignin is a difficult substrate to be degraded by the colon microbiota.[5,6] However, these results should be considered as preliminary and indicative, because to have solid proof of lignin degradation by gut microbiota, isotope-labeled lignin should be used as the substrate.

Several lignans have been previously identified in the extracted P-AE fraction.[18] Lignans are low molecular weight phenolic compounds found in plants, and they are commonly known to have several health-promoting properties, such as antitumor and antioxidant activities.[35] The structure of lignans consists of two β-β-linked phenylpropan units with various hydroxyl and methoxyl substitution patterns in the aromatic rings and other structural modifications. Structures of some lignans, for example, pinoresinol and lariciresinol, exist in lignin,[36] and therefore it is possible that lignans or lignan-like compounds could be released from lignin as a result of microbiological activity, as shown by Begum et al.[36] In this study several lignans were released or formed from the P-AE fraction at significantly higher concentrations than from the fecal control. Syringaresinol is the most abundant lignan in the P-AE fraction,[18] and according to Heinonen et al.[37] it is converted to several other metabolites, and only to a low extent to enterodiol and enterolactone. In this study syringaresinol was shown to disappear during the incubation. Nortracheloge-nin is the second most abundant lignan in the P-AE fraction,[18] but it seemed to be incorporated into the matrix and released by microbial activity over time. 7-Oxomatairesinol was not detected in the fraction, but was probably converted from 7-hydroxymatairesinol. It is possible that a part of the lignans could have been released from lignin structure and a part would have been converted from other lignans, as the gut microbiota is capable of releasing and transforming lignans.

Potential health effects on digestion have been suggested to arise from lignin. Lignin has been shown to be a precursor of the mammalian lignans enterodiol and enterolactone,[36] higher concentrations of which have been associated with a lower risk

of cancers and cardiovascular disease.[38,39] Lignin-enriched DF can adsorb carcinogenic compounds in the colon,[40,41] and the adsorption of the carcinogens by lignin may prevent their absorption from the gut into circulation and thus reduce the risk of cancer.[41] Insoluble fiber is known to deliver antioxidative, phenolic compounds into the gastrointestinal tract,[42] and due to its phenolic structure, lignin also possesses such antioxidative and radical scavenging activity in the lumen.[43]

In conclusion, a lignin-rich fraction originating from BSG did not suppress the conversion activity of gut microbiota in an in vitro colon metabolic model. Neither did it inhibit the growth of beneficial gut bacteria, lactobacilli and bifidobacteria. Moreover, some component in the P-AE fraction enabled the growth of bifidobacteria for a longer time than glucose. Several low molecular weight, lignin-like phenolic metabolites were formed during the fermentation, which suggests partial degradation of lignin in the colon. Most likely, the release of these metabolites occurs only slowly and to a limited extent. More research is needed to confirm the origin of these compounds as other analogous compounds were also present in the P-AE fraction.

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Release of Small Phenolic Compounds from Brewer's Spent Grain and Its Lignin Fractions by Human Intestinal Microbiota in Vitro

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S Supporting Information

ABSTRACT: Brewer's spent grain (BSG), the major side-stream from brewing, is rich in protein, lignin, and nonstarch polysaccharides. Lignin is a polyphenolic macromolecule considered resilient toward breakdown and utilization by colon microbiota, although some indications of release of small phenolic components from lignin in animals have been shown. The aim of this study was to investigate if the human intestinal microbiota can release lignans and small phenolic compounds from whole BSG, a lignin-enriched insoluble fraction from BSG and a deferuloylated fraction, in a metabolic in vitro colon model. The formation of short-chain fatty acid (SCFA) was also investigated. More lignin-related monomers and dilignols were detected from the lignin-enriched fraction than from BSG or deferuloylated BSG. SCFA formation was not suppressed by any of the fractions. It was shown that small lignin-like compounds were released from these samples in the in vitro colon model, originating most likely from lignin.

KEYWORDS: brewer's spent grain, intestinal microbiota, in vitro colon model, lignin, lignan

■ INTRODUCTION

Brewer's spent grain (BSG) is the major side-stream from the brewing of beer. It is composed of the husks and outer layers of malted barley grains together with the residual endosperm remaining after mashing. As such, it is rich in protein and dietary fiber (DF), including arabinoxylan, cellulose, and lignin.[1] So far, the utilization of BSG has been limited to ruminant feed with low commercial value. However, BSG could be a source of nutritional ingredients or platform chemicals, if cost-efficient fractionation techniques can be developed.

As a component of feed, lignin is considered to be poorly digested by rumen microbiota,[2] and thus it most likely remains in the gut lumen, where it could interact with other dietary components or affect conversion activities of gut microbiota. The breakdown of lignin has been demonstrated in the rumen of goats,[3] and lignin was shown to be a precursor of the mammalian lignans enterodiol and enterolactone in rats,[4] suggesting that nonruminants could also be able to degrade lignin. However, the ability of human gut microbiota to degrade lignin has not yet been proven. The in vitro metabolic colon model used in this study is a model designating biochemical changes in introduced components due to fecal microbial enzymes. This model combined with gas chromatographic analysis coupled with mass detection (GC-MS or GC×GC-TOFMS) and metabolite profiling has been used to investigate biochemical conversions of plant foods and their components, carbohydrates, and phenolic compounds.[5−8]

Lignin is a polyphenolic macromolecule acting as the glue between the cellulose−hemicellulose matrix in plant cell walls. Lignin is formed from three monomers: p-coumaryl alcohol,

coniferyl alcohol, and sinapyl alcohol, which are linked together in a branched network structure by radical-induced condensation reactions. The ratio of these alcohols is dependent on the plant species. Lignin units are derived from methoxylated hydroxycinnamic acids, such as ferulic and sinapinic acids, via the phenylpropanoid pathway, and similar substitutions on the phenol ring with guaiacyl (4-hydroxy-3-methoxyphenyl) or syringyl (4-hydroxy-3,5-dimethoxyphenyl) alcohols, respectively.[9] This creates challenges for the identification of monomers that are uniquely lignin-derived, and therefore the removal of ester-linked ferulates might exclude the impact of the formation of methoxylated degradation products from hydroxycinnamic acids.

Lignin could also act as a suppressive component in the colon model. Dose-dependent suppression of short-chain fatty acid (SCFA) formation by tannins, a polymer formed from the flavanols (−)-epicatechins and (+)-catechins, isolated from apples and grapes, has been previously demonstrated in the in vitro colon model, showing lower SCFA concentrations than in fecal control.[7,8] This was possibly caused by inhibition of cell wall degrading enzymes present in the fecal inoculum through the phenolics binding to these enzymes.[10]

The hypothesis of the present study is that gut human microbiota is able to release low molecular weight phenolic compounds, which can potentially be considered as DF

phytochemicals, from lignin. Therefore, lignin-rich fractions were prepared from BSG by enzymatic treatments hydrolyzing carbohydrates and proteins and by an alkaline treatment removing ester-linked ferulates. The obtained fractions were studied using the in vitro metabolic colon model to investigate the microbial release of lignin-derived monomeric and dimeric compounds. The formation of SCFA was also studied to exclude the possibility of suppression of microbial conversion activities. The suppression of SCFA formation was also studied, even though lignin-rich fractions contained a low amount of accessible carbohydrates and therefore were not eminent precursors for SCFA formation.

■ MATERIALS AND METHODS

Preparation of BSG Fractions. BSG was obtained from Sinebrychoff brewery (Kerava, Finland) directly after the mashing process. BSG was composed of 42% carbohydrates, mainly arabinoxylan and cellulose, 19% lignin, 23% protein, 11% lipids, and 4.7% ash.[11] Although most of the glucan in BSG was cellulose, residual amounts of starch (1.3%) and mixed-link β-glucan (0.36%) were also present. A portion of BSG was dried and milled with a Hosokawa Alpine mill with a 0.3 mm sieve (Hosokawa Alpine AG, Augsburg, Germany). The dried and milled BSG was used as one of the samples in the in vitro fermentation. To remove ester-linked ferulates, BSG was incubated in 2 M NaOH at room temperature for 2 h with continuous stirring. After the incubation, the solids were separated by centrifugation, washed thoroughly with distilled water, and neutralized with HCl. Finally, these solids were lyophilized, and the dried material denoted deferuloylated sample (DEFE). Nondried BSG was milled with a Masuko Supermasscolloider MKZA10-15J (Masuko Sangyo Co. Ltd., Kawaguchi-city, Japan) and sequentially digested first with carbohydrases and then with proteases as described previously (Figure 1).[11] The residue after the proteolytic treatment was further digested
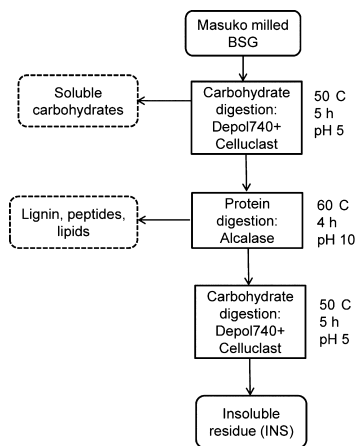
Figure 1. Sequential enzymatic hydrolyses of brewer's spent grain (BSG).

with Celluclast 1.5L (Novozymes, Bagsvaerd, Denmark) (50 FPU/g material) and Depol740L (Biocatalysts Ltd., Cefn Coed, Wales, UK) (5000 nkat of xylanase activity/g material) (5 h, 50 °C, pH 5.0) to remove residual carbohydrates. After the hydrolysis, the solids were separated by centrifugation, washed with distilled water, and lyophilized. The sample was denoted insoluble residue (INS). The composition of BSG and the modified fractions is described in Table 1. The compositions of BSG and its fractions were determined as

Table 1. Compositions of the Fermented Fractions as Mass Percent of Dry Matter

component	initial BSG	insoluble residue (INS)	deferuloylated BSG (DEFE)
carbohydrates	42.2	39.2	53.4
arabinoxylan	22.2	25.7	20.3
glucan	17.1	11.2	30.9
lignin	19.4	40.3	21.9
protein	22.8	6.6	7.6
lipids	11.0	3.1	2.9
ash	4.7	8.7	6.0

described previously.[11] Klason lignin was determined gravimetrically and acid-soluble lignin as UV absorbance of the hydrolysate.[11]

Microscopy Imaging. Light microscopy was carried out as described previously.[12] In brief, samples were embedded in a hydroxyethyl methylacrylate matrix from which 2 μm thick sections were cut. Prior to imaging with the microscope, the sections were treated with chemical dyes Calcofluor and Acid Fuchsin to enable visualization of different components of BSG. Acid Fuchsin stains protein red, and Calcofluor stains β-glucan bright blue. The autofluorescence of lignin and other phenolics is seen as yellow and green. The excitation and emission wavelengths used were 330−385 and >420 nm, respectively.

In Vitro Colon Model. Fermentation of BSG and the modified fractions in the in vitro colon model was performed according to the method of Barry et al.[13] with the following modifications: 200 mg (on dry weight basis) of BSG or its fractions was weighed into bottles (50 mL) and hydrated with 2 mL of medium 1 day before inoculation. Medium was an 0.11 M carbonate−0.02 M phosphate buffer (pH 6.5) including 0.5 g/L L-cystein with the addition of 5 mL of mineral solution.[13] Human feces were collected from five healthy volunteers who had not received antibiotics for at least 6 months and had given written consent. The collection of fecal samples was performed with the approval of and according to the guidelines given by the Ethical Committee of VTT Technical Research Centre of Finland. Freshly passed feces were immediately taken in an anaerobic chamber or closed in a container with an oxygen-consuming pillow (Anaerocult Mini; Merck, Darmstadt, Germany) and a strip testing the anaerobiosis (Anaerotest; Merck). Fecal suspension was prepared under strictly anaerobic conditions. Equal amounts of fecal material from all donors were pooled and diluted to a 20.8% (w/v) suspension, 8 mL of which was dosed to the fermentation bottles to obtain a 16.7% (w/v) final fecal concentration described previously.[5,14] The fermentation experiments were performed in triplicate, and a time course of 0, 2, 4, 6, 8, and 24 h was followed using the same inoculum for all of the substrates. Incubation was performed at 37 °C in tightly closed bottles and in magnetic stirring (250 rpm). Fecal background was incubated without addition of the supplements.

Extraction of Fermentation Samples. Extraction of SCFA to diethyl ether was carried out as described previously.[15] For phenolic compound, lignan, and metabolome analyses, 1 mL of the fermented sample was thawed. One milliliter of 2% NaCl solution was added to break the emulsion and to ensure the separation of the solvent phase. Fifty microliters of 6 M HCl was added to lower the pH to near 1. For the analysis of phenolic compounds and the metabolome, trans-2-hydroxycinnamic acid (Aldrich, St. Louis, MO, USA) was used as the internal standard, and 15 μL (123 mg/L in MeOH) was added to the fermented samples. The samples were subsequently extracted twice with 3 mL of ethyl acetate. The organic phases were collected, combined, and evaporated under nitrogen. For lignan extraction, 20 μL of an ethyl acetate solution containing the internal standards matairesinol-d_6 (430 ng), secoisolariciresinol-d_6 (524 ng), enterolactone-d_6 (316 ng), and dimethylated pinoresinol-d_6 (756 ng) (all prepared at the Laboratory of Organic Chemistry at Åbo Akademi University) was added to 1 mL of the fermented sample. The samples were extracted twice with methanol (3 mL), and the liquid phases were combined. The methanol−water mixture was evaporated under

nitrogen stream. The dried samples were stored under a nitrogen atmosphere at −20 °C until analyzed.

SCFA Analysis. Diethyl ether extracts (2 μL, splitless injection) were analyzed with gas chromatography with flame ionization detector (GC-FID) (Agilent 6890 series, Palo Alto, CA, USA). Analytes were separated on a DP-FFAP capillary column (30 m × 0.32 mm) with a phase thickness 0.25 μm (Agilent). Helium was used as the carrier gas (2.7 mL/min). Both the injector and FID were kept at 250 °C. The temperature program started at 50 °C with a 3 min holding time, then increased at 25 °C/min to 100 °C, finally increasing at 10 °C/min to the final temperature of 240 °C, which was held for 10 min. Compounds were quantitated with corresponding standards. SCFA formation was expressed as a sum of acetic, propionic, and butyric acids. The individual SCFA concentrations were calculated from the averages of three replicates at each time point (0−24 h). The initial SCFA formation rate was calculated as follows: The averages of the sum of acetic, propionic, and butyric acid concentrations (mM) were named SCFA (total). SCFA(total, 0 h) was reduced from the SCFA (toalt, 2 h), and this reduction was divided by 2 h, the time interval.

Metabolomics. The analysis was performed using a two-dimensional gas chromatograph equipped with a time-of-flight mass spectrometer (GC×GC-TOFMS) as previously described.[8] The phenolic acids were quantitated with authentic standards. The following compounds were used as standards: Benzoic acid, 3-hydroxybenzoic acid, 3-(4′-hydroxyphenyl)propanoic acid, and 3-(3′,4′-dihydroxyphenyl)propanoic acid were products from Aldrich (Steinheim, Germany). 4-Hydroxybenzoic acid, 2-(3′-hydroxyphenyl)-acetic acid, and 2-(3′,4′-dihydroxyphenyl)acetic acid were purchased from Sigma (St. Louis, MO, USA). 3-Phenylpropnoic acid and 3,4-dihydroxybenzoic acid were from Fluka (Buchs, Switzerland), and 3-(3′-hydroxyphenyl)propanoic acid was purchased from Alfa Aesar (Karlsruhe, Germany). 4-Methylcatechol (Aldrich), vanillic acid (4-hydroxy-3-methoxybenzoic acid, Fluka), p-coumaric acid (Sigma), and gallic acid (3,4,5-trihydroxybenzoic acid were from Extrasynthése (Genay, France), and ferulic acid was from Sigma-Aldrich (St. Louis, MO, USA). N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) and methoxyamine 2% hydrochloride in pyridine (MOX) from Pierce (Rockford, IL, USA) were used as the derivatization reagents.

The data processing of GC×GC-TOFMS responses has been described earlier.[8] Briefly, the peaks were identified by ChromaTOF software, which matches deconvoluted spectra against an NIST05 mass spectral library. The compounds in different data sets were aligned and normalized using an in-house-developed software, Guineu,[16] for further analyses. Alignment of the data was performed on the basis of retention indices, second-dimension retention times, and spectra.

GOLM Metabolome Database (GMD),[17] the Guineau program,[16] and relevant literature were utilized for second-stage identification of the compounds, which lacked sufficient spectral matches from the NIST05 or in-house-collected libraries. GMD allows searching of the database based on submitted GC-MS spectra, retention indices, and mass intensity ratios. In addition, the database allowed a functional group prediction, which helped characterize the unknown metabolites without available reference mass spectra in the GMD.

Aligned data were filtered using the Guineu program by calculating the fold changes (FC) using the Fold test function describing how many times the test group exceeded the responses of the control group. Aromatic compounds, which showed FC responses >2, were included in the list of further identified compounds and displayed in the heat map. Heat maps created with Guineu showed the 2-based log FC values as red (overexpressed) or blue (underexpressed) color intensities for each time point and each metabolite. Asterisks showed the time point specific significances (∗, $p < 0.05$; ∗∗, $p < 0.01$; ∗∗∗, $p < 0.001$) for each metabolite, and clustering was performed according to the similarity of the profiles.

Lignan Analysis. To the dried methanol extracts was added 1.5 mL of 10 mM sodium acetate buffer (pH 5.0), and the solutions were sonicated for 1−2 min or until the material was completely dissolved. The solutions were then centrifuged for 15 min, and the supernatant was carefully removed and centrifuged again for 15 min. The clear

supernatant was solid-phase extracted using Oasis HLB 30 mg cartridges (Waters Corp., Milford, MA, USA) according to a previously described method.[18] After evaporation of the solvent to dryness, 200 μL of methanol/0.1% acetic acid in Milli-Q water (20:80, v/v) was added, the solution was sonicated for 1−2 min, and 10−20 μL was injected into the HPLC-MS/MS. The HPLC-MS/MS method and conditions were the same as described previously.[19] Quantitation was carried out using standard solutions containing the internal standards and six concentration levels of the analyzed lignans, as described previously.[19] The standard solutions were solid-phase extracted and redissolved as the real samples.

■ RESULTS

Potential degradation of lignin from BSG, INS, and the deferuloylated (DEFE) fraction after incubation in an in vitro metabolic colon model was studied by investigating the metabolite profiles corresponding to the release and conversion of lignans and the formation of phenolic acids. In addition, the formation of SCFAs was studied in the in vitro colon model to estimate residual carbohydrate fermentation and potential suppression of the fermentation by high lignin content.

Composition of BSG and Its Fractions. The compositions of BSG, INS, and DEFE are summarized in Table 1. Carbohydrate content was lowest for INS (39.2%) followed by BSG (42.2%), and DEFE showed the highest content of carbohydrates (53.4%) due to reduction in lignin, protein, and lipid levels by the alkaline treatment. Residual starch (1.3%) and β-glucan (0.36%) were present in BSG, but mostly the glucan fraction consisted of cellulose.[11] Similarly, in the INS and DEFE fractions, predominantly the glucan was cellulose, but trace amounts of starch and β-glucan may have survived the enzymatic and alkaline treatments. Lignin content was highest, as expected, in INS (40.3%), whereas the lignin contents were similar in DEFE (21.9%) and BSG (19.4%) (Table 1). Lipid content was highest in initial BSG (11.0%) and decreased due to the fractionation steps (3.1 and 2.9% for INS and DEFE, respectively). Protein content in BSG was 22.9%, and it was markedly decreased by the fractionation (6.6 and 7.6% for INS and DEFE, respectively). According to Figure 2A, protein in BSG was mostly encapsulated within the aleurone cells. In the INS fraction, protein was hardly visible due to the preceding proteolysis (Figure 2B). The ash content was between 4.7 and 8.7% in the samples.

Metabolite Profiling. After incubation with fecal microbiota, a wide range of metabolites (FC > 2) were detected for all samples: 694 for BSG, 480 for INS, and 572 for DEFE. From these metabolites, the preliminarily identified (NIST05) small aromatic ones were selected, and structural relevance was confirmed during the final identification (GMD, retention indices and mass spectra) for the heat maps (Figure 3). The heat maps visualize the profiles of overexpressed aromatic metabolites formed from BSG, INS, and DEFE during incubation with human fecal microbiota and analyzed by GC×GC-TOFMS using nontargeted metabolite profiling against the fecal control (no sample added). The number of identified aromatic metabolites with statistical significance was remarkably higher in the lignin-enriched INS fraction (Figure 3B) than in the BSG or DEFE fractions (Figure 3A,C).

BSG was a source of eight aromatic metabolites, of which compounds **1−3** (4-methylcatechol, a dilignol, and ferulic acid) showed significant ($p < 0.05$) differences from the fecal control at several time points (Figure 3A). The structure of dilignol **2** (Figure 3A) could not be determined, although an intense m/z 180 ion suggests an enterolactone-type compound.[20] It is
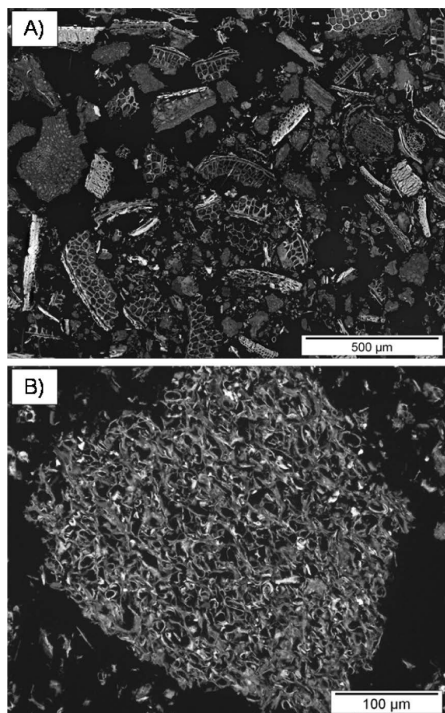
Figure 2. Optical microscopy images of (A) coarse-milled brewer's spent grain (BSG) and (B) insoluble residue (INS). The blue dye indicates cell walls of aleurone and endosperm cells, and the red dye indicates protein. Cell walls containing phenolic compounds are seen as light green due to their autofluorescence.

noteworthy that the other dilignol (compound **6**) appears to have the molar mass of 618, suggesting a tetrakis (TMS) derivative of dihydroxylated enterolactone (or a related compound). The base peak at m/z 179 in its mass spectrum indicates a (mono)hydroxyphenyl compound, but no further structure determination was possible. There was also a guaiacyl compound (number **4**) present, as indicated by the base peak at m/z 297, characteristic of trimethylsilylated compounds with 4-hydroxy-3-methoxybenzyl alcohol structures. Unfortunately, full identification was not possible.

Many of the metabolites originating from the INS fraction (compounds **8**, **13−18**, and **20**) were recognized as dilignols according to their mass spectra and high retention indices (typically >2700) (Figure 3B), whereas compounds **1−3**, **5−7**, **10**, and **26** were methoxylated aromatic compounds including ferulic acid. Compound **9** (4-methylcatechol), phenolic acids (**19**, **21**, and **22**) and compound **25** (4-hydroxymethylcatechol) possessed catechol or other dihydroxyphenyl structures. All or most of these structures can be derived from lignin. It is noteworthy that the aromatic metabolites **2**, **5**, **6**, and **10** in the INS fraction can also be derived from ferulic acid, in addition to lignin. Compound **26** was the only syringyl-type aromatic compound, apparently derived only from lignin. Its final identification was based on published data.[21]

As a whole, eight different dilignols were recognized (Figure 3B). Their full identifications were not possible, but certain

structural features can be distinguished on the basis of a few characteristic ions. Thus, for example, the dilignols **8** and **13** represented compounds with catechol structures (indicated by intense m/z 267 and 179 ions), and compound **15** had a guaiacyl (4-hydroxy-3-methoxyphenyl) unit (intense m/z 209 ion). Three dilignol compounds (**16−18**) had very intense (up to 100%) m/z 179 ion peaks, suggesting (mono)hydroxyphenyl structures in their molecules. Thus, in most cases, clear structural links to lignin macromolecule are apparent.

The four dilignols detected from the DEFE fraction (Figure 3C) were released at later time points. Of them, compounds **1**, **7**, and **9** represent (mono)hydroxyphenyl-type dilignols (intense m/z 179 ion) and compound **2** represents a guaiacyl compound (intense m/z 209 ion). Two of them (compounds **1** and **2**) showed high statistical significance ($p < 0.01$). It is worth noting that some catechol compounds were released from the DEFE fraction as well.

Fold changes between the metabolites originating from the substrates versus from the fecal control were 2.1−7.0 for BSG, 2.0−13.0 for INS, and 2.0−11.8 for DEFE. It seems that the release of lignin-derived metabolites may occur by human microbiota, but slowly and probably only to a small degree, because the FCs were only between 2 and 13. The highest FCs were observed for dilignols, vanillin, methoxybenzenediol, and a guaiacyl compound (Figure 3). FC of the guaiacylpropanoic acid (compound **5**, Figure 3B) was high at both 0 and 24 h but showed lower FC in the middle of the incubation.

Quantitative Analysis of Phenolic Metabolites. Targeted quantitative analysis of the phenolic metabolites from BSG and the INS and DEFE fractions is shown in Figure 4. The four metabolites are shown here because of their structural relevance to lignin (4-methylcatechol) or their connection to feruloylation of lignin and ferulate metabolism in the colon[22] (ferulic acid, 3-hydroxyphenylpropanoic acid, and 3′,4′-dihydroxyphenylacetic acid). Of the metabolites, 4-methylcatechol was formed in significantly higher concentrations from INS and BSG than from the fecal control, whereas the lowest concentrations of the fractions were from the DEFE fraction, which was slightly above the fecal control (Figure 4A). As could be expected, ferulic acid was released in notably higher concentrations from BSG than from DEFE during the incubation with fecal microbiota (Figure 4B).

3-Hydroxyphenylpropanoic acid was present in surprisingly high concentrations already at the beginning of the incubation, also in the fecal control, after which a declining profile was apparent. After the decline, a small increase was observed at 8 h for fecal control and the INS fraction. 3,4-Dihydroxyphenylacetic acid concentration increased significantly for BSG and INS, but this compound was completely absent from DEFE, because the concentrations were mainly below the fecal control. In addition, 3,4-dihydroxyphenylpropanoic acid, 4-hydroxyphenylpropanoic acid, 3,4-dihydroxybenzoic acid, 3-hydroxybenzoic acid, vanillic acid, caffeic acid, and 4-coumaric acid were detected in the fermented samples. However, they were not considered true metabolites because they did not differ significantly from the fecal control, meaning that they most probably originated from the feces, or their concentrations did not significantly change during the incubation.

Quantitative Analysis of Lignans. Lignan concentrations during the incubation in the model were low but differed significantly from the fecal control at certain time points. Syringaresinol showed significantly different concentrations at 0 h for BSG and its fractions (INS > BSG > fecal control >
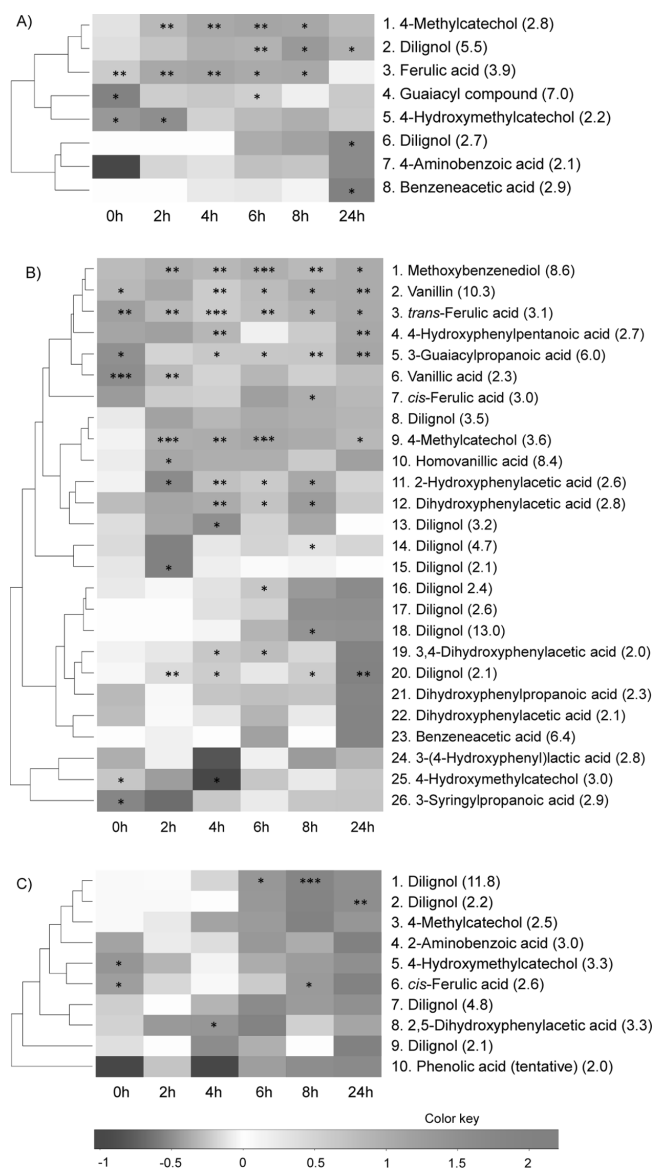
Figure 3. Heat maps of the aromatic metabolites formed in the metabolic in vitro colon model for (A) initial brewer's spent grain (BSG), (B) insoluble residue (INS), and (C) deferuloylated BSG (DEFE). The number after the name in parentheses is the fold change (FC) of the metabolite compared to fecal control (microbiota without substrate). The color key is the 2-log value of the FC. Blue color indicates an underexpression and red color represents an overexpression of the metabolite compared to the fecal control. Significant differences (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) are expressed as asterisks.

DEFE) (Figure 5A). α-Conidendrin concentration from INS was highest at 0 h and differed from the fecal control, and by 8 h DEFE and BSG incubations reached the same level, but fecal control remained at the baseline. Due to high standard deviations of the INS fraction, no significant differences were observed between samples after 0 h (Figure 5B). Matairesinol concentrations from INS and BSG were significantly higher at 2

h compared to the fecal control, whereas DEFE incubation showed intermediary levels (Figure 5C). All lignans were further converted, because their concentrations declined toward the end of the incubation (Figure 5). In addition, secoisolariciresinol, cyclolariciresinol, enterodiol, enterolactone, 7-hydroxyenterolactone, and 7-oxoenterolactone were detected in the fermented samples. However, their concentrations were
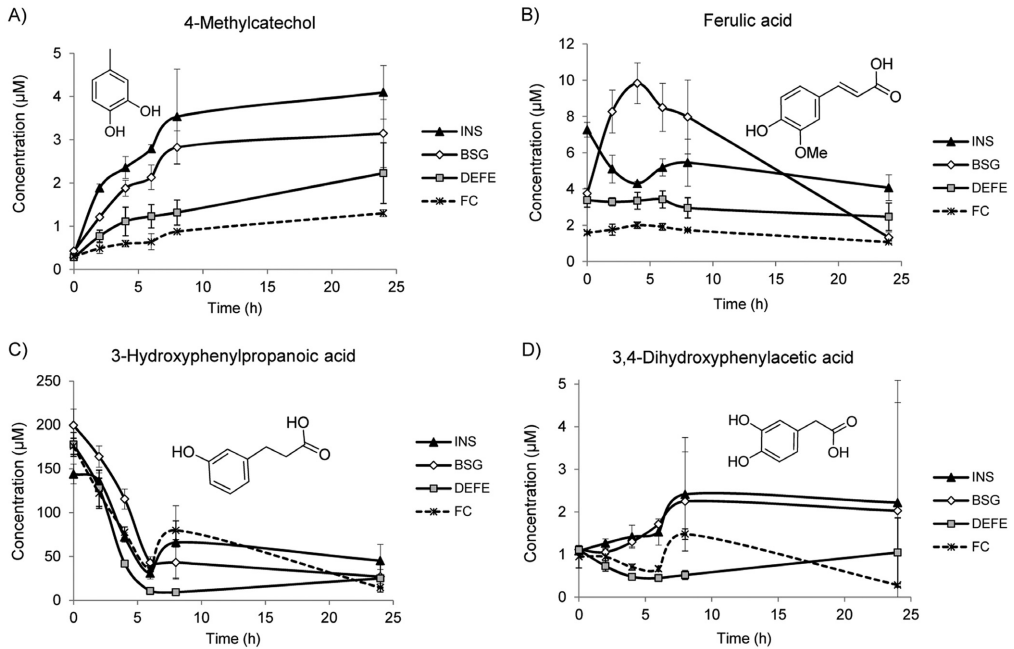
Figure 4. Concentrations of phenolic compounds during the in vitro fermentation of brewer's spent grain and its fractions: (A) 4-methylcatechol; (B) ferulic acid; (C) 3-hydroxyphenylpropanoic acid; (D) 3,4-dihydroxyphenylacetic acid.
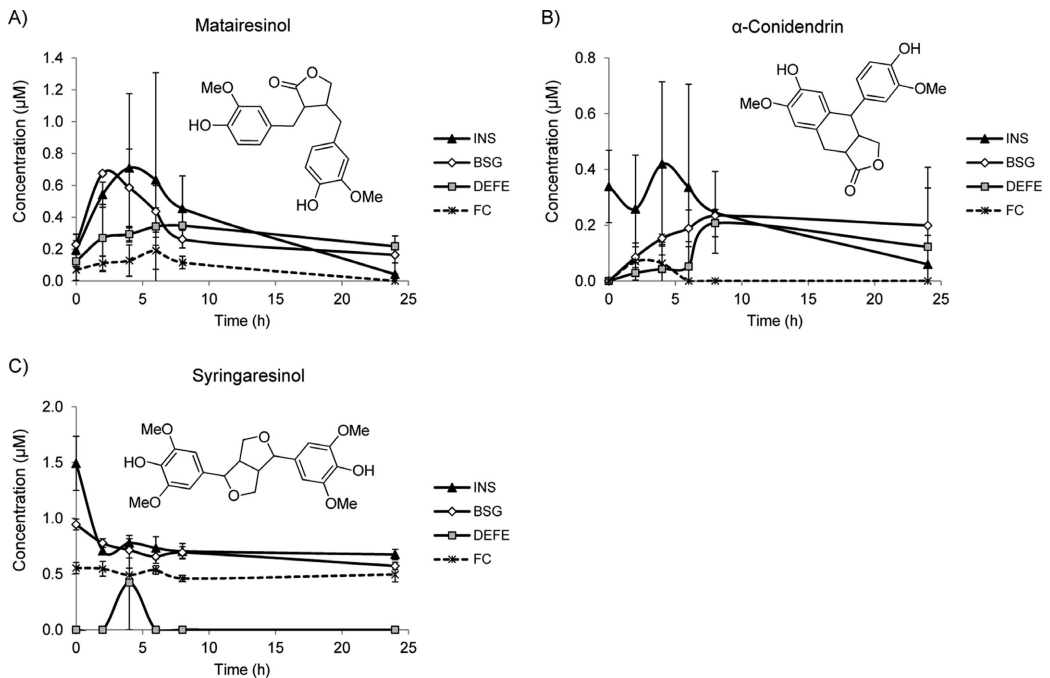
Figure 5. Concentrations of lignans during the in vitro fermentation: (A) matairesinol; (B) α-conidendrin; (C) syringaresinol.

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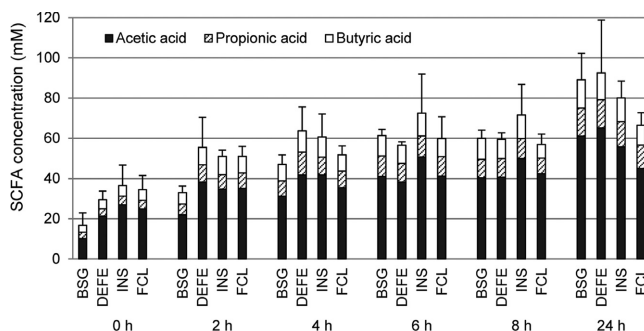
Figure 6. Concentrations of linear short-chain fatty acids formed during in vitro fermentation. The error bar is the combined standard deviation of the three fatty acids.

not significantly different from the fecal control, which means that they most likely originated from the diet of the donors of the feces used in the model and not from the BSG samples.

Formation of SCFA. SCFA formation occurred at a slow rate for all of the substrates (Figure 6). The initial formation rate (0−2 h) was fastest for DEFE (13.0 mM/h) followed by BSG and fecal control (8.2 mM/h). The lowest rate (7.3 mM/h) was observed for INS. There was no indication of suppression of SCFA formation by lignin, because SCFA levels from BSG and its fractions were above those of the fecal control and the carbohydrates originating from the feces and BSG and its fractions were partially fermented. The differences between samples were not significant even in comparison with the fecal control because of high standard deviations between replicates, especially for DEFE and INS fractions.

DISCUSSION

In this study, the emphasis was to study potential release of lignan-like and lignin-derived low molecular weight phenolic compounds from BSG by human fecal microbiota. Release of lignin-derived monomers, phenolic acid conversion products, and formation of SCFAs were analyzed using gas chromatographic techniques combined with identification with authentic standards and mass spectral comparisons with databases.

Conversion of Lignin by Human Intestinal Microbiota. Release of several low molecular weight compounds from BSG and the fractions was detected in the in vitro colon model using human fecal microbiota. Even though, on the basis of their structure, many of the metabolites could have derived either from lignin or from ferulic acid, dilignols and 4-methylcatechol were most pronounced in the lignin-rich INS fraction. The formation of 4-methylcatechol could occur from guaiacyl and syringyl residues by demeth(ox)ylation and partial dehydroxylation, which are common conversion activities of fecal microbiota and have been shown for plant lignans[23,24] and flavonoid metabolites (hydroxylated phenylacetic and propanoic acids).[25,26] The highest concentration of 4-methylcatechol was released from INS, which also had the highest lignin content, suggesting that 4-methylcatechol could originate from lignin.

Identification of Lignin-Derived Metabolites. As a whole, a large number of different phenolic and other aromatic metabolites could be identified or partially characterized. Some structural features (e.g., the presence of catechol or guaiacyl structures) could be linked to the detected dilignols, which

accumulate toward the end of the incubation, and to lignin. Some monomeric compounds can originate either from lignin or from ferulic acid, for example, methoxylated hydroxyphenyl (guaiacyl) compounds. Guaiacylpropanoic acid, homovanillic acid, and vanillic acid can be formed from ferulic acid via reduction of the double bond in the side chain and by shortening of the side chain (α- and β-oxidation), respectively.[22,27] In this study, the structure of one syringyl-type compound (no. **26** in Figure 3B) was identified and was obviously derived only from lignin. Also, vanillin is more likely to originate from lignin, as also known from its commercial production by oxidation of lignin-containing byproduct of the pulping industry.[28] Quantitated 4-methylcatechol showed significantly higher concentrations in INS than in fecal control and is also a structurally promising compound as a released and converted metabolite from lignin. 4-Methylcatechol was formed during incubation with fecal microbiota between 2 and 6 h time points.

As early as in 1929, Csonka et al.[29] anticipated that lignin is degraded in the digestive system of cows (ruminant) and dogs (monogastric) on the basis of the loss of methoxyl groups. Later it was suggested that lignin is degraded during human intestinal transit.[30] Enterolignan conversion from lignin has also been reported in rats: when deuterated synthetic lignin was fed to rats as part of wheat bran diet, deuterated enterolactone was found in the urine, confirming the release of smaller units from lignin and their conversion to mammalian lignan, enterolactone.[4] Degradation of lignin from wheat straw in ruminant goats was associated with excretion of aromatic acids (benzoic and hippuric acids);[3] however, it is possible that the amount of released compounds may be so small that degradation of lignin will not be apparent. Holloway et al.[31] studied fiber digestion in ileostomy patients and healthy control subjects of human volunteers and found no indication of lignin degradation in either group measured by mass balances. The methods for lignin analysis are under discussion, and thus the impact of human microbiota in lignin degradation remains to be studied in the future. The release of monomeric products from lignin by human microbiota is relevant as a part of the contribution of colon-derived circulating metabolites and when the methods, for example, metabolomics of the small molecules are available. There is increased interest in dietary fiber related small molecules released and absorbed from the colon that may have potentially health-promoting systemic effects.

In the targeted analysis, none of the known ferulic acid metabolites such as 3-hydroxyphenylpropanoic acid or 3,4-dihydroxyphenylacetic acid[22] showed significantly different formation from BSG or its fractions compared with the fecal control. The profiles of the known ferulic acid metabolites in this study were exceptionally different from the previous findings from wheat and rye product, which showed clear formation of 3-hydroxyphenylpropanoic acid during incubation with fecal microbiota in the same model[32] and in TNO intestinal models (TIM).[33]

Effect of Fractionation on Conversion Products. Whereas the removal of carbohydrates and protein from BSG in the preparation of the INS fraction increased the lignin content of the insoluble residue, some lignin was solubilized during the proteolytic treatment, which was carried out in mildly alkaline pH.[11] The carbohydrate- and protein-degrading enzymes were chosen on the basis of earlier studies,[11,34] which showed these enzymes to be the most efficient for BSG hydrolysis. The strong alkaline treatment of BSG in the preparation of the DEFE fraction also decreased the lignin content, but, on the other hand, other compounds were simultaneously solubilized by the alkaline conditions and were removed. Therefore, the lignin content in the DEFE fraction was not significantly different from that of the BSG. The alkaline treatment decreased the ferulic acid content in DEFE, as most of the ferulic acid in BSG is ester-bound and can be released with alkali.[35] The targeted analysis of colon model metabolites showed low concentrations of ferulic acid for DEFE, as they were only slightly above the fecal control and remained constant during the incubation, indicating successful deferuloylation of the DEFE fraction (Figure 4B). In the colon model, ferulic acid was released mostly from BSG and to a lesser extent from INS, which could be expected on the basis of the enzymatic hydrolysis of BSG with the feruloyl esterase-containing enzyme cocktail and the mild alkalinity in the proteolytic treatment used in the preparation of the INS fraction.[11] The ferulic acid and its preliminary microbial metabolite 3-hydroxyphenylpropanoic acid showed clearly that very little conversion occurred from INS or DEFE. Also, 3,4-dihydrophenylacetic acid concentration (diferulate metabolite) was low for DEFE. A higher initial SCFA formation rate was seen for DEFE than for BSG or INS, suggesting that the fermentation is retarded if carbohydrate-binding feruloyl bridges are present as in BSG and INS.

Enzymes and altered pH of the hydrolysis solutions in the preparation of the INS fraction had modified the plant matrix structure to make it more susceptible for microbial degradation, shown as promoted initial SCFA formation rate and released lignans and 4-methylcatechol. The strong alkali used in the preparation of the DEFE fraction most probably solubilized the easily released low molecular weight aromatic compounds, such as lignans, which were present in low concentrations in DEFE. It has been shown previously that alkali effectively solubilizes lignans from cereal matrix.[36] Also, mild alkali releases lignans, indicating modification of the matrix as shown earlier.[11]

Formation of SCFA. Garleb et al.[2] stated that dry matter disappearance in an in vitro rumen simulation was higher with low lignin containing fibers than with lignin-rich fibers. It has also been shown in pigs that BSG decreased xylanolytic and cellulolytic enzyme activities in the cecum, and SCFA content in the colon was lower for the group fed BSG than for the control group fed a diet low in fiber.[37] These findings could suggest that BSG may interact with intestinal microbiota and

reduce their effectiveness in fermenting available carbohydrates. In contrast, in this study the proportions of fermentable carbohydrates in BSG and its fractions were quite close to each other. Despite the somewhat different initial SCFA formation rates, there were no significant differences between SCFA concentrations between samples and control. In the case of suppression, samples containing lignin should have shown significantly lower concentration of SCFA as compared with fecal control, as shown for apple and grape tannins.[7,8] However, such an effect was not observed, and it is not likely that enzymatic inhibition occurred to a significant degree. If the lignin were inhibitory, SCFA from fecal inoculum would also have been affected.

The fermentability of BSG and its fractions remained on the same level as in fecal control. In this study the amount of sample was doubled (200 mg) compared with previous experiments (100 mg) studying the carbohydrate fermentation of cereals.[5,14] The high dose of substrate may cause lower fermentation rate and extent as SCFA formation as indicated previously;[13] however, in this study carbohydrates were of resilient nature and comprised a maximum 53% of the weight of the substrate, and thus suppression by excessive carbohydrates is unlikely. It is also known from previous studies that BSG carbohydrates are partially resistant toward hydrolytic enzymes.[11] Removal of easily fermentable carbohydrates by fractionation leaves the lignin-bound resilient carbohydrates in the fractions, and thus the slow fermentation rate of SCFA formation is most likely caused by the poor accessibility of lignin-bound carbohydrates. In the DEFE fraction, in which the ferulic acid cross-links between carbohydrates and lignin were disrupted, the fermentation rate was increased because carbohydrates were more susceptible to microbial fermentation.

Methods. When the colonic degradation of BSG and the INS and DEFE fractions was studied, the substrates were not digested with alimentary enzymes in vitro in the conditions of upper intestine prior to the colon model as in the cases of cereal food samples,[5] because the BSG and its fractions had been extensively digested in the brewing process and contained only minimally starch (1.3%) and less protein (6.6−22.8%) than was left in digested whole meal rye bread (25%) in a previous study.[38] Furthermore, in BSG the protein was shown to be mostly encapsulated in the fiber matrix, so it was considered to be delivered to the colon relatively unchanged (Figure 2A). The fractions contained even less protein, as they were treated with a protease during the processing.

The changes in microbial metabolites in the colon model used in this study depend on the components in the studied substrates, and the microbial composition is not variable during the incubation. The conversions are performed in strictly anaerobic conditions and for precise incubation times in the batch design, which facilitates the detection of metabolite formation as a course of time.

Lignin-Containing Fractions as Dietary Fiber. Lignin is a valuable DF component regardless of its susceptibility to microbial degradation in the colon. Lignin has been shown to adsorb more carcinogenic compounds in vitro in the conditions of the small intestine and colon than nonlignified fiber,[39,40] which could contribute to the cancer-protecting effects of DF. In addition, phenolic compounds within the insoluble fiber are able to quench soluble radicals formed in the gastrointestinal tract,[41] and due to its phenolic structure also lignin possesses such antioxidative and radical scavenging activity in the lumen.[42,43] In addition, if lignin is a source of small bioavailable

compounds, which may be biologically active and partly responsible for the beneficial health effects of DF complex, it comprises a new group of compounds in addition to enterolignans (enterodiol and enterolactone), urolithins, and phenolic acids,[44] which are derived from conversions by the intestinal microbiota and are related to plant food and DF intake. Whether the potentially lignin-derived components have health benefits remains to be studied.

In conclusion, small lignin-derived molecules such as dilignols and catechols were released from BSG and its lignin fractions in the metabolic in vitro colon model. The significance of the release of these small molecules should be evaluated by quantitation of the formed metabolites in respect to lignin degradation and, finally, by measuring the released components from human urine after consumption of lignin-rich diet. It is possible that the physiological effects of lignin as DF component are mostly due to its binding to carbohydrates, inhibiting their fermentation and increasing the bulking potential of DF. Consequently, nonfermentable fibers absorb and remove toxic components and thus reduce the risk of chronic diseases. Lignin is an underestimated DF component, and its characteristics in the digestive tract deserve more attention.

■ ASSOCIATED CONTENT

ⓈSupporting Information

Concentrations (mean ± standard deviation) of quantitated phenolic metabolites and lignans and their significances. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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