Stellingen

1. De aanwezigheid van pectinen in de sojacelwand verhoogt de fermentatiesnelheid van de celwand doordat deze een open structuur veroorzaken, en niet omdat pectinen zelf snel fermenteerbaar zijn.
   (Dit proefschrift)

2. Bij vergelijking van de fermentatiekinetiek van zuivere celwandtypen van verschillende samenstelling, dient de deeltjesgrootteverdeling van deze celwandtypen gelijk te zijn.
   (Dit proefschrift)

3. Celwandonderzoek dient gebaseerd te zijn op de analyse van zowel fysische als chemische celwandkenmerken.
   (Dit proefschrift)

4. Bij de analyse van gasproductieprofielen met een model met meerdere fasen, moet de biologische relevantie van een fase, prevaleren boven de statistische significantie.
   (Dit proefschrift)

5. De *in vitro* cumulatieve gasproductietechniek als zodanig, is veel meer een aanvulling op, dan een vervanging van de *in sacco* methode.

6. Meettechnieken voor de voederwaarde moeten gebaseerd zijn op een conceptueel model van de vertering en niet omgekeerd.

7. De voederwaardering van herkauwers is meer gebaat bij een betere inschatting van de variatie binnen voedermiddelen, dan bij meer verfijnde voederwaarderingssystemen.

8. Voederwaardering is meer gebaseerd op "best professional judgement" dan op harde wetenschappelijke gegevens.
   (Vrij naar: Gerard Borggreve, Instituut voor de Veevoeding 'De Schothorst')

9. Welvaart is geen argument voor verspilling van hoogwaardige grondstoffen van dierlijke oorsprong.
   (Reactie op uitspraak van minister L.J. Brinkhorst, minister van LNV; ANP 09/12/99)

10. Een snelle celwandafbraak is met name interessant voor gedetineerden.

Stellingen behorende bij het proefschrift:
Soya beans and maize: The effect of chemical and physical structure of cell wall polysaccharides on fermentation kinetics.
Harmen van Laar, 10 maart 2000.

Soya beans and maize:
The effect of chemical and physical structure of cell wall polysaccharides on fermentation kinetics

Promotoren: dr. ir. S. Tamminga
Hoogleraar op het vakgebied van de Veevoeding,
in het bijzonder de voeding van herkauwers.

dr. ir. M.W.A. Verstegen
Hoogleraar op het vakgebied van de Veevoeding,
in het bijzonder de voeding van de eenmagigen.

Co-promotor: dr. B.A. Williams
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Soya beans and maize:
The effect of chemical and physical structure of cell wall polysaccharides on fermentation kinetics

Harmen van Laar

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit
dr. C.M. Karssen
in het openbaar te verdedigen
op vrijdag 10 maart 2000
des namiddags te vier uur in de Aula

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Abstract

The analysis of the relationship between cell wall composition and fermentation of endosperm cell walls of soya beans and maize was approached from three different angles. Firstly, the fermentation (rate and extent of fermentation, the sugar degradation pattern, and volatile fatty acid production) of soya bean and maize cell walls was analysed, both *in situ* and *in vitro*. This analysis revealed that *i*) the physical structure of the cell wall (particle size and cell wall thickness) influences cell wall fermentation characteristics, and that *ii*) cell wall polysaccharides from a single cell wall type can be fermented at different rates. Secondly, isolated cell wall polysaccharide fractions, which had been extracted from soya and maize, and subsequently soya and maize cell walls from which polysaccharide fractions had been extracted, were fermented. The fermentation of extracted pectins from soya bean cell walls was slower than expected, whereas the fermentation of extracted arabinoxylans from maize was very rapid. The cell walls from which part of the non-cellulosic polysaccharides (pectins for soya and arabinoxylans for maize cell walls) had been extracted, were more fermentable than the original cell wall. The cellulose-rich cell wall fraction that remained after further extraction of polysaccharides was less fermentable than the original cell wall for soya, whereas for maize the cellulose-rich residue was similar in fermentation to the original cell wall. Lastly, the fermentation of cell walls from different plants, within the same plant group (mono- and dicotyledons), were compared with the fermentation of the soya and maize cell wall, so as to detect the effect of differences in carbohydrate composition on cell wall fermentation. For monocotyledons an increase in particle size decreased rate of cell wall fermentation, other clear relationships between cell wall composition and fermentability were not found. For dicotyledons an increase in sugar content, most likely related to a lower protein content, decreased half-time of gas production. The results are interpreted using the models for mono- and dicotyledon cell walls, proposed by Hatfield (1993).

Key words: soya beans, maize, cell walls, fermentation, polysaccharides

Voorwoord

De totstandkoming van een proefschrift is geen éénmanswerk. Voor dit proefschrift geldt deze stelling zeker. Daarom wil ik de vele mensen die geholpen hebben bedanken.

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Met als missie, de chemische structuuropheldering van celwandpolysachariden en hun functionele eigenschappen, begonnen ruwweg 5 jaar geleden een viertal OIO's bij verschillende onderzoeksgroepen hun zoektocht naar de structuur en eigenschappen van soja- en maïscelwanden. Deze OIO's waren Miranda Huisman, Carel Fransen, Lars Brüll en ondergetekende. Mede-OIO's, van harte bedankt voor de prettige samenwerking. Ik wil niet zeggen dat ik altijd alles van jullie werk heb begrepen, maar ik heb er wel enorm veel van geleerd.

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Zonder een goed fundament valt niet te bouwen, dit fundament werd gelegd door mijn ouders. De stelregel van mijn vader: werken of leren, heb ik wel eens misbruikt door nog wat schoolwerkzaamheden te veinzen, om zo onder een klusje uit te komen. Het is jammer dat mijn vader de verdediging van dit proefschrift niet meer mag meemaken. Pa en Ma, jullie hebben me altijd gestimuleerd verder te leren, zonder jullie was dit niet mogelijk geweest, aan jullie draag ik dit proefschrift op.

Wageningen, januari 2000

Harmen

List of abbreviations

AAE	Acetic Acid Equivalents
AAEY	Acetic Acid Equivalents Yield
ADF	Acid Detergent Fibre
ADL	Acid Detergent Lignin
A/P	Acetic acid to propionic acid ratio
^{13}C CP MAS NMR	^{13}C Cross Polarization Magic Angle Spinning Nuclear Magnetic Resonance
CCW	Combined Cell Wall fractions (whole bean)
CDTA	Cyclohexylene-DinitroTetraacetic Acid
CF	Crude Fat
CP	Crude Protein
CW	Cell Wall(s)
DAPA	DiAmino Pimelic Acid
DM	Dry Matter
DTT	DiThioThreitol
ECW	Cell Wall fraction of Endosperm
GC	Gas Chromatography
GLC	Gas Liquid Chromatography
GLM	General Linear Model (SAS)
HAc	Acetic acid
HBu	Butyric acid
HPr	Propionic acid
HCW	Cell Wall fraction of Hulls
HPLC	High Pressure Liquid Chromatography
ISO	International Organization for Standardization
N	Nitrogen
NDF	Neutral Detergent Fibre
NMR	Nuclear Magnetic Resonance
NSP	Non-Starch Polysaccharides
PF	Pig Faeces
P-GC-MS	Pyrolysis Gas Chromatography Mass Spectrometry
SBM	Soya Bean Meal
SBM CW	Soya Bean Meal Cell Walls
SDS	Sodium Dodecyl Sulphate
SRF	Sheep Rumen Fluid
STW	Stichting Technische Wetenschappen
TS	Total Sugars
UA	Uronic Acids
VFA	Volatile Fatty Acid(s)
WUS	Water Unextractable Solids

Contents

Aan mijn ouders

Chapter 1

General introduction

General introduction

Introduction

In Dutch agriculture today, large quantities of fibre rich residues of seeds used, for production of human foods, are available for use in animal nutrition. Furthermore, large quantities of feedstuffs (soya, maize, tapioca, etc.) are imported. The combination of these factors has made it possible to incorporate many different feedstuffs into the compound feeds used for animal nutrition. In general, knowledge of the digestibility of the different nutrients in these feedstuffs is available through the Dutch Commodity Board for Feedstuffs (CVB, 1999). However, detailed knowledge of the composition and digestion of the fibre fraction (cell walls) of these feedstuffs is generally lacking. This knowledge is needed to make better use of fibre in animal nutrition, in terms of improving both the welfare and health status of the animals, as well as the efficiency of nutrient use. Therefore, this research has been focused on the composition and fermentation characteristics of the cell walls of soya beans and maize, two feedstuffs which are often used in current animal nutrition practice in The Netherlands.

Chemical and physical structure of cell walls

All plant cells are surrounded by a cell wall. The large variety of plants is brought about by the presence of different tissues which contain cell walls varying in thickness, chemical composition and spatial distribution (Wilson, 1993). Whole plants can be divided into a vegetative part, which is composed of tissues such as epidermis, sclerenchyma, mesophyll, parenchyma, collenchyma and phloem (Figure 1A), and generative parts (seeds). The generative parts are also composed of different tissues, like the endosperm and the seed coat, in which different cell wall types may be present. This is illustrated in a drawing of the seed structure of the soya bean and maize kernel (Figures 1B and 1C). The generative parts (seeds), or residues from these parts after production of human foods, constitute a large proportion of the ingredients used in compound feeds.

Cell wall structure

All cell walls are composed of a middle lamella and a primary cell wall. The middle lamella is positioned between the primary cell walls of two adjacent cells. The wall of the growing cell is always composed of a middle lamella and a primary cell wall. After cells have elongated and have reached their final size, secondary cell wall development may start by deposition of polysaccharides on the luminal side of the primary cell wall (Figure 2). Whether a secondary cell wall is formed depends on the tissue type a cell belongs to. Parenchyma cells, for instance, generally have only thin primary cell walls, whereas sclerenchyma cells mostly have a thick secondary cell wall.

At the onset of secondary cell wall development, lignification of the cell wall may start. The presence of lignin gives the plant cell wall extra rigidity, and has important consequences for the

utilization of the cell wall in animal nutrition (Jung and Allen, 1995).

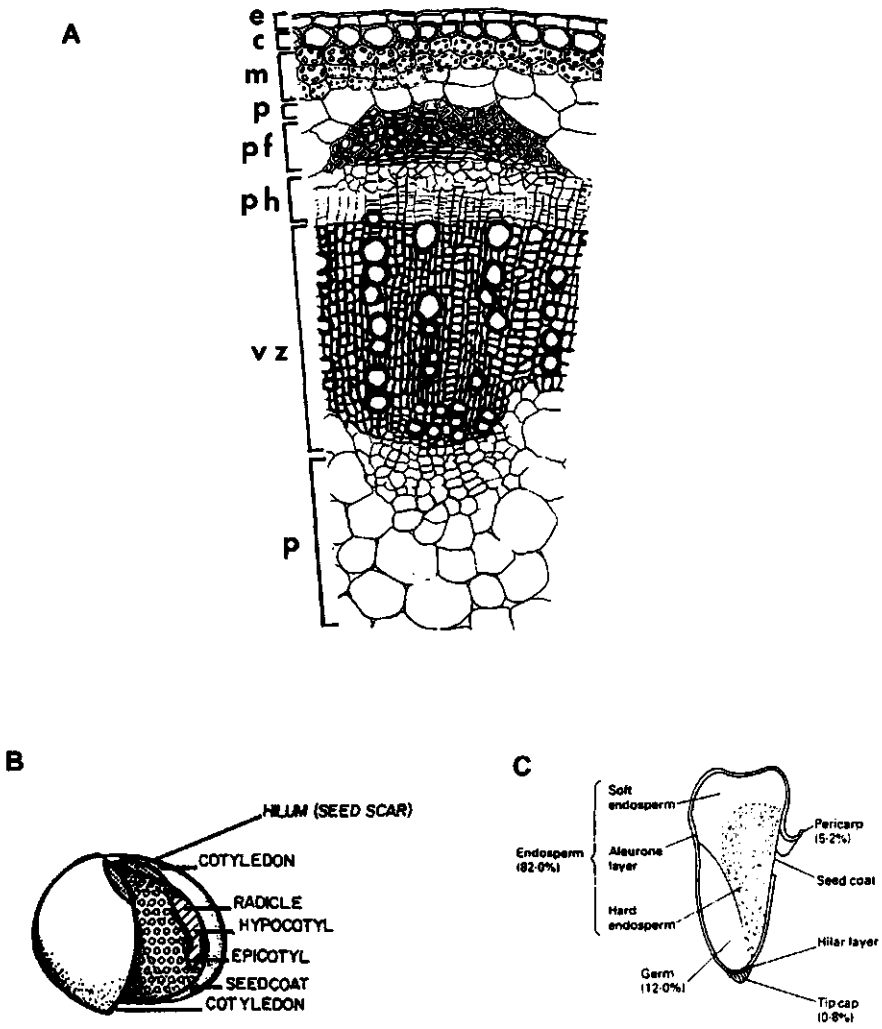

Figure 1: (A) Cross section of the mature stem of a legume (*Stylosanthes scabra* Vog) (e = epidermis, c = collenchyma, m = mesophyll, p = pith parenchyma, pf = phloem fibres, ph = phloem and vz = vascular zone (from: Wilson, 1993)). (B) Soya bean morphology (from: Ensminger *et al.*, 1994). (C) Maize kernel morphology (from: Macrae *et al.*, 1993).

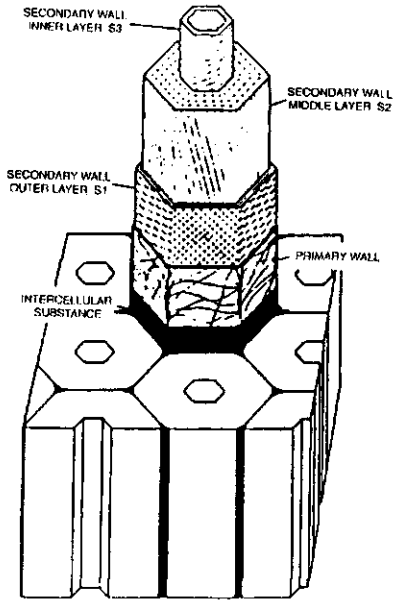

Figure 2: General cell wall structure displaying, primary and secondary cell walls (hardwood) (from: Theander and Aman, 1977).

The composition and structure of both primary and secondary cell walls can differ between plant groups. For example, a marked difference in cell wall composition exists between the cell walls of mono- and dicotyledonous plants (Harris *et al.*, 1997). These differences are already present in the primary cell walls of plant seeds. This study is mainly focusing on the primary cell walls of maize (monocotyledon) and soya beans (dicotyledon), therefore, the composition of the primary cell wall of these two feedstuffs will be discussed.

Primary cell walls of mono- and dicotyledons

The primary cell wall in seeds of monocotyledons (e.g. maize, rice, wheat, etc.) is composed of cellulose, hemicellulose and a little pectin (Selvendran, 1983; Bailey *et al.*, 1976). The actual distribution of cellulose, hemicellulose, and pectin may vary between plant species, but generally, cellulose and hemicellulose form the major part of the cell wall. Other compounds such as phenolic acids and lignin are generally present in very low amounts or are totally absent. The primary cell walls of dicotyledons on the other hand, are completely different from monocotyledon cell walls. Dicotyledon primary cell walls are composed of cellulose, a substantial amount of pectin, and generally only small amounts of hemicellulose (Bailey *et al.*, 1976). The differences in sugar composition between and within some mono- and dicotyledon cell wall types are shown in Table 1. Although these cell wall fractions will probably contain both primary and secondary cell walls, Table 1 does provide an indication of the differences in sugar composition between and within, mono- and dicotyledon cell walls.

Table 1: Sugar composition of the insoluble cell wall material (g kg^{-1} insoluble cell wall sugar) from whole grains of monocotyledons, and protein concentrates of dicotyledons (adapted from: Bach Knudsen, 1997).

	Arabinose	Xylose	Galactose	Glucose	UA[1]
			Monocotyledons		
Maize	213	315	45	348	67
Wheat	234	404	21	287	43
Rye	216	369	36	324	27
Barley	168	382	15	389	31
			Dicotyledons		
Soya	110	110	161	406	148
Lupin	89	133	225	487	44
Faba beans	64	78	14	773	64
Pea	132	93	23	651	93

[1] Uronic Acids; mannose and rhamnose not shown.

Cell wall polysaccharides

Cellulose is a β1-4 linked polymer of *D*-glucose which is present in tightly-packed well-ordered micro-fibrils, held together by hydrogen bonding (Figure 3A) (Eastwood, 1992; Hatfield, 1989). These micro-fibrils can be linked by xyloglucans (hemicellulose) forming a cellulose-xyloglucan network.

For monocotyledon cell walls, the cellulose-xyloglucan network is embedded in a matrix composed of another network of mainly arabinoxylans (Figure 3B). Arabinoxylans are composed of a β1-4 linked backbone of xylose, which can be substituted with arabinose or glucuronic acid (e.g. for wheat: Schooneveld-Bergmans, 1997; for sorghum: Verbruggen *et al.*, 1995). Some of these arabinoxylans are water-soluble (*Glitsø* and Bach Knudsen, 1999). Other polysaccharides such as galactans and arabinans may be present in low amounts. Furthermore, some monocotyledon cell walls (like barley and oats) may contain β-glucans (Eastwood, 1992), which are composed of β1-3 and β1-4 linked glucose, and are generally soluble in water.

For dicotyledon cell walls, the cellulose-xyloglucan network is generally embedded in a matrix formed by a network of mainly pectin, with only small amounts of hemicellulose. Pectin is generally composed of a partly methylated galacturonic acid backbone, which at some places is alternated with rhamnose (Figure 3C). Side chains of arabinans, galactans, or arabinogalactans can be covalently linked to these rhamnose units (Van de Vis, 1994). Small amounts of fucose and xylose may also be present (Huisman *et al.*, 1998). Part of the pectins may be water-soluble, the pectins that are not water-soluble can be rendered insoluble by ionic interactions of the acid group of galacturonic acid with Ca^{2+} or by entwinement with other polysaccharides (Hatfield, 1993).

The primary cell wall of mono- and dicotyledons, when no secondary wall deposition has taken place, generally contains no lignin, but may contain esterified ferulic or *p*-coumaric acids (Figure 3D). Ferulic and *p*-coumaric acid are generally covalently bound (esterified) to the fifth

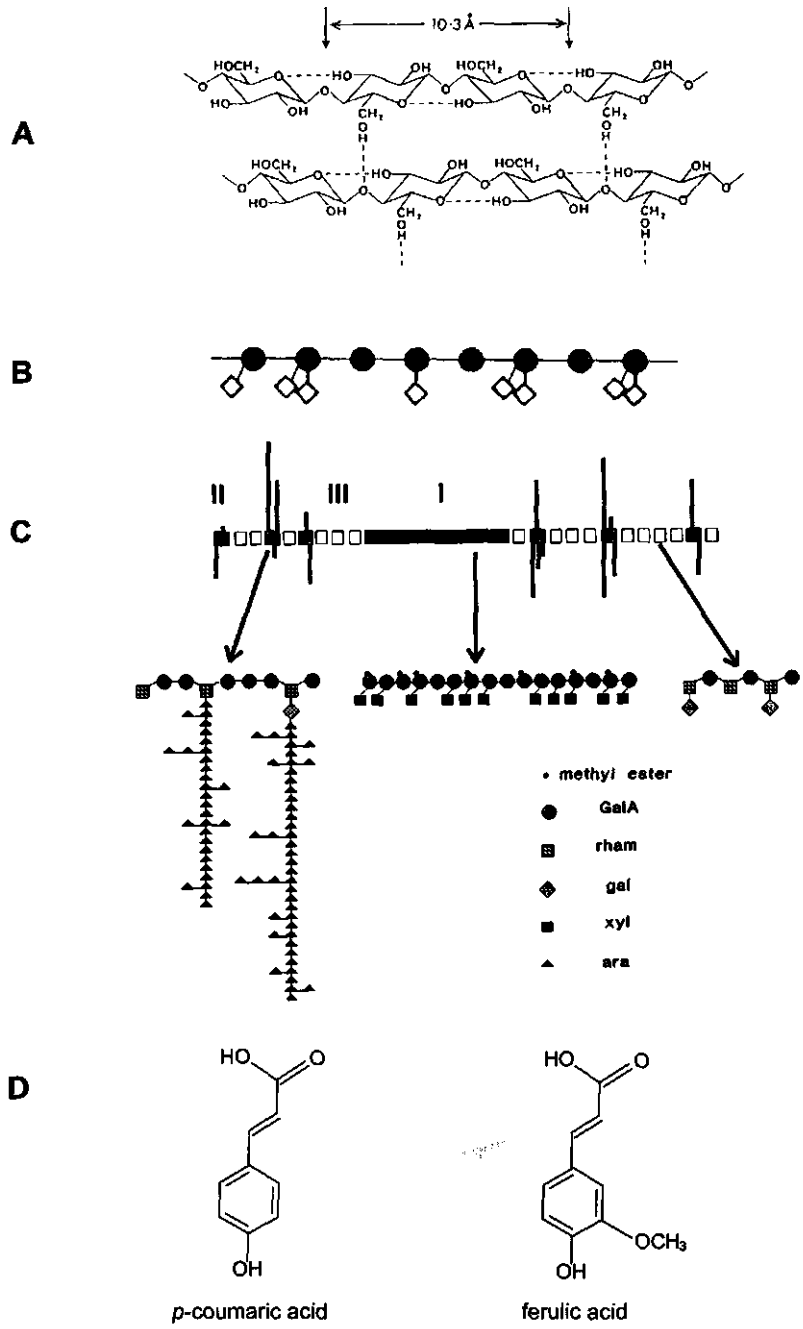

Figure 3: Chemical structures of (A) cellulose (from: Selvendran, 1983); (B) Arabinoxylan from wheat (xylose (•); arabinose (◇)) (from: Verbruggen, 1996); (C) pectin from apple (Schols, 1995), and (D) *p*-coumaric and ferulic acid (from: Ralph and Helm, 1993).

carbon atom of arabinose from arabinoxylans. For monocotyledons, ferulic acid may form di-ferulic cross-links, linking two arabinoxylan polymers, whereas for dicotyledons it may form cross-links between side chains of pectins, which generally will increase the rigidity of the cell wall (Hatfield, 1993).

Cell wall architecture

The cell wall is not just the sum of its polysaccharides; their interactions and arrangement also influence cell wall characteristics (Hatfield, 1993). Figure 4B shows a model of how the polysaccharides are arranged in the primary grass cell wall (monocotyledon). The monocotyledon primary cell wall is densely packed with cell wall polymers, resulting in a very tight structure (Hatfield, 1993). Figure 4A displays a model for the primary cell wall of legumes (dicotyledons). This cell wall has a more open and hydrated structure, in which the space between the cellulose-xyloglucan network is filled with pectin (Hatfield, 1993). The density of the cell wall structure can influence cell wall characteristics such as porosity and pore-size (Carpita and Gibeaut, 1993; Hatfield, 1993). These are the cell wall characteristics which are most likely to influence fermentation in the gastrointestinal tract of animals (Chesson *et al.*, 1997).

Chemical analyses of cell walls

The cell wall fraction is very complex, this means that an accurate and complete analysis of cell walls in feedstuffs is also complex. A complete analysis of the composition of a cell wall, with regard to which polysaccharides are present, and the composition of those polysaccharides, is a very cumbersome procedure (Nevins, 1993), not suited for routine analyses. For routine analyses, methods such as neutral detergent fibre, acid detergent fibre, acid detergent lignin (Van Soest, 1973; Van Soest *et al.*, 1991), crude fibre (NEN, 1988), and the Uppsala method (Theander and Westerlund, 1993) have been developed, and are widely used. The neutral detergent fibre method analyses the total amount of hemicellulose, cellulose and lignin; the acid detergent fibre method analyses the cellulose and lignin content; and the acid detergent lignin method analyses only the lignin content. The crude fibre method gives a measure for cell wall content somewhere between the neutral detergent fibre method and the acid detergent fibre method. None of these methods analyse the pectin content. These methods are fairly crude, dividing the cell wall into hemicellulose, cellulose, and lignin, and do not yield information about the hemicellulose composition (neutral sugars). A more complete method is the Uppsala method, which determines the total fibre content (both water-soluble and -insoluble) and analyses sugar composition and lignin content of the total fibre. This gives an indication of both soluble and insoluble cell wall polysaccharides, including pectins, of which the cell walls are composed. The total of soluble and insoluble cell wall polysaccharides is often also referred to as non-starch polysaccharides (NSP). In this thesis, another method for extracting cell wall material was used, which retains most of the pectin in the cell wall (Huisman *et al.*, 1998). With this method, water-soluble materials, protein and starch are removed by subsequent extractions with different chemicals, to yield a cell wall fraction which contains all water-insoluble cell wall components and which is, therefore, called 'water unextractable solids' (WUS).

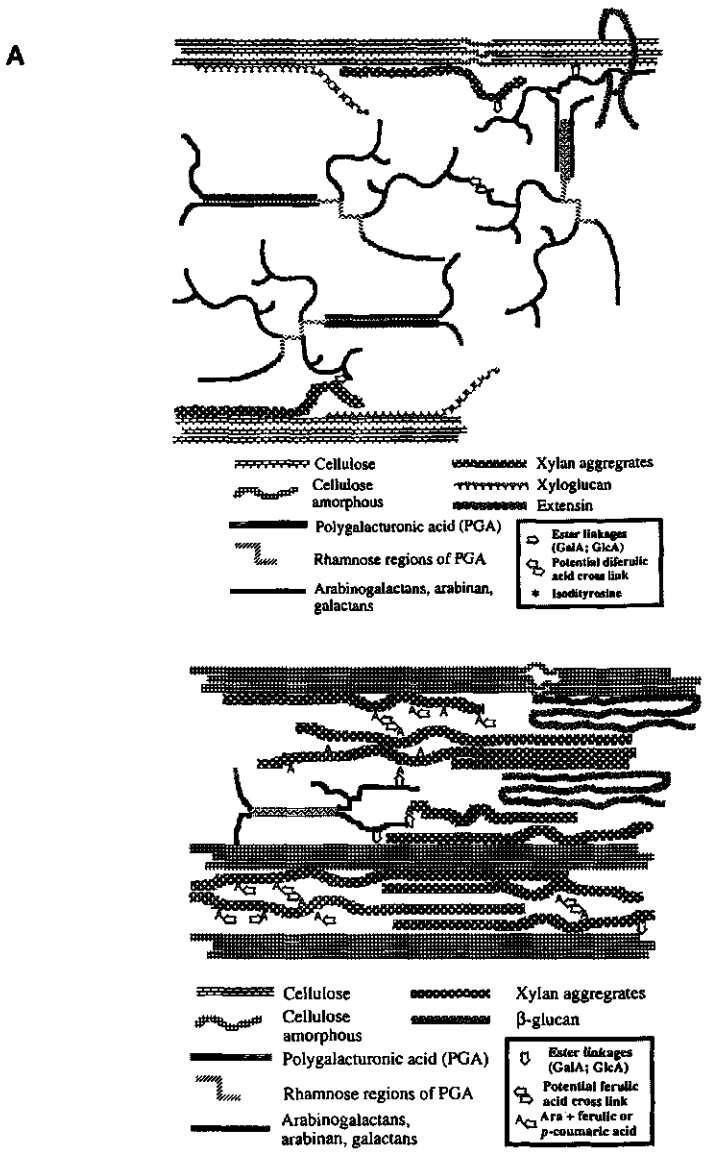

Figure 4: Schematic model for the architecture of (A) a legume (dicotyledon) and (B) a grass (monocotyledon) cell wall (from: Hatfield, 1993).

Effects of plant cell walls in animal nutrition

The role of cell walls in farm animal nutrition is markedly different for ruminants and pigs. These differences are related both to differences in anatomy and functioning of the gastrointestinal tract, and to differences in cell wall characteristics. No mammal can produce enzymes capable of breaking down plant cell walls and rely on micro-organisms for the digestion of plant cell wall material. Thus, in farm animals, plant cell walls are fermented by micro-organisms (microbial enzymes), rather than digested by animal enzymes.

Ruminants

The gastrointestinal tract of ruminants is equipped with a complex stomach composed of the rumen, reticulum, omasum, and abomasum. Especially the rumen, which functions as a fermentation vessel, enables the ruminant to use cell walls as an energy source. Microbial fermentation of cell walls results in the formation of volatile fatty acids, which are absorbed across the rumen wall, and supply a large part of the energy requirement of the ruminant (France and Siddons, 1993). Cell wall material is also important for the regulation of motility and gives structure to the rumen contents, and is, therefore, important for an efficient functioning of the rumen.

Cell walls and digestion

The major part of the cell wall material in the diets of ruminants consists of insoluble cell wall polysaccharides, with a mixture of primary and secondary cell walls, and varying degrees of lignification. Before cell wall material from whole plants (e.g., grasses) can pass from the rumen to the other parts of the gastrointestinal tract they have to be reduced in size, which is achieved by both rumination and fermentation (Kennedy and Doyle, 1993). The rate of fermentation of these cell walls depends on cell wall characteristics such as lignification, cell wall thickness, and tissue type (Chesson, 1993; Jung and Allen, 1995). Unfermented cell walls reaching the small intestines can increase the endogenous protein losses by sloughing off of mucosal cells, thereby influencing the efficiency of protein use (Lammers-Wienhoven *et al.*, 1997). Furthermore, if the cell wall structure is not sufficiently opened before reaching the small intestine, the digestibility of enclosed nutrients, such as protein and starch may be reduced, because these are inside the cell lumen and are shielded from enzymatic digestion by the cell wall. However, because of the efficient fermentation and particle size reduction in the rumen this of only minor importance to ruminants.

The main role of cell wall material in ruminant nutrition is to serve as an energy source for the animal. Fermentation in the rumen produces volatile fatty acids such as acetic, propionic and butyric acid, which can be metabolized by the animal. The amount and proportion of these volatile fatty acids, that are produced, can influence the metabolism of the animal. For instance, acetic acid can be used for production of milk fat (France and Siddons, 1993), whereas propionic acid can be used as a precursor for the synthesis of glucose (Van Houtert, 1993). It is known that the composition of the cell wall fraction can influence the proportions of acetic, propionic, and butyric acid production. Cell walls that are slowly fermentable and contain a large amount of cellulose generally result in the production of more acetic acid (Hungate, 1966), whereas more

rapidly fermentable cell walls result in relatively more propionic acid. An exception are the pectins, which are generally assumed to be rapidly fermentable but have an acetic acid-directed fermentation (Marounek *et al.*, 1995; Sunvold *et al.*, 1995).

Pigs

The gastrointestinal tract of the pig has a completely different anatomy to that of the ruminant. Cell wall fermentation occurs mainly in the large intestine of the pig, which means that cell wall polysaccharides have to pass through the stomach and small intestines, before they can be fermented.

Cell walls and digestion

Several effects of cell walls in the small intestine have been reported. When the cell wall structure is still intact, the cell wall can shield protein, fat and starch which are stored in the cell lumen, from enzymatic digestion in the small intestines (Eastwood, 1992). Thus the digestibility of these nutrients is reduced. Insoluble cell walls can increase sloughing off of mucosal cells into the chyme, thereby increasing endogenous losses (Shulze *et al.*, 1994). Zebrowska and Low (1987) indicated that fibre components of the diet could cause an increased excretion of digestive fluids from the pancreas. The presence of cell walls generally increases the rate of passage through the whole gastrointestinal tract. However, the rate of passage in the small intestines is generally decreased, whereas the passage rate in the large intestines increases (Bakker, 1996). Generally, increasing the amount of cell walls in the diet, will depress ileal digestion of other nutrients, either directly through a decreased absorption, or indirectly through increased endogenous losses (Bakker, 1996).

The major change to the cell walls is due to fermentation in the large intestine, although some hemicellulose can be solubilized at a low pH (Van Soest and Robertson, 1977), which can occur in the stomach. As with ruminants, fermentation in the large intestine of pigs leads to the production of volatile fatty acids, which are absorbed and add to the energy metabolism of the animal. For an adult sow this can yield up to around 30% of the energy required for maintenance (Mason, 1983; Pond, 1987). Furthermore, several beneficial health effects have been ascribed to the fermentation of carbohydrates (such as cell walls) in the large intestine. For instance, the fermentation of protein, which occurs in the large intestine if there is not enough energy for micro-organisms, and may have deleterious effects on animal health, might be reduced by preferential fermentation of carbohydrates (Houdijk, 1998). Also, the production of butyric acid is reported to be beneficial for the intestinal wall (Sakata *et al.*, 1995; Smith and German, 1995). However, one of the main effects on animal health may be, that an active micro-flora in the large intestines reduces the chances for pathogens to proliferate due to an improved colonization resistance (Raibaud, 1992). This effect may in part be mediated through a lower pH, which can reduce the presence of pathogenic bacteria such as *E. coli* (Sutton and Patterson, 1996).

Cell wall fermentation

Much of the earlier research has been conducted with lignified plant material composed of both

primary and secondary cell wall material. In general, fermentation of cell walls is mainly influenced by lignification (Cornu *et al.*, 1994; Jung and Allen, 1995; Lau and Van Soest, 1981). Analysis of cell wall fermentation in these studies is complicated by the presence of different tissue types, which differ in their composition and degradation rate (Chesson *et al.*, 1986; Chesson, 1993). This complicates the analyses of the relationship between composition and fermentation of individual cell wall types. Therefore, work on purified cell wall types, as has been done for grasses (Grabber and Jung, 1991a, b; Gordon *et al.*, 1995) can provide a better description of the factors influencing fermentation on the level of the individual cell wall. However, much is still unknown about the relationship between composition and fermentability of individual cell wall types.

Microbial characteristics

The fermentation of cell walls in animals is determined by the interaction of micro-organisms and cell walls. Therefore, both microbial and cell wall characteristics influence fermentation. The activity of the microbial population in the gastrointestinal tract is mainly influenced by factors such as diets, age of the animal, animal species and several other factors (Dehority and Orpin, 1988). Differences in the microbial population can influence cell wall fermentation, because not all microbes are able to digest every type of cell wall polysaccharide (Stewart and Bryant, 1988). This means that micro-organisms in mono-culture are less effective at breaking down a complex carbohydrate matrix, such as that of a cell wall, than mixtures of different cultures of micro-organisms (Osborne and Dehority, 1989). Furthermore, one microbial species can solubilize polysaccharides, which can subsequently be used and fermented by others (Fondevilla and Dehority, 1994). The cell wall-fermenting micro-organisms in the gastrointestinal tract are part of a complex ecosystem, of which the interactions and complexity are still not completely understood.

Cell wall characteristics

The characteristics influencing cell wall degradation can be separated into physical and chemical characteristics. The main physical characteristics influencing cell wall fermentation are the tissue cell walls are present in (Travis *et al.*, 1997; Wilson, 1993), and the surface area to volume ratio of the cell walls used (Buxton and Redfearn, 1997; Fisher *et al.*, 1989). These two characteristics are directly related to the particle size and cell wall thickness of the cell wall material. Cell walls with a small surface area and a large volume (big particles) are less rapidly fermented than cell walls with a large surface area and small volume (small particles) (Buxton and Redfearn, 1997). The tissue type and tissue particle size are important with regard to the accessibility of the cell wall material. Bacteria generally have to attach to the cell wall to be able to degrade it (Tamminga, 1993; Pell and Schofield, 1993a), therefore, bacteria must be able to physically reach a cell wall inside a particle in order to degrade it. When a cell wall is surrounded by other cell walls in a large particle, first the adjacent cell walls will have to be fermented before fermentation of that specific cell wall can start. Porosity (or pore-size) of the cell wall has also been reported as a possible influence on cell wall fermentation (Chesson *et al.*, 1997), because the size of the pores could determine whether microbial enzymes can penetrate the cell wall. However, the pore-size of most plant cell walls is generally deemed to be too small for

penetration by microbial enzymes (Chesson, 1993), so that cell wall fermentation is mainly limited to the surface of the cell wall.

The main characteristic that influences cell wall fermentation is lignification (Jung and Allen, 1995). However, for unlignified tissues other factors such as crystallinity and the degree of acetylation of polymers (Bailey *et al.*, 1976; Kerley *et al.*, 1988) are reported to influence fermentability. An increase in crystallinity and acetylation would reduce fermentability, because crystalline structures are harder for the microbial enzymes to degrade, and more acetyl groups on polymers would hinder the microbial enzymes. For unlignified cell walls, the main factor determining cell wall fermentation is thought to be the carbohydrate composition of the cell wall. Especially the types and composition of the polysaccharides that form the cell wall influence the cell wall fermentation characteristics. For instance, pectin-rich cell walls are generally found to be more fermentable than pectin-poor cell walls (Hatfield, 1993). Although research on the relationship between cell wall composition and cell wall fermentation has been done for many years (Crampton and Maynard, 1938), many of the underlying principles of this relationship are still unknown.

Measuring cell wall fermentation

In the past years, several methods for analysing fermentation have been developed. These are either *in situ* techniques, incubating feedstuffs in a nylon bag in the rumen of a fistulated animal (Mehrez and Ørskov, 1977) or *in vitro* techniques incubating feedstuffs with rumen fluid (e.g. Tilley and Terry, 1963) or other sources of gastrointestinal microbes. A special case of the *in vitro* techniques is the gas production technique, in which the production of gasses due to fermentation is measured. Several of these techniques have been developed (Cone *et al.*, 1996; Davies *et al.*, 1995; Menke and Ehrensvard, 1974; Pell and Schofield, 1993b), some measure only endpoint gas production, whereas others measure the kinetics of gas production during the whole fermentation period. An example of the latter is the *in vitro* cumulative gas production technique developed by Theodorou *et al.* (1994), which was used for this thesis, to analyse the fermentation characteristics of cell walls. The principle, on which all gas production techniques are based, is that the *in vitro* fermentation is accompanied by the production of gas. This gas is formed either directly by the microbial metabolism of carbohydrates, or indirectly by the release of carbon dioxide from the bicarbonate buffer used in these techniques, due to the production of volatile fatty acids. When measuring gas production, direct and indirect gas production cannot be separated, but because both are directly related to the fermentation of a substrate, the gas production measured at each time point is a measure of fermentative activity.

Objective of this thesis

The fermentation of cell wall material is a complex process, which is influenced by many factors. A considerable research effort has been directed towards the fermentation of cell walls from forages, which generally have a lignified secondary cell wall. Much less attention has been paid to the fermentation of unlignified primary cell walls, like those of soya bean cotyledons and maize endosperm, which are commonly used in compound feeds for both ruminants and mono-

gastric animals. Analysis of the relationship between carbohydrate composition and cell wall fermentation of unlignified materials such as primary cell walls will provide more detailed knowledge of plant cell wall structure and its role in fermentation of plant materials. This knowledge is needed for a better utilization of the fibre fraction of commonly used feedstuffs (such as soya and maize) or fibre-rich by-products (from human food industry) in farm animal nutrition. The objective of the research reported in this thesis was to investigate the relationship between cell wall sugar composition and fermentation, focused on the cell wall material from the soya bean and maize grain.

Outline of the thesis

This thesis describes several *in vitro* experiments (and one *in situ* experiment), which were conducted with the cell walls of soya beans, maize grains and the seeds of other mono- and dicotyledon plants. The results of these experiments are reported in six chapters, which are organized as depicted in Figure 5. Chapter 2 deals with the fermentation characteristics of whole soya bean meal and maize and their cell wall fractions. These were fermented *in vitro* using rumen fluid of sheep, or pig faeces as a source of inoculum. This experiment was broad in objective, investigating differences in microbial population (sheep rumen fluid *vs* pig faeces), feedstuff (maize *vs* soya), and fraction (whole *vs* cell walls). As this experiment was conducted using whole soya beans and maize grain, different cell wall types were still present in the cell wall fractions fermented. Therefore, in Chapter 3, the effect of the presence of different tissues on fermentation characteristics was further investigated, using soya bean hull and soya bean cotyledon cell walls *in situ*, using a fistulated cow and *in vitro* using sheep rumen fluid. Chapter 4 is an addition to Chapter 3, in which a more in-depth analysis using multi-phasic curve fitting and nuclear magnetic resonance (NMR) techniques to analyse the fermentation of cell walls from both hulls and cotyledons of soya beans.

The investigation of the cell wall fermentation of soya bean and maize cell walls using both sheep rumen fluid and pig faeces (Chapter 2), revealed that cell walls were slightly less fermentable using pig faeces, than using sheep rumen fluid. Because of this, and because the cell walls of soya beans and maize are relatively more important in pig nutrition than in ruminant nutrition, the experiments for Chapters 5, 6 and 7 were all conducted using pig faeces as an inoculum. In Chapters 5 and 6, the fermentation characteristics of polysaccharide fractions extracted from the cell walls of soya bean cotyledons and maize, were investigated to study the effect of the different polysaccharides within and outside the cell wall matrix. In Chapter 7, the fermentation characteristics of the cell walls extracted from four monocotyledons (maize, wheat, rye, and rice) and four dicotyledons (soya beans, lupins, faba beans, and peas) were analysed. The idea behind this experiment was to obtain cell walls with generally the same physical characteristics (particle size, cell wall thickness etc.) yet with a different sugar composition. This could clarify the relationship between cell wall composition and fermentation for primary unlignified cell walls.

The results of the different experiments are combined in the general discussion, to provide an overview of microbial fermentation of primary cell walls. The general discussion is primarily

based on Chapters 3, 4, 5, 6, and 7, whereas Chapter 2 has been mainly instrumental in forming the basis of the following chapters.

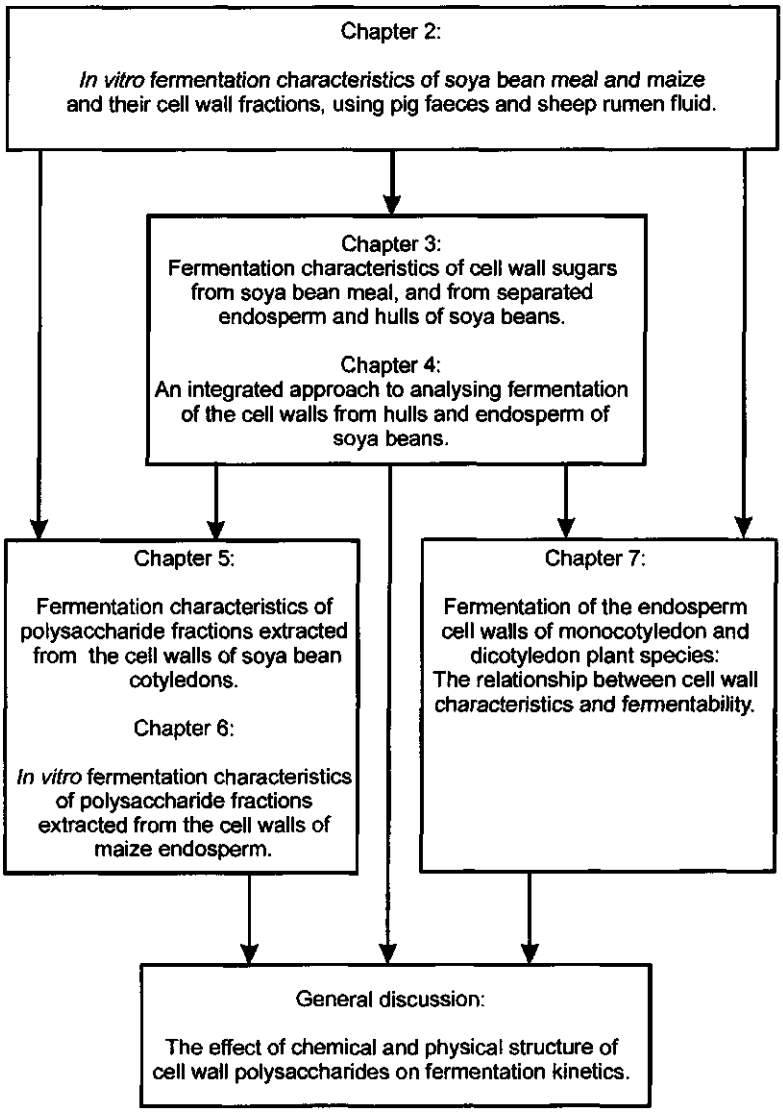

Figure 5: Schematic representation of this thesis.

Chapter 2

In vitro fermentation characteristics of soya bean meal and maize and their cell wall fractions, using pig faeces and sheep rumen fluid*

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In vitro fermentation characteristics of soya bean meal and maize and their cell wall fractions, using pig faeces and sheep rumen fluid

Abstract

A 2×2×2 factorial design was used to investigate the effect of inoculum (sheep rumen fluid (SRF) *vs* pig faeces (PF)), feedstuff (soya bean meal (SBM) *vs* maize grain) and fraction (whole *vs* cell wall (CW) fraction), on fermentation kinetics using the *in vitro* cumulative gas production technique. Maize and SBM were sequentially extracted with water, a detergent, and with α-amylase to prepare CW fractions. Four substrates (whole and CW fractions of both SBM and maize) were used in two separate gas production experiments, one using SRF and one using PF as an inoculum. Gas production was measured during the whole fermentation period, whereas dry matter degradation, sugar degradation, volatile fatty acid production, and diaminopimelic acid content, were measured at specific time points. The gas production profiles of the whole feedstuffs were fairly similar for SRF and PF. However, the gas production profiles of CW were delayed for PF compared to SRF. The total amount of microbial material added with the inoculum was similar, this indicates a better adaptation to fibre degradation of the micro-organisms present in the SRF inoculum, compared to the PF inoculum. The CW of SBM was more rapidly and more extensively fermented than the CW of maize. This may have been due to a difference in CW composition and structure, given that the SBM CW are rich in pectin and poor in hemicellulose, whereas maize CW are the opposite. Microscopic analyses revealed the presence of multiple CW types for the CW fractions of both SBM (two CW types) and maize (at least three CW types). For SBM CW the difference in sugar composition and degradation rates of the two CW types was considered responsible for the difference in degradation rates of the individual CW sugars within the CW fraction of SBM. The maize CW fraction was also composed of multiple CW types, which were degraded at different rates. However, there was no difference in degradation of the individual CW sugars for the maize CW fraction. This indicates that there is not much difference in the sugar composition of the different CW types of maize.

Key words: fermentation, cell walls, gas production, soya bean meal, maize

Introduction

Most of the cell wall (CW) research has been focused on forage CW degradation by ruminal microbes (Chesson, 1981; Engels, 1996). This research has been of great importance for the increased efficiency of plant biomass usage for animal production (Akin, 1993). However, in recent years, CW fermentation and the fermentation process in the large intestine of monogastrics (e.g. pigs) has gained increasing interest (Chabeauti *et al.*, 1991; Houdijk *et al.*, 1998; Pond, 1987). The concentrate diets of monogastrics are often composed of feedstuffs such as maize (*Zea mays*) grain and soya bean meal (*Glycine max*). These two feedstuffs are members of two different plant groups (monocots and dicots), that are distinctly different in their CW composition. The CW of SBM is generally rich in pectins and poor in hemicellulose, whereas the CW of maize is poor in pectin and rich in hemicellulose (arabinoxylans) (Harris *et al.*, 1997).

Fermentation of CW is determined both by the characteristics of the microbial population (Varga and Kolver, 1997), and the plant material used (Buxton and Redfearn, 1997). *In vitro* fermentation of CW, using different sources of micro-organisms, has been investigated by Sunvold *et al.* (1995), they did not find large differences between the fermentative capacity of pig faeces and cattle rumen fluid. There are many plant characteristics relevant to CW degradation on a whole plant basis, including plant structure, tissue structure, particle size, CW thickness, CW composition, and CW fine structure (Buxton and Redfearn, 1997; Weimer, 1996; Wilson, 1993; Wilson and Mertens, 1995). However, on an individual CW basis, fermentability is more influenced by aspects of CW structure, such as the constituent polysaccharides and their sugar composition, degree of acetylation, crystallinity of polymers, cross-linking, and lignification (Bailey *et al.*, 1976; Kerley *et al.*, 1988). The complete analysis of all the factors involved in CW degradation is very cumbersome. A simpler approach to gain insight into the factors governing CW degradation on the level of the individual CW, is to analyse the degradation of the composite sugars of a relatively simple plant part (e.g. seed). The work reported here was designed to analyse *i*) differences in fermentative capacity of the source of inoculum (pig faeces *vs* sheep rumen fluid) and *ii*) the differences in fermentation characteristics between CW originating from two widely used feedstuffs (soya bean meal and maize) with very different CW compositions.

Materials and methods

Design

The experiment was designed as a 2 × 2 × 2 factorial, with the factors: inoculum (sheep rumen fluid (SRF) or pig faeces (PF)), feedstuff (soya bean meal (SBM) or maize), and fraction (whole *vs* cell walls (CW)). Ideally, all factors would have been compared within one gas production run; however, limitations related to the number of fermentation bottles that could be measured per run necessitated splitting the experiment into two separate gas production runs. It was decided not to run replicates in the separate gas production runs (block design), but to separate the experiment into one run with SRF and one with PF. Because the potential differences in fermentative activity between SRF and PF were expected to be larger than potential differences caused by time, the experiment was analysed as a full 2 × 2 × 2 factorial design.

CW extraction procedures

Commercially available SBM and maize were ground over a 0.5 mm sieve and were used to prepare purified soya and maize CW, as described for SBM by Huisman *et al.* (1998). Briefly, 400 g batches of both SBM and maize were sequentially extracted using demineralized water (3 h, room temperature), a solution of 10 g l^{-1} sodium dodecyl sulphate and 1.5 g l^{-1} dithiothreitol (3 h, room temperature), and a maleic acid buffer containing 2 mg l^{-1} α-amylase (Merck art. 16312; pH 6.5 for 16 - 20 h at 30 °C). Before the maleic acid extraction, maize material was wet sieved over a 0.45 μm sieve to remove most of the starch granules. After each extraction step, the materials were centrifuged at 11,000 × g for 30 min, the supernatant discarded, and the pellet subjected to the subsequent extraction step. The final residue was freeze-dried and left to air-equilibrate for at least 3 h. The material obtained in this way was considered to be representative

of the CW fraction.

In vitro gas production and chemical analyses

Two *in vitro* gas production runs (Theodorou *et al.*, 1994) were conducted, one with sheep rumen fluid (SRF) and one with pig faeces (PF) as inoculum. In both runs (SRF and PF), four materials were used as substrates: whole SBM and maize and their respective CW fractions. SRF was collected from three rumen-fistulated sheep, fed medium quality grass hay, which had been fasted overnight. PF was collected from four mature sows fed a commercial diet containing soya beans, soya bean meal and barley, without additional copper or antibiotics.

Approximately 1.1 g dry matter (DM) from each substrate was weighed into 100 ml serum bottles. One day prior to inoculation, 82 ml of medium B (Lowe *et al.*, 1985) was added to the serum bottles containing the substrate. To prepare the inoculum, SRF and PF were diluted with medium B (SRF, 1:1; PF, 1:4), mixed in a blender for 60 seconds and strained through a double layer of cheesecloth. All bottles were inoculated with 5 ml of inoculum and incubated at 39 °C. For each substrate, fermentation was stopped after 0, 6, 12, 24, 36, 48 and 144 h of incubation, by autoclaving (10 min, 110 °C). Bottles were then stored at −18 °C for further analysis. The number of bottles (minimum of two, maximum of 8 bottles) for each combination of substrate and incubation time was chosen based on an estimated total residue of 1 g DM. For 144 h of incubation three fermentation bottles were used. After storage, the contents of each bottle were centrifuged at 11,000 × g for 30 min. The supernatants were analysed for volatile fatty acids (VFA) using GLC (Packard 419, CE Instruments, Milan, Italy; glass column filled with chromasorb 101, carrier gas N_2 saturated with methanoic acid, 190 °C with isocaproic acid as the internal standard). The pellets (residues) were freeze-dried and pooled by inoculum, substrate, and fermentation time.

Residues and substrates were analysed for composite sugar composition using HPLC (column: Dionex PA-10, with PA-10 guard) with Pulsed Amperiometric Detection (detector: Dionex ED-40) after hydrolysis with sulfuric acid (1 h 7.3 M, and 3 h 1 M) using allose as an internal standard. Uronic acids were analysed with a colorimetric *m*-hydroxydiphenyl assay, using a spectrophotometer at 520 nm (Blumenkrantz and Asboe-Hansen, 1973). Diamino pimelic acid (DAPA) content, in residues and substrates, was analysed with an amino acid analyser using post-column derivatization with ninhydrin (Biotronik LC 5001, Eppendorf-Biotronik, Maintal, Germany) after hydrolysis under reflux (6 M HCl at 108 °C, 22 h). Furthermore, Kjeldahl nitrogen (ISO, 1979) was analysed. Substrates were additionally analysed for DM (ISO, 1983), ash (ISO, 1978), and fat (ISO, 1996) content.

Bacterial isolations from inocula

To analyse the bacterial DM content, SRF and PF were centrifuged at 550 × g for 15 min to pelletize large particles (originating from the feed), after which the supernatants were centrifuged again at 11,000 × g for 30 min to pelletize the bacteria (procedures adapted from Cecava *et al.* 1990; and Hsu and Fahey, 1990). To analyse the total DM content (bacterial and other DM) both SRF and PF inocula were centrifuged at 11,000 × g for 30 min. after which the pellets were collected. The pellets of both the bacterial DM and total DM collection were freeze-dried and analysed for nitrogen, diamino pimelic acid (DAPA) and composite sugars as described.

Gas production calculations

Pressure and volume of gas production were measured for each bottle at different time intervals after inoculation, using a pressure transducer and a syringe (Williams *et al.*, 1996; Theodorou *et al.*, 1994). As volume measurements by syringe are less accurate, than pressure measurements with the transducer, the volume data were smoothed by using the linear regression of measured volume on measured pressure, to recalculate cumulative gas production.

Gas production profiles (ml g^{-1} OM), were fitted to a multi-phasic modified Michaelis-Menten equation (Equation 1; Groot *et al.*, 1996), for the bottles fermented to 144 h. The improvement in fit when adding an additional phase, was analysed with an *F*-test which analysed the reduction in sum of squares (due to a better fit), relative to the decrease in degrees of freedom (more parameters) (Motulski and Ransnas, 1987).

$$Y = \sum_{i=1}^{n} \frac{A_i}{1 + \left(\frac{C_i}{t}\right)^{B_i}} \qquad \text{(Eq. 1)}$$

Y Cumulative gas production (ml g^{-1} OM);
A_i Asymptotic gas production for phase i (ml);
B_i Smoothness factor for phase i;
C_i Time at which half of the gas is produced for phase i (h);
i Number of phases in the gas production;
t Time (h).

Mathematical analyses of degradability

For DM and individual sugars, the fractional rate of breakdown (k_d, % h^{-1}) for each substrate × inoculum combination was calculated using Equation 2 (Mertens, 1993; Robinson *et al.*, 1986). The amount of each component (e.g. DM or an individual sugar) present at 0 h of incubation was set at 100%, representing the potentially degradable and the undegradable fraction of the specific component. The amount of component present at the other time points was scaled accordingly. When there was more material present at 6 h than at 0 h of incubation (which was the case for some sugars, due to experimental and analytical error), the amount at 6 h was set to 100%, and the other time-points scaled accordingly.

$$Y_t = Fr + (100 - Fr) \times e^{(-k_d \times (t - T_0))} \qquad \text{(Eq. 2)}$$

Y_t % component present in the residue relative to 0 h of incubation;
Fr Undegradable fraction relative to the potentially degradable and undegradable fraction (%);
k_d Fractional rate of breakdown (% h^{-1});
t Time (h);
T_0 Lag time (h).

Statistical analyses

Parameter fittings and VFA production results were analysed as a full 2 × 2 × 2 factorial design (Model 1), using the GLM procedure of SAS (1989).

$$Y = \mu + Inoculum_i + Feedstuff_j + Fraction_k + Inoculum_i \times Feedstuff_j + Feedstuff_j \times Fraction_k + Inoculum_i \times Fraction_k + Inoculum_i \times Feedstuff_j \times Fraction_k + \varepsilon_{ijkl} \quad \text{(Model 1)}$$

Y	Result;
μ	Mean;
$Inoculum_i$	Effect of inoculum source (i = SRF or PF);
$Feedstuff_j$	Effect of kind of feedstuff (j = SBM or maize);
$Fraction_k$	Effect of fraction (k = whole feedstuff or CW);
ε_{ijkl}	Error.

Microscopic evaluation

When subjected to polarized light under a light-microscope, different CW types colour differently making it possible to distinguish between different CW types. Using this principle, SBM CW and maize CW were studied to relate CW structures to seed morphology. Furthermore, residues after incubation were studied to analyse the disappearance of the CW structures present.

Results

The yield of CW from SBM and maize was 14.8% and 7.7%, respectively. The DM, crude protein, fat content, and sugar composition of the substrates are given in Table 1. The total recovery of sugars in the CW fraction from SBM was 47.8%, with recoveries of 95.4% (xylose), 76.2% (arabinose), 70.9% (uronic acids), 47.3% (galactose) and 28.6% (glucose) for individual sugars. Removal of oligo-saccharides (predominantly galactose and glucose) and part of the pectins (predominantly galactose and uronic acids) during CW isolation probably accounted for the low recovery of uronic acids, galactose, and glucose. The total recovery of sugars in the CW fraction from maize was 7.1%, with recoveries of 72.6% (xylose), 70.8% (arabinose), 57.2% (galactose), 17.8% (uronic acids), and 2.2% (glucose) for individual sugars. Possible particle loss during wet sieving (xylose, arabinose) and removal of starch (glucose) were probably responsible for the relatively low recoveries. The low recovery of uronic acids was probably due to the analytical procedure used to determine uronic acids, which tends to overestimate uronic acids in carbohydrate rich feedstuffs (starch in maize).

Gas production results

Figure 1 shows the gas production (ml g^{-1} OM) profiles for SBM, maize, and their CW fractions, for both the SRF and PF inoculum. For SRF, SBM CW appeared to be more rapidly degraded than maize CW, whereas whole maize was more rapidly degraded than SBM. For the PF inoculum, the gas production profiles followed a similar pattern, although the difference between SBM CW and maize CW appeared larger than for SRF. The most striking difference between the gas production profiles of SRF and PF was that PF had a longer lag phase, for the CW fractions relative to the whole feedstuffs, than SRF.

Table 2 shows the fitted curve parameters for both a mono-phasic and a di-phasic model for

Table 1: Dry matter (DM, g kg^{-1}), organic matter (OM), crude protein (CP), and crude fat (CF) content (g kg^{-1} DM) and sugar composition (g kg^{-1} DM in polymeric form) of whole soya bean meal (SBM), whole maize grain, and their cell wall fractions (CW).

		DM	OM	CP	CF	Ara[1]	Xyl	Gal	Glc	UA	TS[1]
SBM	whole	925.6	934.5	546.5	8.8	27	11	82	103	42	285
	CW	875.0	965.8	35.4	0.0	139	70	261	199	202	920
Maize	whole	885.3	985.6	99.9	4.4	20	27	6	734	34	860
	CW	893.8	986.8	55.5	2.8	185	256	46	213	80	797

[1] Respective sugars: Arabinose, Xylose, Galactose, Glucose, Uronic Acids and Total Sugars (including rhamnose, fucose, mannose).

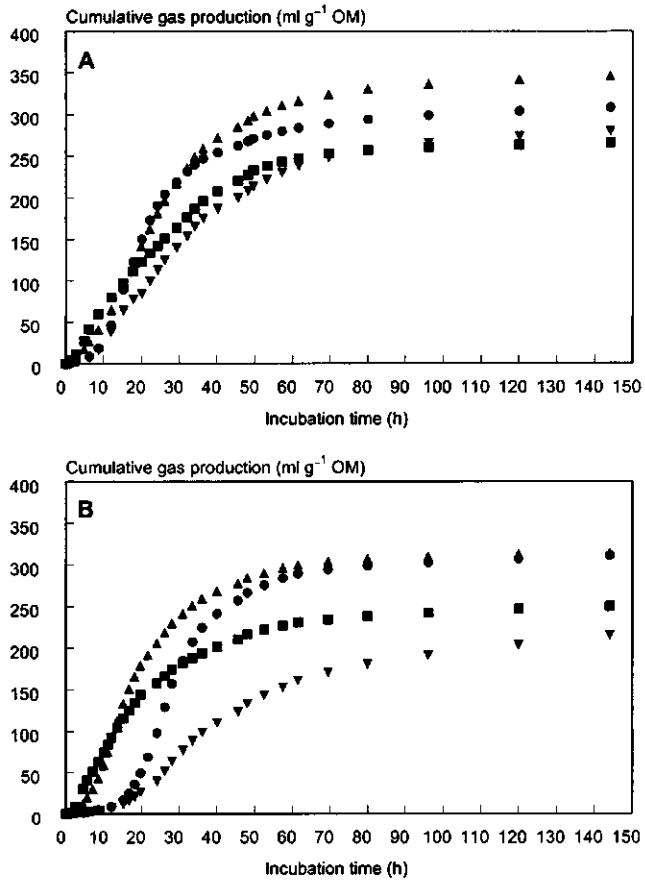

Figure 1: Cumulative gas production per gram of organic matter (OM) for soya bean meal (SBM, ■), maize grain (▲), and their cell wall fractions (SBM CW (●); maize CW (▼)), for incubation with (A) sheep rumen fluid and (B) pig faeces inocula.

Table 2: Asymptotic gas production (A, ml g^{-1} OM), smoothness factor (B), and half-time (C, h) for the fermentation of soya bean meal (SBM), maize, and their cell wall fractions (CW) with sheep rumen fluid (SRF) or pig faeces (PF) as inoculum, when fitted with the mono-phasic or the di-phasic model.

			Mono-phasic			Di-phasic					
Inoc.	Feedst.	Fraction	A	B	C	A_1	B_1	C_1	A_2	B_2	C_2
SRF	SBM	whole	293	1.6	23.3	154	1.7	11.6	114	3.8	35.2
		CW	298	2.8	20.4	178	4.2	17.6	137	2.0	33.1
	Maize	whole	353	2.2	23.5	139	1.3	23.1	220	2.9	24.4
		CW	290	2.1	30.2	52	9.3	12.8	235	2.4	35.4
PF	SBM	whole	261	1.5	17.8	160	3.6	11.2	95	4.4	35.6
		CW	301	4.3	28.4	135	7.4	25.9	177	3.0	33.3
	Maize	whole	315	2.3	18.0	252	2.8	14.9	62	3.7	40.3
		CW	214	2.6	40.0	45	7.2	28.5	182	2.2	49.8
		SEM[1]	6.0	0.06	0.62	28.5	1.67	1.66	29.5	0.5	3.2
Inoculum			***	***	***	n.s.	n.s.	***	**	n.s.	***
Feedstuff			n.s.	***	***	n.s.	n.s.	**	**	n.s.	n.s.
Fraction			***	***	***	***	***	***	**	***	*
Inoculum × feedstuff			***	***	n.s.	*	n.s.	n.s.	**	n.s.	***
Feedstuff × fraction			***	***	***	***	n.s.	***	n.s.	n.s.	**
Inoculum × fraction			n.s.	***	***	*	n.s.	***	*	n.s.	n.s.
Inoc. × feedstuff × fraction			***	***	n.s.	n.s.	n.s.	***	n.s.	n.s.	n.s.

* $P < 0.1$; ** $P < 0.05$; *** $P < 0.01$; n.s. not significant.

[1] Standard Error of the Mean.

SBM, maize, and their CW fractions. For each individual fermentation bottle, fitting a di-phasic model significantly improved the fit ($P < 0.05$). Fitting a third phase did not significantly improve the fit for most bottles with the SRF inoculum, but did for PF. However, for a comparison of substrate degradation, the mono-phasic fit is most informative. For the mono-phasic fit, SRF had a slightly higher asymptotic gas production (A-parameter) with a slightly shorter half-time of gas production (C-parameter) compared to PF. However, the significant inoculum-by-fraction interaction shows, that for whole feedstuffs the half-time of gas production was actually slightly shorter for PF than for SRF, whereas the half-time was considerably longer for CW with the PF inoculum. For the di-phasic fit, the large standard error (SEM) shows that the parameter fitting was more variable. Only for whole maize did the division of gas between the two phases seem to be different between PF and SRF. For the other substrates, the division of gas between phases was fairly similar.

Residue and VFA production

Table 3 shows the residue, the pattern of VFA production, and other fermentation characteristics for the 144 h fermentation. The amount of residue was not different between the inocula.

Maize had a greater residue than soya, and CW fractions had higher residues than whole feedstuffs. The pattern of VFA production differed only slightly between inocula (although

Table 3: Percentage of DM remaining (Res), VFA production patterns (% of acetic (HAc), propionic (HPr) and butyric acid (HBu)), acetic acid to propionic acid ratio (A/P), acetic acid equivalents produced (AAE, mmol g^{-1} OM), acetic acid equivalents yield (AAEY, mmol g^{-1} DM digested), and gas yield (gas Y, ml g^{-1} DM digested) for the fermentation of soya bean meal (SBM), maize grain, and their cell wall fractions (CW) using sheep rumen fluid (SRF) or pig faeces (PF) as an inoculum.

	Feedst.	Fraction	Res	HAc	HPr	HBu	A/P	AAE	AAEY	Gas Y
SRF	SBM	whole	17.8	57.4	31.2	11.4	1.8	9.7	10.7	272
		CW	20.5	52.4	37.1	10.6	1.4	10.6	12.3	341
	Maize	whole	22.3	43.7	34.0	22.3	1.3	9.2	11.2	406
		CW	34.8	60.0	33.5	6.5	1.8	9.3	13.4	382
PF	SBM	whole	14.2	52.2	32.2	15.6	1.6	10.6	11.2	245
		CW	20.5	51.5	37.9	10.6	1.4	10.9	12.9	346
	Maize	whole	21.9	53.9	25.4	20.6	2.1	9.7	11.9	364
		CW	41.7	56.9	31.6	11.5	1.8	8.7	14.1	317
		SEM[1]	1.4	0.4	0.4	0.2	0.03	0.4	0.4	9.8
Inoculum			n.s.	n.s.	***	***	***	n.s.	*	***
Feedstuff			***	n.s.	***	***	***	***	**	***
Fraction			***	***	***	***	***	n.s.	***	***
Inoculum × feedstuff			**	***	***	n.s.	***	n.s.	n.s.	***
Inoculum × fraction			**	***	***	***	***	n.s.	n.s.	n.s.
Inoc × feedstuff × fraction			n.s.	***	***	***	***	n.s.	n.s.	*

* $P < 0.1$; ** $P < 0.05$; *** $P < 0.01$; n.s. not significant.

[1] Standard Error of the Mean.

significant for HPr and HBu). Fermentation of whole maize resulted in a very high production of HBu. When comparing the CW fractions, SBM CW had a HPr directed fermentation, whereas the fermentation of maize CW produced more HAc. The proportion of HBu seemed slightly higher for maize CW with the PF inoculum than with the SRF inoculum. The A/P ratios for the CW fractions were the same for both SRF and PF, indicating that the VFA production pattern was determined more by substrate than by source of inoculum.

Figure 2 shows the change in the pattern of VFA production with time. Most of the changes in VFA production occurred in the first 48 h of fermentation. In all cases (except maize CW with SRF) the percentage of HAc produced dropped whereas HPr and HBu production became relatively larger. However, CW fractions appeared to have a lower HBu percentage compared to whole substrates, whereas the largest increase in HBu production was for whole maize.

Composite sugar degradation

The lag time, k_d and undegradable residue (*Fr*) for the degradation of DM and sugars is shown in Table 4. These data have not been statistically analysed, because there is only one value for each combination of inoculum, feedstuff and fraction. The full model (Model 1), therefore, uses all degrees of freedom, leaving none for an error term.

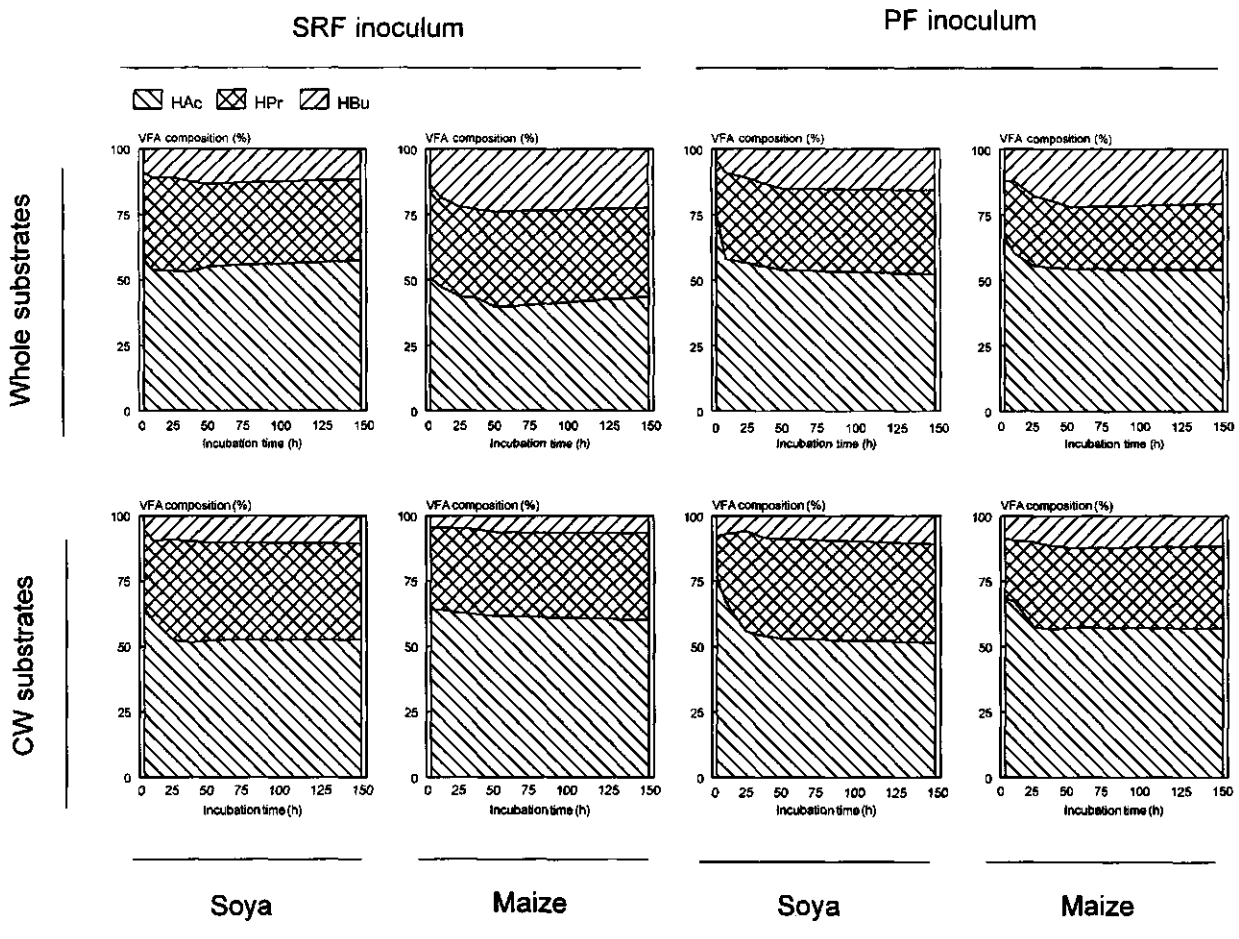

Figure 2: VFA production patterns of acetic acid (HAc), propionic acid (HPr), and butyric acid (HBu) for the fermentation of whole soya beans and maize and their cell wall (CW) fractions using sheep rumen fluid (SRF) or pig faeces (PF) as inoculum.

Table 4: Undegradable residue (*Fr*, %), fractional rate of breakdown (k_d, % h^{-1}), and lag time (T_0, h) for dry matter (DM), individual sugars, and total sugars (TS) for the degradation of soya bean meal (SBM), maize grain, and their cell wall fractions (CW), using sheep rumen fluid (SRF) or pig faeces (PF) as inoculum.

	Feedst.	Fraction		DM	Ara[1]	Xyl	Gal	Glc	UA	TS
SRF	SBM	whole	*Fr*	40.5	2.6	13.3	3.7	8.5	11.2	9.5
			k_d	4.7	36.5	30.3	35.5	10.2	25.0	16.0
			T_0	4.1	3.6	4.9	0	5.7	4.0	2.8
		CW	*Fr*	31.1	3.0	21.0	2.0	19.0	12.3	15.2
			k_d	13.0	17.0	13.6	44.0	3.7	13.7	15.2
			T_0	4.8	5.1	5.4	5.9	1.3	5.0	5.2
	Maize	whole	*Fr*	30.5	29.3	5.5	0	1.6	29.3	5.25
			k_d	8.1	10.6	1.9	0.4	9.6	10.6	9.5
			T_0	4.0	0	0	0	4.7	0	4.2
		CW	*Fr*	37.8	17.4	18.0	20.4	18.8	22.0	18.7
			k_d	3.7	5.4	4.1	3.8	4.5	4.4	4.4
			T_0	1.3	1.4	1.3	1.5	2.9	0	1.3
PF	SBM	whole	*Fr*	40.3	5.1	20.1	4.2	10.8	11.6	11.4
			k_d	5.0	25.9	26.2	29.6	10.2	24.5	17.5
			T_0	6.1	9.1	10.2	9.1	7.1	10.4	8.8
		CW	*Fr*	33.1	5.7	22.6	2.1	26.5	8.8	13.5
			k_d	9.4	20.2	9.9	17.0	5.2	12.6	11.2
			T_0	10.5	10.8	9.5	10.7	9.0	10.9	10.0
	Maize	whole	*Fr*	35.1	59.1	81.7	x	5.7	21.8	12.2
			k_d	7.6	21.4	24.6	x	8.2	7.6	8.0
			T_0	4.7	10.2	19.9	x	4.4	5.4	4.7
		CW	*Fr*	48.9	26.3	29.1	32.2	28.4	32.9	28.7
			k_d	3.4	3.5	3.3	2.5	4.2	3.6	3.4
			T_0	7.3	5.3	1.2	3.4	7.1	5.5	3.4

[1] Respective sugars: Arabinose, Xylose, Galactose, Glucose, Uronic Acids and Total Sugars (including rhamnose, fucose, mannose).

The undegradable residue differed considerably between the sugars of whole SBM, whole maize, and SBM CW. For maize CW, the undegradable residue was fairly similar for all sugars, and seemed higher for the PF inoculum.

For whole SBM and SBM CW, the k_d for the different sugars was highest for galactose and arabinose, with lower values for uronic acids and xylose, and the lowest for glucose. A similar pattern was also found in previous work with whole and fractionated SBM (Van Laar *et al.*, 1999; Chapter 3). The k_d values for the sugars of SBM (whole and CW fraction) were ranked similarly for both the SRF and PF inoculum. For whole maize, the k_d was quite variable between sugars, but for maize CW the k_d of the different sugars was fairly constant for both the SRF and PF inoculum.

Table 5: Amount of dry matter (DM) in inoculum (g l^{-1}), crude protein content (CP), diamino pimelic acid content (DAPA) and sugar composition (g kg^{-1} DM) of total (inoc.) and bacterial (bact.) DM of the sheep rumen fluid (SRF) and pig faeces (PF) inocula.

	DM	CP	DAPA	Ara[1]	Xyl	Gal	Glc	UA	TS
SRF inoc.	15.4	416.2	2.6	3.1	8.3	8.4	30.5	10.0	70.6
SRF bact.	3.5	453.3	3.3	0.9	2.1	9.3	21.5	10.6	57.5
PF inoc.	27.9	280.6	1.6	8.8	10.6	9.2	26.7	15.7	83.7
PF bact.	6.4	279.0	1.9	2.7	1.0	5.6	6.6	8.1	35.7

[1] Respective sugars: Arabinose, Xylose, Galactose, Glucose, Uronic Acids and Total Sugars (including rhamnose, fucose, mannose).

The lag time was slightly shorter for the SRF inoculum compared to PF. For all CW fractions the lag times were fairly comparable to the original whole feedstuffs. With the SRF inoculum the lag times were similar for the sugars within a substrate. This was also the case for whole SBM and SBM CW with PF, but for whole maize and maize CW, the lag times appeared to differ between sugars.

Bacterial analyses

The composition of inocula DM and bacterial DM is shown in Table 5. The inocula were composed mainly of crude protein, though also contained a maximum of 8.4% sugars in the DM. Therefore contamination of the substrate by sugars originating from the inoculum was very low. The contamination of CW fractions with glucose (most abundant contaminating sugar) originating from the PF inoculum was estimated to be between 2 to 3%, whereas it was between 1 to 2% for the SRF inoculum.

Figure 3 displays the amount of DAPA produced during fermentation. For SBM CW the amount of DAPA produced increased to approximately 1 mg g^{-1} DM at 24 h of fermentation, after which it decreased again. There were considerable differences in the amount of DAPA production between the different substrates. The SBM CW produced the largest amount of DAPA, which at its peak at 24 h was equivalent to about 322 mg bacterial DM per gram substrate DM for the SRF inoculum. This was calculated by dividing the DAPA production at its peak at 24 h (Figure 3), by the DAPA content for SRF bacteria (Table 5).

Microscopic evaluation

The microscopic analyses of SBM CW revealed the presence of two major CW types, a thick CW type and a thin CW type. By a comparison with whole soya beans, these CW types were shown to originate from the hull (thick CW type) and the endosperm (thin CW type) of the soya bean, as described by Van Laar *et al.* (1999; Chapter 3). It was also found that the thin CW material was degraded faster than the thick CW material. Therefore, the proportion of thick CW within the residue increased during fermentation.

For maize CW the microscopic analyses indicated the presence of different CW types. However, it proved to be much more difficult to relate the different CW structures from maize CW to the structures present in the maize grain. Some CW structures from the bran could easily

be traced back to the maize grain, however, other CW types (at least two) were harder to trace back. Bran CW structures appeared to be less degradable than other 'thinner' CW types present.

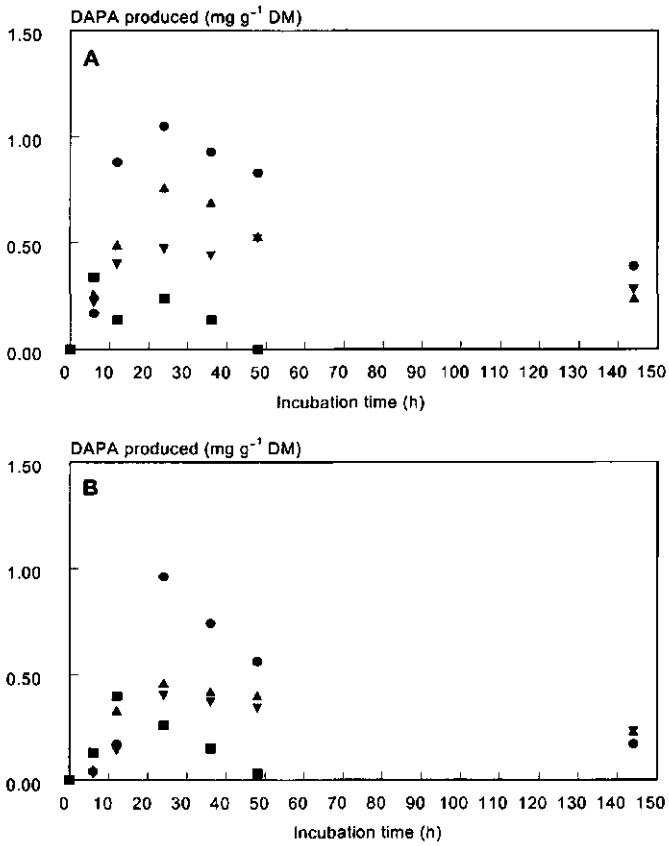

Figure 3: Diamino pimelic acid (DAPA) produced (mg g^{-1} DM) from the incubation of soya bean meal (SBM, ■), maize grain (▲), and their cell wall fractions (SBM CW (●); maize CW(▼)), during incubation with (A) sheep rumen fluid and (B) pig faeces inocula.

Discussion

Substrate composition

The whole feedstuffs were commercially available maize and SBM. Maize is a starch rich, monocotyledonous plant, and soya a protein rich, dicotyledonous plant. These two plant groups are known for their differences in CW composition (Harris *et al.*, 1997). The maize CW and SBM CW material used contained only small amounts of protein and fat and was composed

mainly of CW polysaccharides. Judging from the high content of arabinose, glucose, and xylose, the maize CW polysaccharides were mainly cellulose and arabinoxylans with very little pectin, as reported by Harris *et al.* (1997). However, the SBM CW was composed mainly of arabinose, galactose, uronic acids, and glucose and, therefore, most likely consisted of cellulose, very little hemicellulose, and a large amount of pectins, as also found by Huisman *et al.* (1998). Thus, these two CW had marked differences in CW sugar composition, but also differed in CW polysaccharide structure.

Gas production results

The fermentation patterns of whole SBM and whole maize were fairly similar for SRF and PF. However, the main difference between the fermentation using SRF and PF seemed to be that CW fractions were less readily fermented by the PF inoculum. The total extent of fermentation for SBM CW with SRF and PF was similar, whereas for maize CW the extent of fermentation was lower with PF. The difference in the delay of CW fermentation is most likely a direct indicator of the difference in adaptation to fibre fermentation of SRF and PF. This because the amount of nitrogen and DAPA added with the inoculum, and thus probably the number of bacteria added, were fairly similar for SRF and PF (Table 5). Furthermore, it is likely that the SRF inoculum was better adapted to fibre fermentation, because of the fibrous nature (hay) of the sheep diet. The PF inoculum would then be less adapted to fibre degradation, although it should be adapted to soya fibre, because soya was present in the diets of the pigs.

The fermentation of whole maize and SBM involves a basic comparison between a carbohydrate-rich feedstuff and a protein-rich feedstuff, respectively. The gas production profiles would suggest that whole SBM was less fermentable; however, the DM residues (Table 3) indicate that whole SBM was more fermentable. This means that for SBM, either, not all DM (protein) which was solubilized was fermented, or that fermentation of this DM (protein) did not produce gas. The latter possibility is supported by Cone and Van Gelder (1999) who found, that the fermentation of protein yields less gas than the fermentation of carbohydrates. Therefore, when comparing the gas production curves of feedstuffs with very different protein contents this effect must be taken into account.

The protein content of the CW fractions of maize and SBM were fairly similar and gas production can therefore be used as an indicator of CW fermentation. With both SRF and PF as inocula, SBM CW was fermented more rapidly and more extensive than maize CW. Although possible (unknown) differences in particle size (Buxton and Redfearn, 1997) may influence this difference in fermentability, the main cause is thought to be the difference in CW composition. This is because pectin rich CW (SBM) are generally thought to be more fermentable than hemicellulose rich CW (Hatfield, 1993).

CW sugar degradation and microscopic evaluation

The results of the CW sugar degradation must be interpreted in combination with the data of the microscopic evaluation. The microscopic evaluation revealed that the CW fractions of SBM and maize CW were composed of multiple CW types. For SBM CW there were two main CW types, one originating from the hull (a thick CW), and one originating from the cotyledon (a thin CW) of the soya bean seed. The difference in degradation characteristics combined with a difference

in sugar composition between the CW types, was responsible for the differences in degradation of the individual sugars within SBM or SBM CW (Table 4) as described by Van Laar *et al.* (1999; Chapter 3). Briefly: the thin cotyledon CW, rich in galactose and arabinose, were rapidly degraded, hence the rapid degradation of galactose and arabinose. However, the thick hull CW, rich in glucose and xylose, were slowly degraded, hence the slow degradation of xylose and glucose. Chesson (1993) has also described this principle for forage materials.

In maize, multiple CW tissue types were present, which varied in thickness. Some CW tissues originating from the maize bran were easily identifiable and were thick CW fragments, which were not well fermented. However, the CW from the endosperm was much thinner and more rapidly fermented. Similar to SBM CW, maize CW had different tissues with different degradabilities. However, this did not lead to a difference in degradation rate for the individual sugars within the maize CW fraction. This would suggest that the different CW tissues within the maize grain have a similar sugar composition.

VFA composition

The VFA production pattern over time (Figure 2) showed, in most cases, a drop in the proportion of HAc, whereas the proportion of HPr and HBu increased. Thus, VFA production during fermentation was subject to change, possibly caused by a changing microbial population and a changing substrate composition. Therefore, analysing the VFA production of a fermentation end-point, is not necessarily valid to study the whole fermentation process over time.

The relatively large proportion (Table 3) of HBu production for maize may have been related to the fermentation of starch, as indicated by Hungate (1966). The difference in VFA production profile between SBM and maize CW, as indicated by the A/P ratio, was consistent between inocula and was therefore, most likely related to CW characteristics. The slightly higher HPr production could be reflective of the more rapid fermentation for SBM CW, whereas the slower fermentation of maize CW could be the cause of the higher HAc production (Groot *et al.*, 1998; Van Houtert, 1993). However, the A/P ratio for SBM CW was rather low compared to other research (2.0; Van Laar *et al.*, 2000b; Chapter 4) and was expected to be slightly higher due to the pectin-rich nature of SBM CW (Howard, 1961; Marounek *et al.*, 1985).

Bacterial contamination

The DAPA concentrations of 3.3 g kg^{-1} and 1.9 g kg^{-1} in the microbial DM for SRF and PF, respectively, are in the range of concentrations (1.9 to 3.0 g kg^{-1}) reported by Olubobokun *et al.* (1988), Hsu and Fahey (1990), and Caine *et al.* (1999) for bacterial isolates from the rumen and small intestines (pigs). However, the crude protein and DAPA content of the bacterial isolates of SRF is much higher than for those of PF, whereas the ratio of crude protein to DAPA is fairly similar (137 and 147 for SRF and PF, respectively). This indicates that the bacterial isolate of PF is probably contaminated with DM, which does not originate from bacteria.

The total amount of DAPA in the residues (Figure 3) illustrated an increase in bacterial material due to bacterial growth in the first 24 h. After 24 h the amount of bacterial material decreased again, probably due to microbial turnover (Cone *et al.*, 1997). There was a marked difference in DAPA production between the substrates. The SBM CW led to the production of the largest amount of DAPA. This is probably because of its high rate and extent of fermentation.

Van Houtert (1993) discussed how a rapid fermentation results in a higher microbial yield due to a decreased use of energy for maintenance. The low DAPA production for whole SBM is probably related to the low amount (relative to whole maize and CW materials) of carbohydrates, and, thus, contains less energy for microbial growth.

There were considerable differences in DAPA production between the different substrates using SRF, with the estimated maximal amount of bacterial DM produced being 322 mg g^{-1} substrate DM for SBM CW. Because the bacterial isolates for PF were considered to be contaminated with DM, not originating from microbes, only the bacterial DM production for SRF could be calculated. The value of 322 mg microbial DM produced g^{-1} SBM CW DM, would mean a microbial DM production of approximately 412 mg g^{-1} truly digested DM. This is somewhat higher than would be possible from the results of Van Houtert (1993) who reported efficiencies up to 300 mg g^{-1} fermented organic matter. However, an efficiency of 557 mg g^{-1} truly fermented substrate was reported by Blümmel *et al.* (1997). Therefore, the efficiency of microbial DM production found in the present study may be high, but similar efficiencies have been reported before.

The bacterial DM contained only 57.5 g kg^{-1} and 35.7 g kg^{-1} of carbohydrates (total sugars) for SRF and PF, respectively (Table 5). Therefore, although the efficiencies of microbial DM production were very high, the contamination of the residue with sugars from bacterial DM was low, with a maximum of 3% for glucose with SBM CW and SRF. The same goes for the contamination with carbohydrates from the inoculum, which for the sugar with the highest contamination (glucose) was around 3%. The total contamination of residue, with glucose from both inoculum and bacterial DM, would be a maximum of 6%. A maximal contamination with glucose of 6% for SBM CW with SRF is too small to have caused the large differences in degradation characteristics relative to the other sugars. Thus, the influence of contaminating sugars from inoculum and the production of microbial DM, on the calculation of the sugar degradation characteristics, was considered to be negligible.

Conclusions

The main difference in fermentation between the SRF and PF inoculum was the less rapid degradation of CW material by the PF. The PF micro-organisms are probably not as well adapted to CW fermentation as the micro-organisms from SRF. The fermentation of whole SBM and maize was similar for both PF and SRF. The CW fractions of SBM and maize were composed of multiple CW types, which were different in their degradation characteristics. Furthermore, the composition of the maize and SBM CW fractions was markedly different. The maize CW was rich in arabinoxylans and the SBM CW was rich in pectin. This difference in CW composition may have been the cause of the more rapid and extensive fermentation of SBM CW, compared with maize CW. For SBM CW, the two CW types present had different sugar compositions and different degradation characteristics, causing differences in rate of degradation of the CW sugars in the SBM CW fraction. Although the maize CW fraction consisted of different CW types, with different degradation characteristics, there were no differences in the degradation rates of the individual sugars within the maize CW fraction. This indicates that the different CW types most

likely had the same sugar composition. The contamination of the substrate with carbohydrates originating from the inoculum or from microbial growth was negligible and did not disturb calculation of the degradation characteristics of the individual sugars.

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Chapter 3

Fermentation characteristics of cell wall sugars from soya bean meal, and from separated endosperm and hulls of soya beans*

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Fermentation characteristics of cell wall sugars from soya bean meal, and from separated endosperm and hulls of soya beans

Abstract

Two experiments were conducted to investigate the degradation of cell wall sugars from soya bean meal (*in situ*), and soya bean endosperm and hulls (*in vitro*). Soya bean meal, soya bean endosperm, and soya bean hulls were extracted with different chemicals to obtain the cell wall fraction. Soya bean meal cell walls were incubated in the rumen of a fistulated cow. The individual cell wall sugars were degraded at different rates: galactose (13.6% h^{-1}), arabinose (7.8% h^{-1}), uronic acids (5.1% h^{-1}), xylose (3.5% h^{-1}) and glucose (3.2% h^{-1}). Microscopic evaluation of the cell walls and degraded material revealed the presence of two cell wall types, with distinctly different degradation characteristics: one originating from the hull (thick, slowly degraded) and one from the endosperm (thin, rapidly degraded). Furthermore, the cell wall sugar composition of endosperm and hull cell walls was different, most markedly for galactose (267 *vs* 12 g kg^{-1}) and glucose (125 *vs* 490 g kg^{-1}). The degradation of endosperm and hull cell walls was measured *in vitro* by use of *in vitro* cumulative gas production. Degradation rates of the individual cell wall sugars for hull cell walls were similar (ranging from 2.4 to 4.6% h^{-1}). For endosperm cell walls the degradation rates of the individual sugars were different, with the same ranking as in the *in situ* experiment (ranging from 20.9 to 7.0% h^{-1}). It was concluded that for soya bean meal cell walls, the cell wall sugar degradation pattern was influenced by the presence of two cell wall types (hull and endosperm cell wall), which differed in their rate of degradation and sugar composition. The difference in cell wall sugar degradation pattern between hull and endosperm cell walls was likely caused by a combined effect of particle size and cell wall thickness.

Key words: cell walls, degradation, neutral sugars, soya bean meal

Introduction

Knowledge of the composition of the plant cell wall and its digestion in ruminants and monogastrics is needed to be able to optimize utilization of plant cell wall components. Since cell walls cannot be hydrolysed by the digestive enzymes of the mammalian gastrointestinal tract (Jung, 1997), cell wall digestion can only occur by microbial degradation (microbial enzymes). This is true for both ruminants and monogastrics, even though microbial degradation of cell walls occurs in different anatomical regions of the gastrointestinal tract.

Plant-based feedstuffs can be composed of different cell wall types, ranging from thin primary cell walls to thick secondary cell walls, both of which can be lignified. Microbial degradation of cell walls is influenced by the polysaccharides present, their composition, crystallinity (Bailey *et al.*, 1976), degree of acetylation (Kerley *et al.*, 1988) and cross-linking amongst other factors. Primary cell walls of dicotyledonous plants present in non-structural plant tissues (such as in soya bean endosperm), are thin and unlignified, and are composed mainly of cellulose, pectins, and some hemicellulose (Chesson, 1993). The secondary cell wall is formed by deposition of

polysaccharides on the luminal side of the primary cell wall. These polysaccharides are usually richer in hemicellulose compared to primary cell wall polysaccharides (Chesson, 1993). The primary cell wall becomes lignified when deposition of secondary cell wall layers begins (Engels and Schuurmans, 1992). Lignification of secondary cell walls has important consequences for its degradation (Lau and Van Soest, 1981; Cornu *et al.*, 1994).

Most of the research on the composition and microbial degradability of cell walls has been done for forage crops such as grasses (Burrit *et al.*, 1984; Engels, 1996), alfalfa (Titgemeyer *et al.*, 1991) and straw (Chesson, 1981), which are rich in lignified secondary cell walls. Much less attention has been paid to cell wall degradation of concentrate feedstuffs, which are relevant for both ruminants and monogastrics. Both the amount and type of cell wall polysaccharides are important for the ability of gastrointestinal microbes to degrade the primary cell wall.

Determination of the exact content and composition of polysaccharides in cell walls is a very cumbersome and complex matter (Nevins, 1993). A crude, but easy measure of cell wall polysaccharides is to determine the content and composition of the cell wall sugars. Although sugar content and composition do not yield detailed knowledge on the exact fine structure of the cell wall polysaccharides, it does give an indication. These cell wall polysaccharides can roughly be divided into cellulose (glucose), hemicellulose (xylose, arabinose, galactose, glucose) and pectins (uronic acids, galactose, arabinose). Two experiments were conducted to investigate the composition and microbial degradation of primary cell walls from soya beans. The first experiment investigated cell wall sugar degradation of soya bean meal and isolated cell walls in a rumen incubation experiment (*in situ*). The second experiment investigated the *in vitro* fermentability of cell walls isolated from the hull and endosperm of soya beans.

Materials and methods

Cell wall preparation

In situ *experiment*

Untoasted industrially defatted soya bean meal (SBM) was ground over a 0.5 mm sieve and used to prepare purified soya bean meal cell walls (SBM CW) (Huisman *et al.*, 1998). For this purpose, two 400 g batches of ground SBM were sequentially extracted using demineralized water (3 h, room temperature), a solution of 10 g l^{-1} sodium dodecyl sulphate (SDS) and 1.5 g l^{-1} dithiothreitol (DTT) (3 h, room temperature), and with a maleic acid buffer containing 2 mg α-amylase (Merck art. 16312 (from porcine pancreas)) (pH 6.5 for 16 - 20 h at 30 °C). After each extraction step, the material was centrifuged at 11,000 × g for 30 min, the supernatant discarded, and the pellet was subjected to the subsequent extraction step. The final residue was freeze-dried and left to air-equilibrate for three hours. The material obtained in this way is supposed to be representative of the cell wall fraction of SBM. For more detailed extraction procedures see Huisman *et al.* (1998).

In vitro *experiment*

Untreated, full-fat, Argentinean soya beans were soaked overnight in iced water. After soaking,

the beans were manually dehulled, and separated into hulls and endosperm. These were freeze-dried, ground over a 0.5 mm sieve, and extracted with petroleum ether 40-60 in a Soxleth extractor to remove fat. Cell wall material (CW) was extracted from defatted hulls and endosperm as described for the *in situ* experiment. This resulted in two types of cell wall material: hull cell walls and endosperm cell walls (HCW and ECW). HCW and ECW were combined to reconstitute the cell wall fraction of the original bean: combined cell walls (CCW).

Incubation and chemical analysis

In situ *experiment*

Four g dry matter (DM) of air-dry ground (0.5 mm sieve) SBM or SBM CW was weighed into nylon bags (Nybolt PA 15/10; pore-size 15 μm/open surface area 10%) of 10 × 19 cm, which were sealed on two sides. To minimize loss of particulate matter, due to the relatively small particle size (0.5 mm sieve), it was decided to use bags with a 15 μm pore-size, instead of 41 μm normally used in Dutch *in situ* trials, because the 41 μm pore-size led to a 25% DM loss after simple washing (Van Laar, unpublished data). A fistulated non-lactating dairy cow was used for rumen incubation, and was fed a ration of 1.7 kg DM concentrate and 7.4 kg DM grass silage per day (CVB requirements, 1993). The cow was adapted to this diet for 10 days before the start of the incubation period. Bags were incubated in the rumen for 0, 3, 6, 12 and 24 h. After incubation, the bags were submerged in water, washed in a washing machine and freeze-dried. Bags containing SBM CW residues were washed twice to minimize contamination with rumen material. Bags incubated for 0 h were not incubated in the rumen, but were only washed and subsequently freeze-dried to determine the washable fraction of the incubated material. After freeze-drying, all bags were allowed to air-equilibrate for at least three hours, and then weighed. Residues were pooled based on material and incubation time.

SBM, SBM CW, and their residues were analysed for dry matter (DM), ash, and nitrogen (N). DM was determined by drying to a constant weight at 103 °C following ISO method 6496, and ash by combustion at 550 °C according to ISO method 5984. N was determined with the Kjeldahl method with $CuSO_4$ as the catalyst, following ISO method 5983. SBM and residues were additionally analysed for Neutral Detergent Fibre (NDF) according to Van Soest *et al.* (1991) modified by Goelema *et al.* (1998) and Acid Detergent Fibre (ADF) according to Van Soest (1973). Neutral sugar composition of SBM, SBM CW and their residues, was determined by gas chromatography (GC) according to Englyst and Cummings (1984), using inositol as internal standard. The samples were pre-treated with 7.3 M sulphuric acid (1 h, 30 °C) followed by hydrolysis with 1 M sulphuric acid for 3 h at 100 °C. Sugars were analysed as their alditol acetates. Uronic acids were determined by a colorimetric *m*-hydroxydiphenyl assay using a spectrophotometer at 520 nm (Blumenkrantz and Asboe-Hansen, 1973).

In vitro *experiment*

The three substrates (ECW, HCW, and CCW) were assessed for their fermentability according to the *in vitro* cumulative gas production technique described by Theodorou *et al.* (1994). Approximately 0.5 g DM of ECW, HCW, and CCW was weighed into serum bottles (size: 100 ml). Bottles were inoculated using rumen fluid of sheep fed, medium-quality hay. Sufficient

replicates were used, so that bottles containing substrate could be removed after 0, 9, 12, 24, 36, 48 and 144 h of inoculation. For each combination of fermentation time and substrate, the number of bottles used was determined based on an estimated total residue of approximately 1 g DM.

Fermentation was stopped by autoclaving the bottles at 110 °C for 10 min, thereafter bottles were stored at −18 °C pending residue collection. After thawing, each bottle was rinsed into a centrifugation tube with demineralized water and centrifuged at 11,000 × g for 30 min. The pellet was resuspended in demineralized water and centrifuged again. The residue was brought into a 50 ml plastic bottle, freeze-dried, and the total DM determined. The residues were left to air-equilibrate, after which the amount of air-dry matter was determined enabling the calculation of the DM content of these air-dry residues. Subsequently, air-dry matter samples were pooled by substrate and fermentation time, reground in a porcelain mortar and analysed for neutral sugar composition. After pretreatment as described for the *in situ* experiment, neutral sugars were analysed by HPLC (High Pressure Liquid Chromatography, Dionex PA-10 column and PA-10 guard column) with Pulsed Amperiometric Detection, using allose as an internal standard. The use of an HPLC method for analysing neutral sugars in the *in vitro* trial, instead of the before-mentioned GC method in the *in situ* trial, was for purely practical reasons, related to availability of equipment. This, however, should not interfere with the analyses of the results, as is also indicated by Lebet *et al.* (1997).

Mathematical evaluation

In situ *experiment*

To calculate the degradation characteristics of SBM and SBM CW (*in situ*), and ECW, HCW and CCW (*in vitro*) the fractional degradation rate (k_d) and lag time (T_0) were estimated using Equation 1 (Robinson *et al.*, 1986; Mertens, 1993):

$$Y_t = Y_0 \times e^{(-k_d \times (t - T_0))} \qquad \text{(Eq. 1)}$$

Y_t Residue at time t as a percentage of Y_0;
Y_0 Amount of starting material (100%);
k_d Fractional degradation rate (h^{-1});
T_0 Lag time (h).

Y_t is expressed as a percentage of component (e.g. dry matter (DM) or crude protein (CP)) present after 0 h of incubation (unwashable fraction). The fractional degradation rate (k_d) represents the percentage of the potentially degradable unwashable material, which is degraded per hour. The lag time is the time during which degradation has not yet started. This model does not estimate a possible indigestible fraction, because cell walls (Mosterd, 1992; DePeters *et al.*, 1997) and protein (Van Straalen and Tamminga, 1990) of SBM and SBM CW were assumed to be totally digestible after prolonged incubation.

For SBM residues (*in situ*), there was an increase in percentage of residue from 0 to 3 h, possibly due to inflow of ruminal material, which complicated calculation of degradation

characteristics. The addition of inoculum in the *in vitro* experiment led to a slightly increased percentage of residue present during the lag time. To reduce the complicating effects of this increase, on the calculation of the rate of degradation, all residues for both the *in situ* and *in vitro* experiment were expressed relative to the timepoint with the highest residue, which was set to 100%. Residues from prior to the timepoint with the highest residue, were also set to 100%. For the DM component (*in vitro*), an undegradable fraction was incorporated into the model for correct estimation of the degradation characteristics.

Calculations were carried out using the Gauss Marquard method of the NLIN procedure of SAS version 6.11 for Windows (SAS, 1989).

Microscopic evaluation

To relate cell wall structures in the SBM CW to the intact soya bean, both SBM CW and intact soya beans were studied using light microscopy. All residues after incubation were also examined using light microscopy. When subjected to polarized light, different cell wall types can colour differently. On the basis of this principle it was possible to distinguish between different types of cell walls. Also ECW, HCW and CCW and residues were studied using light microscopy procedures.

Results

In situ *experiment*

The composition of SBM and SBM CW used for rumen incubation is shown in Table 1. The yield of CW from the extraction of SBM was 15.7% on a DM basis. The recovery of total sugars for the extraction of CW from SBM was 54%, but ranged from > 90% (arabinose, xylose) to 70% (uronic acids), 54% (galactose) and 35% (glucose) for individual sugars.

Table 1: Dry matter (DM), organic matter (OM), crude protein (CP), crude fat (CF), neutral detergent fibre (NDF), acid detergent fibre (ADF), and acid detergent lignin (ADL) content (g kg^{-1}) and sugar composition (g kg^{-1}) of soya bean meal (SBM) and soya cell walls (SBM CW) (*in situ* experiment), and of cell walls from soya bean hull (HCW) and endosperm (ECW) (*in vitro* experiment).

	DM	OM	CP	CF	NDF	ADF	ADL[1]	Ara[2]	Xyl	Gal	Glc	UA	TS
SBM	935	872	516	n.a.	71	45	n.a.	20	9	70	79	29	222
SBM CW	925	886	41	n.a.	n.a.	n.a.	n.a.	134	55	237	175	151	798
HCW	938	913	48	4.7	779	665	11.6	55	95	12	490	111	788
ECW	913	881	44	6.6	300	177	12.9	133	41	267	125	154	760

n.a. Not analysed.

[1] ADL values were calculated from composition of original hull and endosperm material.

[2] Respective sugars: Arabinose, Xylose, Galactose, Glucose, Uronic Acids and Total Sugars (including rhamnose, fucose, mannose).

Table 2: *In situ* degradation: washable fraction (S, %), fractional degradation rate (k_d, % h^{-1}) and lag time (T_0, h) for components of soya bean meal (SBM) and soya cell walls (SBM CW).

		DM[1]	OM	N	NDF	ADF	HE[2]	OM1[3]	OM2[4]
SBM	S	73.1	72.3	79.1	14.8	23.6	0	62.4	74.3
	k_d	10.3	10.4	10.0	6.4	4.9	10.0	10.9	18.8
	T_0	4.0	4.0	4.8	2.8	3.4	2.9	3.1	3.9
SBM CW	S	13.0	12.4	4.7	x	x	x	12.8	x
	k_d	5.2	5.2	1.0	x	x	x	5.7	x
	T_0	1.1	1.1	6.0	x	x	x	1.2	x

[1] Respectively: Dry Matter, Organic Matter, Nitrogen, Neutral Detergent Fibre, Acid Detergent Fibre.

[2] HE (hemicellulose) was calculated as NDF – ADF.

[3] OM1 was calculated as OM – Crude Protein.

[4] OM2 was calculated as OM – Crude Protein – NDF.

Table 3: *In situ* degradation: washable fraction (S, %), fractional degradation rate (k_d, % h^{-1}) and lag time (T_0, h) for sugars of soya bean meal (SBM) and soya cell walls (SBM CW).

		Ara[1]	Xyl	Gal	Glc	UA	TS
SBM	S	72.0	79.7	41.1	34.8	64.4	46.5
	k_d	22.9	9.0	29.7	7.3	16.9	12.9
	T_0	4.2	4.4	3.0	3.6	4.4	3.2
SBM CW	S	72.7	88.2	79.2	93.9	75.2	81.3
	k_d	7.8	3.5	13.6	3.2	5.1	5.9
	T_0	2.2	0	2.3	0	1.7	1.0

[1] Respectively: Arabinose, Xylose, Galactose, Glucose, Uronic Acids, and Total Sugars (including rhamnose, fucose, mannose).

Washing out of oligosaccharides (for galactose and glucose) and part of the pectins (for galactose and uronic acids) is held responsible for the low recovery of uronic acids, galactose and glucose. For SBM, the amount DM disappeared at 0 h (washable fraction) was 73.1%. This is slightly more than the amount of soluble DM of 59% indicated by Huisman *et al.* (1998) using the same batch of untoasted soya bean meal. This 14% difference may, therefore, indicate that some insoluble material might have been lost by washing. Contrary to expectations, there was an increase in percentage of material left, after 3 h of incubation, compared to 0 h. This increase has also been witnessed in other experiments with finely ground untoasted soya bean meal (Van Laar, unpublished data), and was mainly due to an increase in the total amount of crude protein present. This crude protein may have originated either from microbial matter or ruminal material entering the nylon bags, which were not removed during washing procedures or from soya protein, which was not washed out. The washable fraction (DM basis) of SBM CW was 13.0%, which, because of the nature of SBM CW (insoluble cell wall), is composed of particles smaller than 15 μm. For SBM CW, there was no increase in the amount of DM after 3 h incubation as found for SBM. Furthermore, the amount of nitrogen (N) in the bags remained almost constant, whereas the amount of total DM decreased.

Table 2 presents the washable fraction, the fractional degradation rate and the lag time for components of SBM and SBM CW. The fractional degradation rates for all SBM components were higher than for SBM CW. The hemicellulose (HE) and the protein-free organic matter (OM1) degradation rate of SBM, were similar to the DM, OM and N degradation of SBM. The OM2 is the protein-free organic matter minus the NDF fraction, which since the fat and starch content of SBM is low, is approximately equal to the pectin fraction. The OM2 had the highest rate of degradation of all components of SBM. The degradation rates of SBM CW, DM, OM and OM1 were fairly similar.

The washable fraction, fractional degradation rate, and lag time, for sugars from SBM and SBM CW are shown in Table 3. The fractional degradation rate for all sugars was higher for SBM than for CW. Table 3 indicates a difference in degradation rate for the different sugars in both SBM and CW. For both SBM and SBM CW, glucose had the lowest, and galactose the highest degradation rate. The ranking of sugar degradation rates in SBM CW and SBM is similar. The difference in degradation rates of the sugars is also shown in Figure 1. This figure shows the percentage of each sugar left, compared to the 0 h of incubation for CW. As in Table 3, galactose disappeared the fastest, followed by arabinose, uronic acids, xylose and glucose.

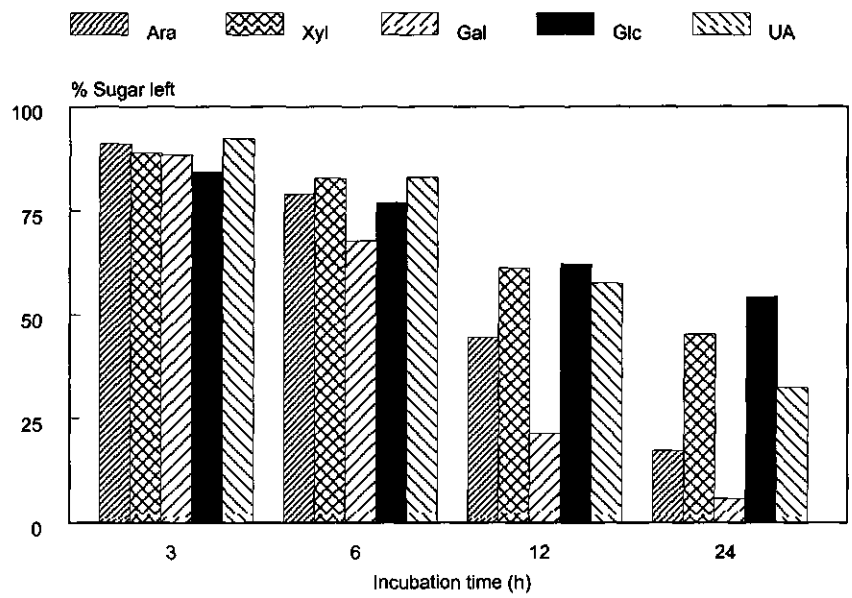

Figure 1: *In situ* experiment: percentage of individual sugar left in residue, relative to the amount of individual sugar present at 0 h, for the incubation of SBM CW (Ara: Arabinose; Xyl: Xylose; Gal: Galactose; Glc: Glucose; UA: Uronic Acids).

In vitro *experiment*

Whole soya bean consisted of 8.0% hull on a DM basis. Endosperm and hull yielded 13.6% and 62.3% CW on a DM basis, respectively. The DM of the reconstituted cell wall fraction of the original bean (CCW) was, therefore, composed of 71.5% and 28.5% of ECW and HCW, respectively. Table 1 shows the composition of ECW and HCW. The ADL was calculated from the composition of the original endosperm and hull, since determination of ADL in cell wall material was not reproducible. Table 1 shows a large difference in the content of galactose and glucose for ECW and HCW.

Since there was virtually no degradation of CCW, HCW and ECW sugars up to 12 h, the percentage of sugar left in the residue for these substrates (Figure 2), is expressed relative to 12 h of incubation. The pattern for CCW was similar to that in the *in situ* experiment, with glucose disappearing the slowest and galactose the fastest. For HCW, all the individual sugars seemed to be degraded at a similar rate, whereas for ECW the sugars were degraded at different rates. These findings are also illustrated in Table 4, which shows the degradation characteristics for the dry matter and composite sugars of ECW, HCW and CCW.

Light microscopy was used to check whether the ECW was contaminated with HCW and *vice versa*. Both ECW and HCW were composed of virtually pure endosperm and hull cell walls, respectively.

Microscopic results

Microscopic evaluation of SBM CW revealed the presence of two distinctly different cell wall types: a thick-walled cell wall type (which coloured blue/yellow in polarized light) and a thin-walled cell wall type (which coloured red). These cell wall types could also be distinguished when looking at the hull (only thick cell walls) and the endosperm (only thin cell walls) of the intact soya bean. Microscopic examination suggested, therefore, that the soya CW was composed of cell wall material originating from the hull and the endosperm of the soya bean. However, after 24 h of digestion, the main cell wall type left in the residue, was the thick cell wall originating from the hull, whereas the thin-walled cell wall had almost completely disappeared. These results led to the separation of the hull and endosperm of the soya bean, and sugar analyses of the hull and endosperm CW, as described in the *in vitro* experiment.

Discussion

In situ *experiment*

The sugar composition of SBM CW was similar to that reported by Schols *et al.* (1993). For SBM, arabinose, xylose and uronic acids were at a similar level compared to Schols *et al.* (1993), though galactose and glucose were much higher in the present experiment. Glucose and galactose however, are present in the oligosaccharides of SBM (Coon *et al.*, 1990). The sugars of these oligosaccharides were included in the analysis of the original material of this paper, but not in the analysis of Schols *et al.* (1993).

The crude protein level of SBM CW (40.8 g kg^{-1}) was in agreement with Selvendran (1983) who stated that the cell wall of dicotyledonous plants contains about 3 to 6% protein. The yield

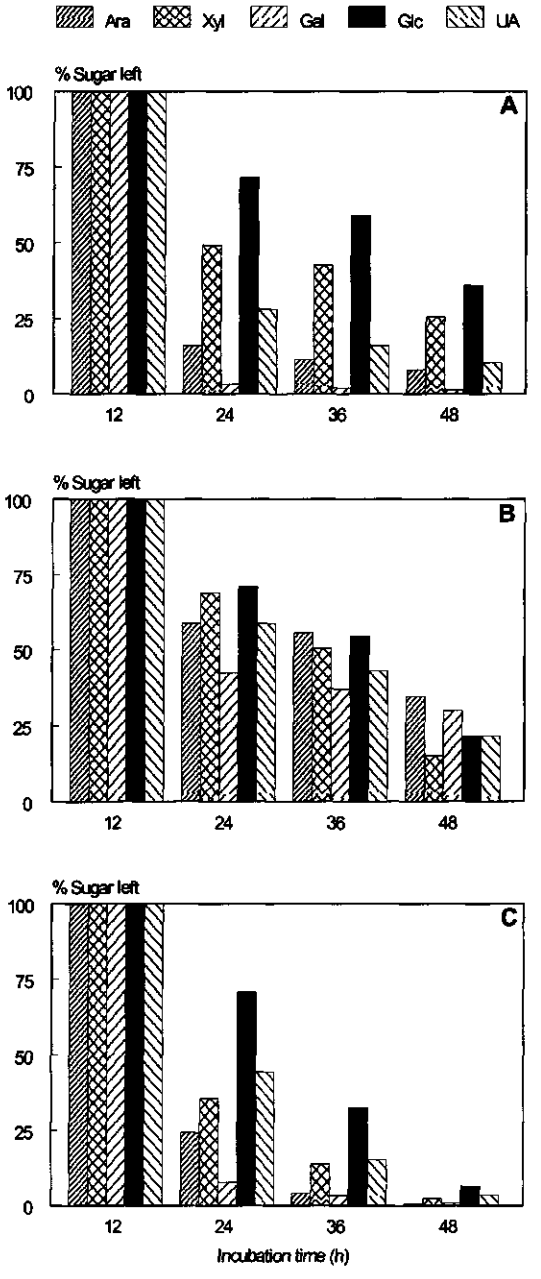

Figure 2: *In vitro* experiment: percentage of individual sugar left in residue, relative to the amount of individual sugar present at 12 h, for the incubation of CCW (A), HCW (B) and ECW (C) (Ara: Arabinose; Xyl: Xylose; Gal: Galactose; Glc: Glucose; UA: Uronic Acids).

Table 4: *In vitro* degradation: undigestible fraction (*Fr*, %), fractional degradation rate (k_d, % h^{-1}) and lag time (T_0, h) for dry matter (DM) and sugars of combined cell walls (CCW), hull cell walls (HCW) and endosperm cell walls (ECW).

		DM	Ara[1]	Xyl	Gal	Glc	UA	TS
CCW	*Fr*	25.1	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.
	k_d	6.1	13.4	4.0	27.9	2.6	7.0	5.8
	T_0	11.2	11.2	11.1	11.5	12.2	6.7	10.7
HCW	*Fr*	21.5	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.
	k_d	3.2	2.4	4.6	4.2	3.9	4.3	3.4
	T_0	11.8	8.9	16.3	10.5	16.1	4.5	11.7
ECW	*Fr*	23.3	n.e.	n.e.	n.e.	n.e.	n.e.	n.e.
	k_d	7.2	12.1	8.6	20.9	7.6	7.0	9.1
	T_0	11.6	11.7	11.5	11.6	19.6	7.1	11.3

[1] Respective sugars: Arabinose, Xylose, Galactose, Glucose, Uronic Acids, and Total Sugars (including rhamnose, fucose, mannose).

n.e. Not estimated.

of CW (15.7%) from SBM was similar to that of Huisman *et al.* (1998), using the same batch of SBM and identical extraction procedures. The yield of CW was more than would have been expected from the amount of NDF present. However, this difference is attributable to the pectins present in soya cell walls, which are present in the CW fraction, but are not included in the NDF fraction.

The degradation rate of DM and nitrogen for SBM were similar, most likely because a large part of the SBM consisted of protein. The nitrogen degradation rate was similar to that of raw untoasted soya beans (10.2% h^{-1}) and only slightly higher than that of SBM (8.3% h^{-1}) reported by Van Straalen and Tamminga (1990). The degradation rate of NDF, ADF and hemicellulose were higher than values of 3.2% h^{-1} and 3.5% h^{-1} reported for SBM NDF by Mosterd (1992) and 3.8% h^{-1} for soya hull NDF by DePeters *et al.* (1997).

The higher degradation rate of sugars for SBM compared to soya CW may have been due to the delay of the start of the fermentation which resulted in a higher T_0 which can lead to a mathematical increase in k_d. The higher k_d in this case would not be caused by an empirical effect, but by design of the mathematical model. However, the decrease in the rate of degradation may also have been caused by changes in the cell wall fraction during preparation of CW. This latter mainly in relation to the water content of the cell wall, since preparation of CW includes freeze-drying which can dehydrate the cell wall, thus decreasing degradability of the cell wall sugars (F.M. Engels, Wageningen University, personal communication).

Differences in degradation rates between the sugars for soya CW must be interpreted using both the results of microscopic evaluation and sugar composition analysis, for both hull and endosperm cell walls. Microscopic evaluation showed that the soya CW is composed of two cell wall types: a thin endosperm cell wall and a thick hull cell wall. This means that the original commercial SBM had not been completely dehulled prior to cell wall extraction procedures. Furthermore, it was discovered that at 24 h of digestion, virtually all thin cell wall material had

disappeared while only thick cell wall material originating from the hull was left. This is in accordance with research of Chesson (1993) and Chesson *et al.* (1986) who found that primary cell wall is completely degraded after 12 h of rumen incubation. Table 1 confirms that the sugar compositions of endosperm and hull cell walls were different. The hull cell wall contained more glucose and xylose, and less galactose, arabinose and uronic acids than the endosperm cell wall. This explains the rapid galactose disappearance from the soya CW, since virtually all galactose is associated with the endosperm cell walls, which are degraded faster than hull cell walls. The disappearance of each sugar seemed to be related to the amount present in endosperm and hull cell walls. For a sugar that was present in different amounts in hull and endosperm cell walls, the degradation rate of this sugar in SBM CW, was influenced by the difference in degradation rate between hull and endosperm cell walls.

In vitro *experiment*

The overall high values for T_0, generally more than 10 h, indicated a slow start of fermentation (Table 4). The most important reason for this is likely to have been the nature of the material itself: pure insoluble soya cell walls, without easily degradable soluble sugars. Since there was no soya bean meal in the sheep diet the rumen microbes would need time and energy to adapt their enzyme systems, and to attach to this new and unfamiliar substrate (Van Soest, 1982).

The sugars were virtually completely degraded after 144 h, therefore, an undegradable fraction was only calculated for the DM component of the substrates. The undegradable fraction of DM was similar for ECW, HCW and CCW, indicating that the total degradability of the substrates was the same. The dry matter degradation of ECW, HCW and CCW (Table 4) was in the order as expected from the microscopic results of the *in situ* experiment. The ECW had the highest degradation rate, HCW the lowest, and the mixture CCW was in between. There were probably different mechanisms behind the difference in HCW and ECW degradation. It has long been known that particle size has an effect on rate of degradation (Dehority and Johnson, 1961). Microscopic evaluation clearly showed a larger particle size of HCW compared to ECW particles, and indicated that the cell walls of HCW were thicker than for ECW. Both factors are likely to have influenced degradation rate. Since digestion takes place at the surface of particles, the ratio of surface area to inner material is an important factor in degradation rate (Chesson, 1993). Beyond particle and cell wall structure, the cell wall composition, and even its fine structure may also influence degradation rates of HCW and ECW. Table 1 shows differences in composition of HCW and ECW, which were mainly associated with the carbohydrate fraction of the cell walls. HCW is mainly composed of cellulose (glucose), with little hemicellulose and some pectins (uronic acids), while ECW had a much lower cellulose content and contained much more pectin. Which of the factors: particle size, cell wall thickness or cell wall composition, had the most influence in terms of cell wall degradation could not be concluded from this experiment. However, Chesson (1993) stated that particle size and cell wall thickness have a far greater influence than cell wall composition.

The sugar degradation pattern of CCW (Figure 2A; Table 4) was similar to that of soya bean CW from the *in situ* experiment (Figure 1), which was due to the different degradation rates of endosperm and hull cell walls, combined with the difference in sugar composition. However, for the pure cell walls of HCW (Figure 2B) the sugar degradation pattern was completely different,

to that of the mixed CCW cell walls. For HCW, all sugars were degraded at a similar rate. This would suggest that there was no specific degradation of any cell wall sugar. Thus for HCW, all cell wall polysaccharides are degraded at similar rates, which is in accordance with Chesson (1993). Although from Table 4 the degradation rate of arabinose seems lower than for the other sugars, the highest difference is only 2.2%. This difference is small enough (see also Figure 2B) to have been caused by the relative inaccuracy of the HPLC method, and the relatively small number of data points available to calculate the degradation rate. It was, therefore, concluded that the degradation rates for the cell wall sugars of HCW were similar. The sugar degradation pattern for ECW, however, was completely different from that of HCW, since for ECW the cell wall sugars were degraded at different rates (Figure 2C, Table 4). It seems therefore, that specific degradation of cell wall polysaccharides is possible for endosperm cell walls.

In situ *and* in vitro *experiments*

The degradation of monomeric cell wall sugars has been studied with various materials consisting of different cell wall types (Ben-Ghedalia and Miron, 1984; Miron and Ben-Ghedalia, 1995; Titgemeyer *et al.*, 1991). In these studies the cell wall sugars, glucose and xylose, are usually reported to be less digestible (at the ruminal or total tract level) while arabinose and galactose are relatively more digestible, which is in agreement with what was found in the present experiments. However, the analysis of monomeric sugar degradation in intact plant tissues is complicated by the presence of different tissue types, differing in their degradation rate and sugar composition (Chesson *et al.*, 1986; Grabber and Jung, 1991a, b; Chesson, 1993). This difference in tissue type was confirmed by microscopic evaluation of the cell walls of soya bean endosperm and hull. Differences in sugar digestibility during incubation were the result of the presence of multiple cell wall types differing in degradation rate and sugar composition.

To investigate monomeric sugar degradation within one specific cell wall type, individual cell wall types have to be collected as has been done for grasses by Grabber and Jung (1991a, b) and Gordon *et al.* (1985). For grasses, such cell wall separations are quite laborious, while for cell wall research on beans, a manual fractionation of the bean into hull and endosperm might suffice. Some research (Grabber and Jung, 1991a, b; Chesson *et al.*, 1986) does indicate preferential degradation of cell wall sugars for primary cell wall material, but not for cell walls containing a secondary cell wall. This was confirmed by the results of the *in vitro* experiment, where a difference in the degradation rates of cell wall sugars was observed for ECW, while for HCW all the cell wall sugars were degraded at essentially the same rate. The cause of this difference is likely to have been a combination of particle size and cell wall thickness. For large particles and thick cell walls, microbes are assumed to only be able to degrade a thin layer of the cell wall, because the enzymes cannot penetrate the cell wall any further. In this thin layer, subjected to degradation, polysaccharides might be degraded at different rates. However, this thin layer needs to be completely degraded before enzymes can reach the material below, as undigested material could otherwise block the degradation of material below. In this model, preferential degradation of primary cell wall polysaccharides is possible if the primary cell wall is thin enough to allow specific degradation of polysaccharides without the undegraded material inhibiting breakdown. This still does not address the effect of cell wall composition on degradation, this could theoretically be resolved by milling cell walls, differing in composition, into

particles of the same size, but smaller than the size of one cell wall. This would assure that the only effect on cell wall degradation would be from the composition of the cell wall and not from its particle size.

Conclusions

The analysis of cell wall monosaccharide degradation in SBM is influenced by the presence of two cell wall types, differing in their rate of degradation and sugar composition. This leads to a difference in the degradation rates of the different cell wall sugars. This difference does not reflect preferential degradation of sugars from the cell wall, but is caused by the different properties of the two cell wall types present. This was supported by the results of ECW and HCW degradation. HCW was degraded at a slower rate than ECW, which may have been due to differences in particle size, cell wall thickness, and/or cell wall composition.

The hull cell wall sugars were all degraded at essentially the same rate, while the endosperm cell wall sugars were degraded at different rates. This difference may be explained by differences in cell wall thickness and particle size between ECW and HCW.

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Chapter 4

An integrated approach to analysing fermentation of the cell walls from hulls and endosperm of soya beans*

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An integrated approach to analysing fermentation of the cell walls from hulls and endosperm of soya beans

Abstract

To study the fermentation kinetics of soya bean cell walls, hulls and endosperm (cotyledons) were manually separated from soya beans and extracted to obtain their respective cell wall fractions (hull cell walls, HCW, and endosperm cell walls, ECW). The ECW and HCW fractions were combined (71.5% and 28.5%, respectively) to reconstitute the cell walls (combined) of the original whole bean (CCW). The three cell wall fractions were fermented using an *in vitro* gas production technique with rumen fluid as an inoculum. In addition to neutral sugar analyses, cell wall composition and structure were analysed by ^{13}C Cross Polarization Magic Angle Spinning NMR, light microscopy, and particle size distribution analysis. The ECW was a pectin-rich thin cell wall present in small particles, whereas the HCW was a cellulose-rich, thick cell wall present in large particles. Although there were large differences in cell wall composition, the major determinant of HCW and ECW dry matter degradation rate (3.2 and 7.2% h^{-1}, respectively) seemed to be particle size. It was not possible to assess the contribution of cell wall composition to the differences in degradation rates. Gas production profiles of ECW, HCW, and CCW were analysed using a multi-phasic curve fitting approach. The gas production profile of HCW was essentially mono-phasic while those of ECW and CCW were di-phasic. For ECW, the division of gas production between phase one (65.2%) and phase two (34.8%) was in good agreement with a division of degradation into, degradation of pectin (60-70% of cell wall) and non-pectin (30-40% of cell wall) cell wall polysaccharides. Furthermore, it appeared feasible to predict the gas production profile of CCW from the gas production profiles of the individual components (ECW and HCW).

Key words: cell walls, degradation, gas production, particle size, multi-phasic analysis

Introduction

Plants are complex organisms containing multiple tissue types, each harbouring a potentially different cell wall. These cell walls may differ in both external (e.g. thickness) and internal (e.g. arrangement and composition of polysaccharides) structures (Chesson, 1993; Wilson, 1993). These factors are of importance when analysing the fermentability of cell wall material by microbes from the gastrointestinal tract of farm animals. This fermentability of plant cell walls is of direct relevance to an efficient use of fibrous materials in animal nutrition. Many studies have focused on the degradation of specific tissues in whole plants, or in cross sections of plants, using light microscopy to study cell wall fermentability (e.g. Akin, 1989; Engels, 1996). These studies often focused on differences in degradation between different tissue types in the plant as a whole. Therefore, it was difficult to link the degradation of the different cell wall types with cell wall composition. Some studies (Miron *et al.*, 1997; Titgemeyer *et al.*, 1990) have focused on the degradation of cell wall material by analysing the chemical composition of whole plant material during degradation. Other studies (Grabber and Jung, 1991a) have investigated the cell wall

composition and degradation characteristics of isolated tissues. However, to better understand the principles of cell wall degradation, research should not be confined to either a microscopic (physical) or chemical approach. Results of physical and chemical analyses should be integrated to yield a holistic view of cell wall degradation.

Light microscopy and composite sugar analyses of cell walls are well-established methods for the analysis of cell wall composition (Engels, 1996). Less common, although reported previously, is cell wall analysis using ^{13}C Cross Polarization Magic Angle Spinning Nuclear Magnetic Resonance (^{13}C CP MAS NMR) (Newman, 1996; Jarvis and Apperley, 1990). This method analyses the cell wall structure on an 'atomic basis', analysing the nature and relative amount of linkages of carbon atoms. As a general rule, but less frequently applied for cell wall materials, particle size plays an important role in the kinetics of any form of degradation of an insoluble material. Thus, for cell wall material, the analysis of particle size may contribute to an explanation of differences in degradation between cell wall tissues (Dehority and Johnson, 1961; Robles *et al.*, 1980).

Common fermentation experiments, where fermentation is stopped at different time points after which the residues are analysed, yield marginal information on fermentation kinetics. Much more detailed information can be obtained using a method which records the production of gases during fermentation. The gas production technique, as described by Theodorou *et al.* (1994) can provide a more indepth analysis of cell wall fermentation.

This chapter describes the integration of physical characteristics (NMR, light microscopy, and particle size analyses) and chemical composition (sugar composition) of cell walls, with results of a cell wall fermentation experiment using the gas production technique. Portions of this experiment, dealing with the degradation of cell wall sugars, were published separately (Van Laar *et al.*, 1999).

Materials and methods

Cell wall preparation

Untreated, full fat Argentinean soya beans were soaked overnight in ice water. After soaking, the beans were manually separated into hulls and endosperm (cotyledons). Cell walls (CW) were isolated as described by Huisman *et al.* (1998). Briefly, the soya bean hulls and endosperm were freeze-dried, ground over a 0.5 mm sieve, and extracted with petroleum ether 40-60 in a Soxlet extractor to remove fat. To obtain CW material, defatted hulls and endosperm were sequentially extracted using demineralized water (3 h, room temperature) and a solution of 10 g l^{-1} sodium dodecyl sulphate and 1.5 g l^{-1} dithiothreitol (3 h, room temperature). After gelatinization (pH 5, 85 °C, 1 h), starch was removed using a maleic acid buffer containing 2 mg l^{-1} porcine α-amylase (Merck art. 16312) (pH 6.5 for 16 - 20 h at 30 °C). After each extraction step, the material was centrifuged at 11,000 × g for 30 min, the supernatant discarded, and the pellet was subjected to the subsequent extraction step. The final residue was freeze-dried and left to air-equilibrate for 3 h. The material obtained in this way represents the cell wall fraction of hulls (HCW) and endosperm (ECW). The HCW and ECW fractions were combined to reconstitute the cell wall fraction of the original bean (CCW).

Experimental procedures

The three substrates (ECW, HCW, and CCW) were assessed for their fermentability according to the *in vitro* cumulative gas production technique described by Theodorou *et al.* (1994). Approximately 0.5 g dry matter (DM) of ECW, HCW, and CCW was weighed into 100 ml serum bottles. To these bottles, 82 ml of semi-defined medium, supporting growth of most micro-organisms from the rumen (Lowe *et al.*, 1985), was added. Bottles were inoculated with 5 ml of rumen fluid from sheep fed medium-quality hay, which had been strained through a double layer of cheesecloth and diluted with medium (1:1). Sufficient replicates were used so that bottles containing substrate could be removed after 0, 12, 24, 36, 48, and 144 h of incubation. For 144 h of incubation, 4 bottles were used for every substrate, and 4 bottles were used as a blank, which contained only medium and inoculum. For all other combinations of fermentation time and substrate, the number of bottles used was determined based on an estimated total residue of approximately 1 g DM (minimum of 2, maximum of 8 bottles depending on substrate and fermentation time). For each bottle, gas production was measured by recording pressure and volume of gas produced at regular intervals during fermentation.

Fermentation was stopped by autoclaving the bottles at 110 °C for 10 min, thereafter the bottles were stored at −18 °C pending residue collection. After thawing, the contents of each bottle was rinsed into a centrifuge tube using demineralized water and centrifuged at 11,000 × g for 30 min. The pellets were resuspended in demineralized water and centrifuged again. The residues were transferred to a 50 ml plastic bottle, freeze-dried, and residual DM was determined (freeze-drying). Subsequently, samples were pooled by substrate and fermentation time and reground with a porcelain pestle and mortar. The supernatants from the centrifugation procedure were pooled by bottle and brought up to a volume of 250 ml with demineralized water. A subsample of 10 ml was taken and 0.5 ml of phosphoric acid (85%) was added, after which the sample was stored at −18 °C pending volatile fatty acid (VFA) analyses.

Chemical analyses

The original soya bean endosperm, soya bean hulls and their cell wall materials (HCW, ECW) were analysed for DM, ash, nitrogen (N), crude fat, neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL), and monosaccharide composition. Fermentation residues were only analysed for sugar composition. Dry matter was determined by drying to a constant weight at 103 °C (ISO method 6496), ash by combustion at 550 °C (ISO method 5984), and N was determined by the Kjeldahl method with $CuSO_4$ as the catalyst (ISO method 5983). Crude fat was determined according to ISO method 6492. Concentration of NDF was determined according to Van Soest *et al.* (1991) modified as described by Goelema *et al.* (1998). Concentrations of ADF and ADL were determined according to Van Soest (1973). Neutral sugar composition was determined by HPLC (Dionex PA-10 column and PA-10 guard column) with Pulsed Amperiometric Detection, using allose as an internal standard, similar to Lebet *et al.* (1997). The samples were pre-treated with 12 M sulfuric acid (1 h, 30 °C) followed by hydrolysis with 1 M sulfuric acid (3 h, 100 °C). After hydrolysis, uronic acids were determined by a colorimetric *m*-hydroxydiphenyl assay using a spectrophotometer at 520 nm (Blumenkrantz and Asboe-Hansen, 1973). VFA in fermentation liquids were analysed using gas chromatography (Packard 419, CE Instruments, Milan, Italy; glass column filled with chromasorb 101, carrier gas

N_2 saturated with methanoic acid, 190 °C with isocaproic acid as the internal standard).

Microscopic, particle size and solid state NMR analyses

Incubation materials (HCW, ECW, and CCW) and residues after fermentation were subjected to microscopic analyses using light microscopy with polarized light. When subjected to polarized light, primary and secondary plant cell walls can be distinguished based on a difference in colour.

To analyse the particle size distribution, HCW and ECW were analysed with a Coulter laser LS 130 particle size analyser (Keetels, 1995), using the Fraunhofer optical mode. However, this method assumes that all particles are spherical, which in the case of cell wall material is not the case. The Coulter counter measures both the number of particles, and the volume and surface area of specific particle size classes.

The ECW and HCW residues after fermentation, were analysed using ^{13}C Cross Polarization Magic Angle Spinning Nuclear Magnetic Resonance (^{13}C CP MAS NMR). ^{13}C CP MAS NMR yields an NMR spectrum, in which each type of carbon atom in the material is represented by its own specific signal. This signal depends on the type and substitution pattern of the monosaccharide the carbon atom belongs to. The NMR spectra were recorded at ambient temperature on a Bruker DMX 300 spectrometer (NSR center, University of Nijmegen) operating at 300 and 75 MHz for ^{1}H and ^{13}C, respectively (Fransen *et al.*, 1999).

Calculations and statistics

Gas production

To smoothen the gas production profiles, the gas volume data for each bottle were regressed against the gas pressure data yielding a volume-pressure relationship for each individual bottle. Subsequently this relationship was used to calculate the gas volume produced for each pressure, which were added per measurement time to give the cumulative gas production. Gas production up to 10 h was very variable among bottles with a maximum production of 20 ml g^{-1} of total sugar (TS). Most probably this gas production was due to activity of the inoculum itself, because the blank bottles had similar gas productions up to 10 h. Fermentation of the substrate did not begin until 10 to 12 h post inoculation as indicated by the VFA production and DM degradation results. The gas production from the inoculum can be corrected for, either by fitting an extra phase (see gas production curve fitting), or by assuming no gas production prior to 10 h. Because the former would result in additional complications during curve fitting (more phases are more difficult to fit) it was decided to use the latter method of correction. Therefore, gas production prior to 10 h was assumed not to be associated with the substrates and omitted.

Curve fitting of gas profiles

For each bottle fermented for 144 h, gas production profiles (Y, ml g^{-1} of TS) were fitted to mono-, di- and tri-phasic models as described in Equation 1 (Groot *et al.*, 1996):

$$Y = \sum_{i=1}^{n} \frac{A_i}{1+(C_i/t)^{B_i}} \qquad \text{(Eq. 1)}$$

Y Total gas production (ml g^{-1} of TS);
A_i Asymptotic gas production for phase i;
B_i Switching characteristic factor for phase i;
C_i Half-time for asymptotic gas production for phase i;
t Time (h).

For each phase, the maximal fractional rate of substrate degradation (R_M) was calculated, using Equation 2 adapted from Groot *et al.* (1996):

$$R_M = \frac{(B-1)^{(B-1)/B}}{C} \quad \text{(Eq. 2)}$$

R_M Maximal fractional rate of substrate degradation;
B Switching characteristic of gas production equation (Equation 1);
C Half-time of gas production (Equation 1).

When comparing the fits for models with different number of phases, the difference in number of parameters between these models has to be taken into account. The statistical comparison between the mono-, di-, and tri-phasic models was done with an F-test (Equation 3) as described by Motulsky and Ransnas (1987), instead of simply comparing the sum of squares of the different models. The P-values for the comparison between curves with different numbers of phases were obtained by consulting a standard table using (df1–df2) and df2 as degrees of freedom.

$$F = \frac{(SS1 - SS2)/(df1 - df2)}{SS2/df2} \quad \text{(Eq. 3)}$$

F F value for the comparison of two specific curves;
SS1 Sum of squares of fit for the model with fewer parameters;
SS2 Sum of squares of fit for the model with more parameters;
df1 Degrees of freedom for the model with fewer parameters;
df2 Degrees of freedom for the model with more parameters.

Parameter fittings and other results within each fermentation time were analysed with Model 1 using the GLM procedure of SAS (1989). Differences between individual substrates were analysed by a multiple comparison test (Tukey), using the LSMEANS statement of SAS (1989).

$$Y_{ij} = \mu + Substrate_i + \varepsilon_{ij} \quad \text{(Model 1)}$$

Y_{ij} Result;
μ Mean;
$Substrate_i$ Effect for substrate i (i = ECW, HCW or CCW);
ε_{ij} Error (j = replicates).

VFA and gas production in relation to DM degradation

The relationships between VFA or gas production with DM degradation were analysed with Model 2 using the GLM procedure of SAS (1989):

Comparing the di-phasic *C*-values from CCW and ECW, and the mono-phasic *C*-value from HCW (Table 2) it appears that the first phase of CCW (composed of ECW and HCW) is almost identical to the first phase of ECW, while the second phase of CCW seems to coincide with the second phase of ECW and the mono-phasic HCW model. To determine whether the fitted gas production curve of CCW could be calculated from those fitted for ECW and HCW, the gas production curves for the different phases of ECW and HCW were multiplied by their relative proportions in CCW (28.5% HCW and 71.5% ECW) and added. Phase one of CCW was calculated from the first phase of ECW, and phase two of CCW was calculated from the second phase of ECW and the mono-phasic HCW. The results of this procedure are illustrated in Figure 2. The calculated phases and total gas production curve were in good agreement with the fitted one, although for phase one there is a slight difference at the beginning, and for phase two a slight difference at the end of the curve.

Fermentation results for each time point

The percentage of DM remaining, the VFA production, and gas production after incubation times of 24, 36, 48, and 144 h are reported in Table 4. After 144 h of fermentation the residues for ECW, HCW and CCW were similar, though the VFA production profiles were markedly different between the substrates. The amounts of gas produced were slightly different for the three substrates, though the yield (ml gas g^{-1} DM digested) was similar. During fermentation (from 24 to 144 h) the percentage of DM remaining in the residue was not similar, illustrating the different fermentation kinetics of the substrates. The VFA production profile during fermentation shifted from acetic acid to propionic acid production, which can be seen by the lower A/P ratio (mmol acetic acid / mmol propionic acid produced).

Microscopic results and particle size distribution

The ECW and HCW had different cell wall structures, the HCW was composed of large particles with thick cell walls, while ECW was composed of smaller particles with thinner cell walls, as has been reported in Van Laar *et al.* (1999, Chapter 3). This is illustrated in Figure 3. The thin

Table 3: Sum of squares and *F*-values for the fit of the mono-, di-, and tri-phasic models (SS1, SS2, and SS3, respectively).

	SS1 (df[1] = 41)	SS2 (df = 38)	SS3 (df = 35)	*F*-value[2] 1 *vs* 2	*F*-value 2 *vs* 3
ECW	4986[b]	256[a]	75[a]	234[b]	28[a]
HCW	396[c]	99[b]	69[a]	38[c]	5[b]
CCW	10425[a]	168[ab]	87[a]	775[a]	11[b]
SEM[3]	515	24	5	45	3.4

[1] Degrees of freedom (df) for specific model, equals number of observations minus number of parameters in model.

[2] An *F*-value larger than 2.4 for this combination of df is highly significant ($P < 0.01$).

[3] Standard Error of the Mean.

[abc] Means with different superscripts within one column differ significantly ($P < 0.05$).

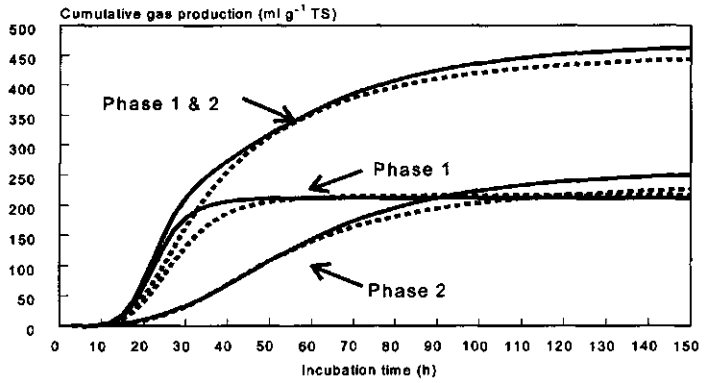

Figure 2: Measured total gas production curve and the separation into two phases for CCW (solid line) and the total calculated gas production curve; and separation into two phases (dashed line, calculated from the individual gas production profiles of the endosperm and hull cell walls).

Table 4: Percentage of DM remaining (residue), acetic acid equivalents produced (AAE, mmol g^{-1} total sugars (TS)), percentage of acetic (HAc), propionic (HPr) and butyric acid (HBu), acetic acid to propionic acid ratio (A/P), acetic acid equivalents yield (AAEY, AAE mmol g^{-1} DM digested), gas production (ml g^{-1} TS), and gas yield (ml g^{-1} DM digested), for the three substrates at the different incubation times.

		DM residue	AAE	% HAc	% HPr	% HBu	A/P ratio	AAEY	Gas prod.	Gas yield
24 h	ECW[1]	52.6^{b}	7.6^{a}	58.9^{b}	25.2^{a}	15.9^{a}	2.4^{ab}	12.5^{a}	142^{a}	228^{a}
	HCW	75.9^{a}	4.2^{b}	69.6^{a}	22.9^{a}	7.5^{b}	3.1^{a}	14.7^{a}	7^{b}	248^{a}
	CCW	53.2^{b}	7.6^{a}	59.9^{b}	28.8^{a}	11.3^{ab}	2.1^{b}	13.0^{a}	167^{a}	285^{a}
	SEM[2]	3.7	0.7	0.7	1.6	1.9	0.2	0.6	20	23
36 h	ECW	39.6^{c}	11.3^{a}	57.2^{b}	31.9^{a}	10.9^{a}	1.8^{b}	15.0^{a}	291^{a}	386^{a}
	HCW	65.1^{a}	7.1^{b}	70.1^{a}	23.1^{b}	6.7^{b}	3.1^{a}	16.3^{a}	124^{c}	287^{b}
	CCW	47.0^{b}	10.0^{a}	58.6^{b}	31.2^{a}	10.2^{a}	1.9^{b}	15.0^{a}	238^{b}	361^{a}
	SEM	1.5	0.6	0.6	0.6	0.2	0.1	0.9	10	12
48 h	ECW	30.9^{c}	12.2^{a}	55.9^{c}	32.8^{a}	11.4^{a}	1.7^{c}	14.2^{a}	345^{a}	400^{a}
	HCW	42.9^{a}	9.11^{c}	67.4^{a}	26.2^{b}	6.3^{c}	2.6^{a}	12.9^{b}	258^{c}	369^{b}
	CCW	39.8^{b}	10.8^{b}	58.3^{b}	31.5^{a}	10.2^{b}	1.9^{b}	14.4^{a}	306^{b}	409^{a}
	SEM	0.6	0.2	0.6	0.4	0.3	0.0	0.3	4	5
144 h	ECW	21.9^{a}	14.3^{a}	54.3^{c}	33.8^{a}	11.9^{a}	1.6^{a}	14.6^{a}	453^{ab}	465^{a}
	HCW	24.4^{a}	12.5^{c}	65.6^{a}	26.2^{c}	8.2^{c}	2.5^{b}	13.6^{b}	437^{b}	474^{a}
	CCW	21.3^{a}	13.8^{b}	59.5^{b}	29.9^{b}	10.6^{b}	2.0^{c}	14.1^{ab}	464^{a}	475^{a}
	SEM	1.0	0.1	0.6	0.7	0.3	0.1	0.2	5	8

[1] Cell walls endosperm, hull and combination, ECW, HCW and CCW, respectively.

[2] SEM, Standard Error of the Mean.

[abc] Means with different superscripts within fermentation time and column differ significantly ($P < 0.05$).

'red' cell walls of ECW and the large solid 'blue' particles of HCW are clearly visible. In HCW, some thin 'red' cell wall material seems to be present. Further microscopic analyses proved that these thin 'red' cell wall fragments in the HCW are a part of the soya bean hull, and not a contamination with ECW. For HCW, it is hard to distinguish between different cell walls within a 'blue' particle. Therefore, the absolute cell wall thickness cannot be determined. For the 'red' cell walls in the HCW material a more clearly visible cell structure is present. When studying the degradation of CCW at different time points, microscopic analyses revealed that after 24 to 36 h of fermentation the main cell wall type present originated from HCW. This means that virtually all ECW had rapidly disappeared from the CCW material.

The difference in particle size between ECW and HCW is illustrated in Figure 4. The particle size distribution for HCW was clearly shifted towards larger particles, compared to ECW. For cell wall degradation, the ratio of volume to surface area is important since it can be a major determinant of degradation rate. This ratio is represented by the d3/2 value, which is the total amount of measured volume in a sample, divided by the total amount of measured surface area. For HCW and ECW the d3/2 values were 27.7 and 10.0, respectively.

NMR results

The results of the solid-state NMR analysis of ECW for the different fermentation times are shown in Figure 5A(1-5). During fermentation, the peaks between 60 and 110 ppm gradually decreased in intensity, indicating the disappearance of sugar components from the material (Fransen *et al.*, 1999). The slightly more rapid decrease of the peaks at 74 and 106 ppm relative to the peak at 70 ppm was probably caused by the difference in degradation rates of the different monosaccharides present in ECW. This is in agreement with the more rapid degradation of arabinose and galactose, as was found previously (Van Laar *et al.*, 1999). The peaks at 170 to 180 ppm and 10 to 35 ppm, representing carboxyl groups (–CO–O–) and –CH2– groups, respectively, increased during fermentation. This can be explained by a relative increase in the amount of protein from microbial origin, which has been found in other experiments (Van Laar *et al.*, 2000, Chapter 2). For HCW (Figure 5B) only the NMR pattern of the original material is shown, which was similar to NMR patterns found for cellulose (Fransen *et al.*, 1999). During fermentation the peaks between 60 and 110 ppm decreased in intensity because of the disappearance of cellulosic material due to fermentation, but did not change relative to each other. Furthermore signals from microbes increased, as for the fermentation of ECW.

VFA production related to DM degradation

The relationship between dry matter fermentation and VFA production (acetic acid equivalents (AAE): 1 mole of HAc, HPr, or HBu equals 1, 1.5, and 2 moles of AAE, respectively) was analysed by linear regression. Because fermentation did not begin until 12 h after inoculation, the data points up to and including 12 h of inoculation were omitted. The regression was calculated in two ways, firstly by forcing the intercept through the origin, and secondly by estimating the intercept for each substrate. The regression parameters for each substrate, for both regressions, are given in Table 5. The slopes of the regression lines, without intercept, were not significantly different. For the regression lines with an intercept, HCW and ECW were significantly different ($P < 0.05$) for both intercept and slope. Figure 6 shows the regression lines for the models with a

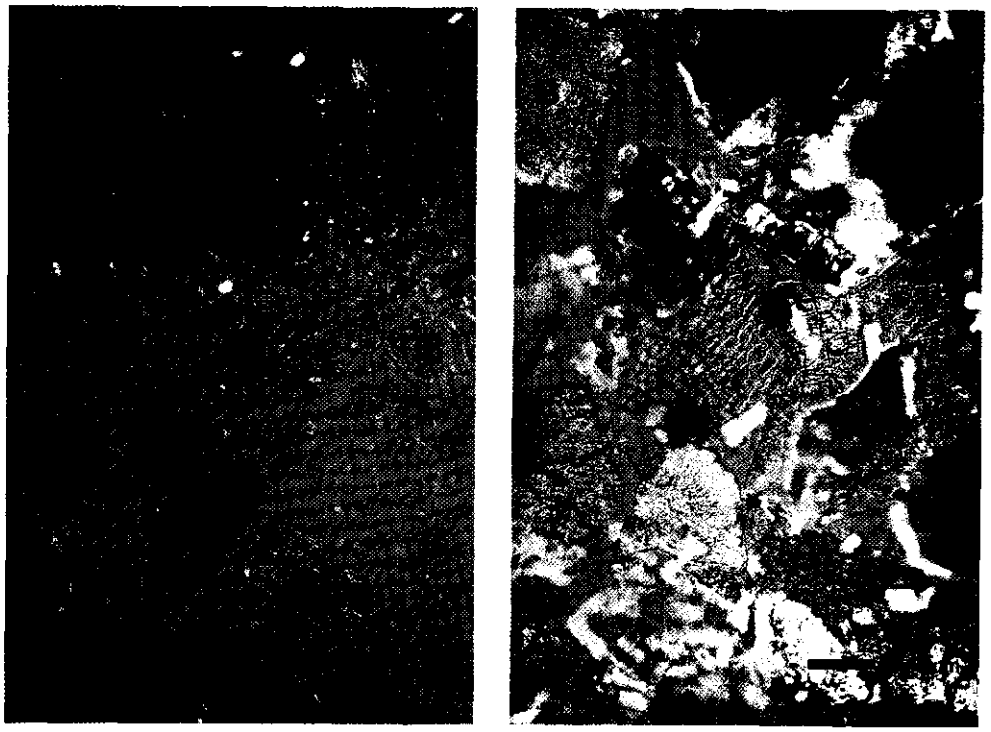

Figure 3: Micrographs of ECW (A) and HCW (B).

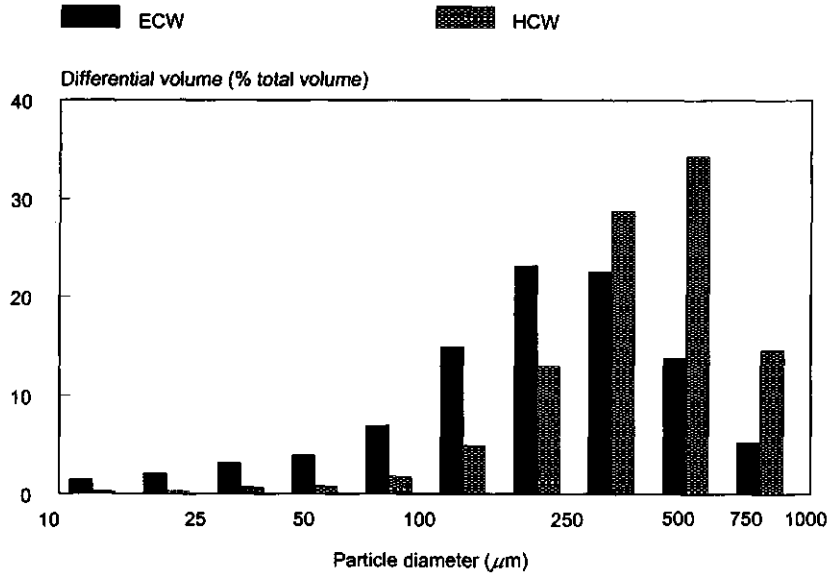

Figure 4: Particle size distribution of soya bean endosperm (ECW) and hull (HCW) cell wall isolates.

fitted intercept.

Discussion

Cell wall structure and composition

The degradation results (gas production) for ECW and HCW have to be interpreted in light of the structure and composition of both cell wall types. Therefore, tentative structures for ECW and HCW are suggested that integrate the information gathered by particle size, NMR, microscopic, and sugar composition analyses.

Endosperm cell walls (ECW)

The ECW are basically unlignified (Table 1) and mainly composed of polysaccharides plus small amounts of protein (4%) and fat (1%). Judging from the ADF and glucose content, up to 20% of the cell wall is likely to be composed of cellulose, although some of the glucose might be present in non-cellulosic polysaccharides similar to those found in other beans (Pritchard *et al.*, 1973; Umadevi *et al.*, 1981). Hemicellulose (NDF–ADF) appears to represent 10 to 15 % of the cell wall. The main component (60 to 70 %) of the ECW appears to be pectin, judging from the NDF content (Table 1). This large pectin fraction consists of a rhamnogalacturonan backbone substituted with side-chains of arabinans, galactans, and arabinogalactans as has previously been described for soya beans and cotyledons of other dicots (Brillouet and Riochet, 1983; Van de Vis, 1994). Therefore, most of the arabinose and galactose of the ECW would be structurally associated with (galact)uronic acid through the pectin molecule. NMR analyses of ECW confirms that the majority of the material is composed of carbohydrates, with peaks between 60 and 110 ppm (Fransen *et al.*, 1999).

The 'external' cell wall structure of ECW (Figure 3) seems to be mainly thin-walled primary cell wall from cotyledon tissue. The term 'thin-walled' is used in relation to the thicker cell walls (or particles) of the HCW, because absolute cell wall thickness was not measured. The micro graphs (Figure 3) combined with the measurement of particle size distribution (Figure 4) and the d3/2 value of 10, indicate the somewhat 'delicate' nature of the ECW material, being composed of relatively small particles which are composed of thin cell walls.

Table 5: Parameters for the linear regression of VFA production (mmol acetic acid equivalents) against degraded DM (g) for soya bean endosperm (ECW), hull (HCW) and combined cell walls (CCW), estimated without and with intercept.

Substrate	Coefficient (no intercept)	Intercept	Coefficient (with intercept)
ECW	14.6^{a}	-0.75^{b}	16.7^{a}
HCW	13.8^{a}	0.44^{a}	12.2^{b}
CCW	14.2^{a}	-0.21^{ab}	14.9^{ab}

[ab] Means with different superscripts within one column differ significantly ($P < 0.05$).

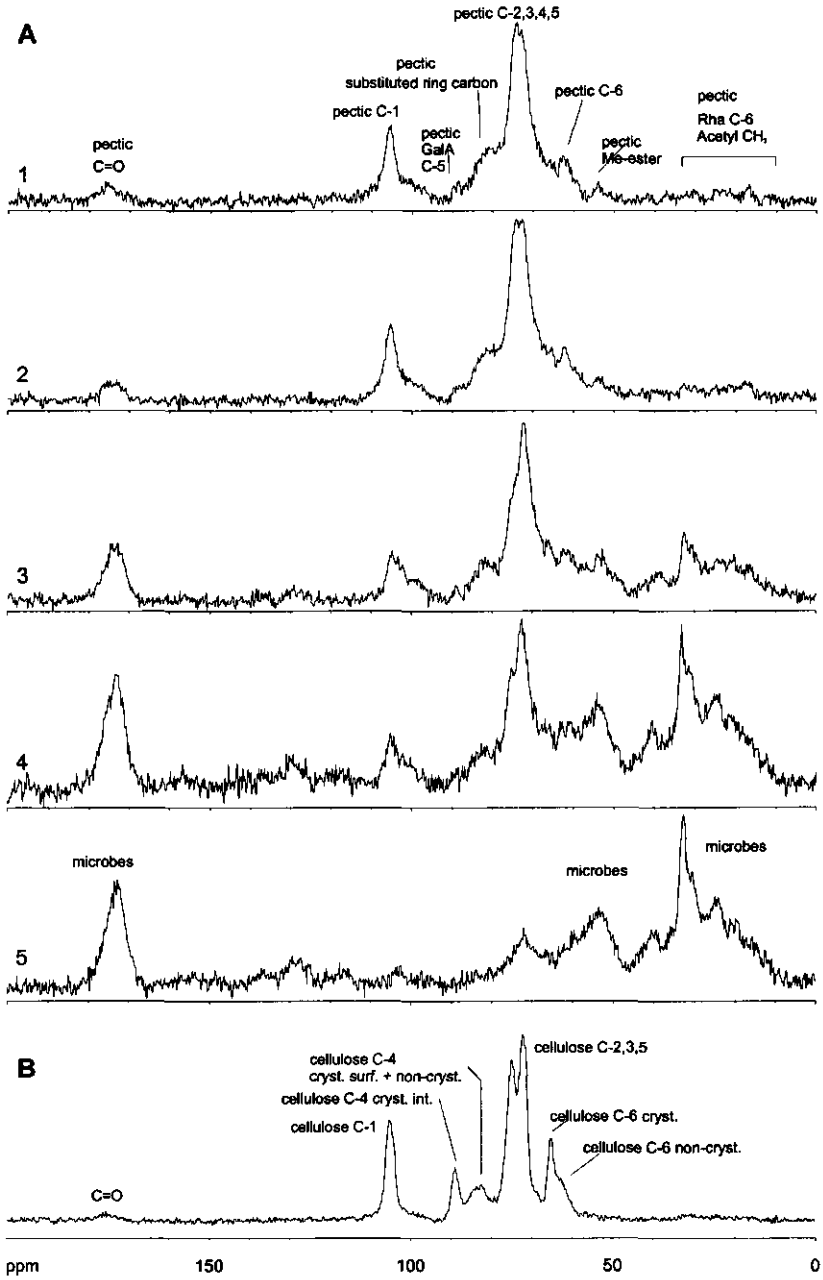

Figure 5: ^{13}C CP MAS NMR spectra (A1-5) for ECW at 0, 12, 24, 36 and 48 h of incubation and (B) for HCW at 0 h of incubation (see also Fransen *et al.*, 1999).

Hull cell walls (HCW)

Judging from the ADL content (Table 1), HCW is basically unlignified, and composed primarily of polysaccharides, although low levels of lignin can be found when determining Klason lignin (Jung, personal communication). The ADF and glucose content of the HCW material indicate that the cellulose content was 50 to 65% of the cell wall. The cellulosic nature of HCW is supported by NMR analyses. The NMR pattern is very similar to results from literature using pure celluloses (Fransen *et al.*, 1999). Comparable levels of glucose (cellulose) and lignin have been found in soya bean hull (Whistler and Saarnio, 1957), pea hulls (Weightman *et al.*, 1995), and in lupin hulls of different varieties (Brillouet and Riochet, 1983). Because the difference between NDF and ADF is only about 10 percentage units, HCW (like ECW) contains only a small amount of hemicellulose. These hemicelluloses could, in part, be composed of xylans, which have been identified in soya hulls (Aspinall *et al.*, 1966). Because the amount of NDF and total cell wall sugars were almost equal, the pectin content of HCW was low, although some uronic acids were present, probably in little substituted pectin.

Whereas the ECW material appeared to be composed mainly of one cell wall or tissue type, the HCW was composed of three tissue types (lancunate parenchyma cells, pillar cells and palisade cells) as described by Weightman *et al.* (1995) citing from Gassner (1973). Comparing the 'blue' and 'red' tissues types found in HCW with data from Weightman *et al.* (1995), the 'red' cell walls were from lancunate parenchyma and pillar cells, whereas the 'blue' cell walls were palisade cells. The predominant cell wall type seemed to be the 'blue' thick cell wall (palisade cells). When combining these results with those of particle size distribution analysis, the HCW generally consists of relatively large particles, which are composed of cells with a thick wall.

Degradation of CW material

ECW versus *HCW degradation*

The final total volume of gas produced from ECW and HCW was similar (Table 4 and Figure 1). This is not surprising since both cell wall types were basically unlignified and, therefore, had a high and similar total degradability. However, the kinetic profiles by which the final gas production was reached were very different. For ECW, maximal gas production was reached rapidly, while for HCW gas production was much slower. This difference in fermentation rate was also reflected in the differences in fractional degradation rates of DM for HCW and ECW. It is well known that particle size has a marked effect on rate of degradation (Dehority and Johnson, 1961; Robles *et al.*, 1980). Therefore, the difference in degradation rates between HCW and ECW must be considered in light of particle size effects. For this, one can compare the volume to surface ratio (d3/2 value) of particles, with the fractional degradation rates (k_d) of DM (HCW: d3/2=27.7; k_d=3.2% h^{-1} and ECW: d3/2=10.0; k_d= 7.2% h^{-1}). The ratio of d3/2 values for HCW *vs* ECW was 2.7 while for the k_d the reciprocal of the HCW:ECW ratio was 2.25. This implies that HCW particles were 2.7 times larger, and HCW dry matter was degraded 2.25 times slower than for ECW. Therefore, it appears that particle size can explain the difference in degradation rates.

Gas production phases and cell wall fermentation

Fitting multiple phases to gas production curves is a tool to obtain more insight in the underlying fermentation characteristics of complex substrates. By comparing the statistically fitted phases with the composition of substrates during fermentation, it is possible to attribute a gas production phase to the fermentation of a specific portion of the substrate (Beuvink and Kogut, 1993). The mono-phasic gas production fitted to HCW data supported the conclusion that HCW was being homogeneously degraded, as was concluded from the similar fractional degradation rates for the individual sugars of HCW (Van Laar *et al.*, 1999; Chapter 3). Chesson (1993) suggested that secondary wall degradation proceeds in a homogeneous manner, where all polysaccharides are degraded simultaneously. For ECW, the fractional degradation rates of the composite sugars of cell wall polysaccharides differed and those sugars associated with the pectin molecule degraded most rapidly. Differential degradation rates of cell wall components from isolated primary cell walls have also been demonstrated by Chesson *et al.* (1986) for hemicellulose *versus* cellulose. From the di-phasic gas production curve for ECW, it would appear that the first phase was primarily associated with the degradation of pectin (galactose, arabinose and uronic acids). In the second phase of ECW gas production hemicellulose and cellulose were digested (xylose and glucose). This was supported by the close agreement between the amount of gas produced in each phase (phase one: 65.2%, phase two: 34.8%) and the composition of the ECW (60 to 70% pectin and 30 to 35% cellulose plus hemicellulose). Fitting of different phases to gas production curves is a relatively new approach, although there are several reports in literature (Beuvink and Kogut, 1993; Groot *et al.*, 1996; Schofield *et al.*, 1994). From the current experiment it appears that with relatively pure cell walls of well-defined types, interpretation of gas phases within a gas production curve is feasible.

To further explore the possibilities of fitting different phases, we attempted to estimate the gas production curve for CCW from the curves for ECW and HCW (Figure 2). The curves estimated from the separate curves of ECW and HCW seemed to agree well with the fitted curve (phase one, phase two and phase one and two) of CCW. This is a further indication that fitting multiple phases to gas production curves, combined with adding curves or phases from pure substrates, can lead to a greater understanding of the fermentation of complicated substrates (e.g. whole plant material). Based on the same principle of additivity, subtraction of gas production curves has also been tried by other authors (Hall *et al.*, 1998; Schofield and Pell, 1995) by analysing the fermentation kinetics of different fibre fractions in feedstuffs.

VFA production

The production profiles of VFA (HAc *vs* HPr *vs* HBu) were different for HCW and ECW. The relatively high A/P ratio for all time points (Table 4) for HCW is typical for slower digesting, more fibrous materials (Hungate, 1966). Therefore, it was expected that the A/P ratio of ECW would be much lower. However after 24 h of fermentation this was not the case. During fermentation the A/P ratio of ECW drops much more than for HCW. This might have been associated with the fermentation of pectins from ECW, because pectin fermentation can produce a high A/P ratio (Marounek *et al.*, 1985; Sunvold *et al.*, 1995; Howard, 1961). After disappearance of the pectin the fermentation of hemicellulose and cellulose determined the VFA production pattern. This could explain the sharp drop in A/P ratio associated with ECW after

extended fermentation. It appears that the VFA production patterns can be explained by degradation patterns of cell wall polysaccharides.

In addition to the production profile of VFA, the efficiency of VFA production may be an indicator for an altered microbial metabolism. From the acetic acid equivalents yield (mmol acetic acid g^{-1} DM digested) (Table 4), it can be concluded that the production of VFA from DM was significantly ($P < 0.05$) less efficient for HCW than for ECW after both 48 and 144 h of fermentation. This reduced conversion efficiency of dry matter to VFA was supported by data presented in Table 5 and Figure 6. These show a significantly steeper slope for the linear relationship between acetic acid equivalents produced and DM digested for ECW compared to HCW. From the differences in VFA production profile and efficiency of acetic acid equivalent production, it appears likely that the microbial metabolism (or population) might have been different for the fermentation of HCW and ECW. The impact of this effect on the differences in degradation rates and gas production is unclear.

Conclusions

The differences in composition and degradation of HCW and ECW show that the tissues within the same plant vary tremendously with regard to their cell walls. The ECW material was composed of thin pectin-rich cell walls present in relatively small particles. The HCW material was composed of thick cellulose-rich cell walls present in relatively large particles. The difference in particle size between HCW and ECW explained the majority of the differences observed in cell wall degradability. Therefore, the impact of cell wall composition on cell wall degradability is still unknown. With regard to fermentation pattern, the components of HCW were fermented more homogeneously, with a mono-phasic gas production, whereas for ECW the degradation occurred in two phases. For ECW, it appeared that first the sugars associated with pectin were degraded, after which the degradation of hemicellulose and cellulose predominated. When studying different cell wall tissues, techniques such as particle size distribution analyses, ^{13}C CP MAS NMR, and even basic light microscopy provide valuable tools for an integrated approach to cell wall degradation research. Furthermore, fitting different phases to gas production curves is also valuable for a better understanding of the degradation of complex substrates. It seems possible to add gas production phases for single substrates to explain the gas production of a combined substrate.

Acknowledgements

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Chapter 5

Fermentation characteristics of polysaccharide fractions extracted from the cell walls of soya bean cotyledons*

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The primary cell wall of the soya bean cotyledon is composed of pectin (55 - 70%) (Huisman *et al.*, 1998), little hemicellulose and some cellulose. A model for the architecture of the primary legume (soya is a legume) cell wall has been given by Hatfield (1993). In this model, a cellulose/xyloglucan network is embedded in pectin. Pectin in the legume cell wall could determine the pore-size of the cell wall (Carpita and Gibeaut, 1993). Generally, pectins have been found to be rapidly and completely fermentable (Van Soest *et al.*, 1991). However, the effect of pectins in the cell wall, or the removal of pectins from the cell wall on cell wall fermentation, is not clear. Removal of pectin from the cell wall might alter the cell wall architecture, and in turn cell wall fermentability. Investigation of the fermentation characteristics of pectins, and of cell walls from which pectins have been removed, could help elucidate the relationship between pectin in the cell wall matrix and cell wall fermentability.

Extraction of pectin from cell walls has been found to be most efficient when using cyclohexane-*trans*-1,2-diamine-*N,N,N',N'*-tetraacetate (CDTA) (Eriksson *et al.*, 1997), although other extraction procedures have also been used (Hatfield and Weimer, 1995). Subsequent extraction with alkali solutions of increasing molarity can further extract pectin and hemicellulose polysaccharide fractions from the cell wall (Huisman *et al.*, 1998). Whereas extraction procedures can elucidate the chemical structure of cell walls, they provide no information on the fermentation of polysaccharides within the cell wall matrix. Therefore, this experiment was designed to investigate the differences in fermentation of the polysaccharides in the cell walls from the cotyledons of soya beans, in order to study the relationship between cell wall composition and cell wall fermentation. To be able to do this, chemical extraction of soya bean cotyledon cell walls was combined with an *in vitro* gas production technique, to analyse fermentation characteristics of the extracted polysaccharides.

Materials and methods

Cell wall preparation

Untreated, full-fat Argentinean soya beans were soaked overnight in iced water. After soaking, the beans were manually separated into hulls and endosperm (cotyledons). Cell walls (CW) were isolated from the cotyledons, using a method adapted from Huisman *et al.* (1998). Briefly, the cotyledons were freeze-dried, ground over a 0.5 mm sieve, and extracted with petroleum ether 40-60 in a Soxlet extractor to remove fat. Defatted material was then sequentially extracted, using demineralized water (3 h, room temperature) to remove soluble material, a solution of 10 g l^{-1} sodium dodecyl sulphate and 1.5 g l^{-1} dithiothreitol (3 h, room temperature) to remove protein, and using a maleic acid buffer containing 2 mg l^{-1} bacterial α-amylase (Boehringer Mannheim 161 764; pH 6.5 for 16 - 20 h at 30 °C) to remove starch. After each extraction step, the material was centrifuged at 11,000 × g for 30 min, the supernatant discarded, and the pellet subjected to the subsequent extraction step. The material obtained in this way is considered to be representative of the CW fraction of the soya bean cotyledon.

Cell wall fractionation

To fractionate the cotyledon CW into its polysaccharide fractions, CW material was extracted

using a procedure adapted from Huisman *et al.* (1998), based on Redgewell and Selvendran (1986). In short: CW material was treated with 0.05 M 1,2-cyclohexylene-dinitrotetraacetic acid (CDTA) and 0.05 M NH_4-oxalate in 0.05 M NaAc-buffer (pH 5.2, 70 °C, 1 h) to extract pectins (Extract 1). The residue was subsequently extracted with demineralized water (room temperature, 1 h), and this water extract was combined with Extract 1. Subsequently the material was extracted with 0.05 M NaOH (2 °C, 1 h; Extract 2: mainly pectins), with 1 M KOH + 20 mM $NaBH_4$ (room temperature, 2 h; Extract 3: pectins and hemicellulose), and with 4 M KOH + 20 mM $NaBH_4$ (room temperature, 2 h; Extract 4: mainly hemicellulose). After each extraction, solubilized polymers were separated from the insoluble residue by centrifugation (19,000 × g; 30 min), and a sub-sample of the pellet was taken (Residues 1 to 4), while the rest of the pellet continued in the subsequent extraction step. All residues and extracts were acidified to pH 5.2 using glacial acetic acid, extensively dialysed against demineralized water, and freeze-dried. Extract 1 was first dialysed against 0.1 M NH_4Ac buffer (pH 5.2), before the dialysis step against demineralized water. These extraction procedures resulted into nine substrates: CW, Residues 1 to 4, and Extracts 1 to 4.

The extractions were designed to first extract pectin and subsequently hemicellulose from the CW material. Therefore, the composition of the extracts was thought to shift from mainly pectin, to a mixture of pectin and hemicellulose, for the sequential Extracts 1 to 4 (Huisman *et al.*, 1998). For the sequential residues (CW material to Residue 4), the pectin and hemicellulose content were expected to decrease whereas cellulose content would increase.

Assessment of fermentability

The nine substrates were assessed for their fermentability according to the *in vitro* cumulative gas production technique described by Theodorou *et al.* (1994). Approximately 0.5 g dry matter (DM) of each substrate was weighed into 100 ml serum bottles. To these bottles, 82 ml of semi-defined medium, supporting growth of most micro-organisms from the rumen (Lowe *et al.*, 1985), was added. Bottles were inoculated with 5 ml of an inoculum prepared from the mixed faeces of four pigs fed a maize diet, containing no added antibiotics or copper, which had been diluted 1:5 with saline (9 g l^{-1} NaCl), mixed in a blender for 1 min, and strained through a double layer of cheesecloth. Fermentation was stopped at 0, 16, 24, 36, 48 and 167 h after inoculation. For all bottles containing substrate, the number of bottles removed at each time point (minimum: 2, maximum: 7) was sufficient to yield an estimated total residue of 1 g DM. Two bottles containing only medium and inoculum were also removed at each time point. For 167 h of fermentation four bottles were used for every substrate. For each bottle, gas production was measured by recording pressure and volume of headspace gas at regular intervals, during fermentation.

Fermentation was stopped by autoclaving the bottles at 110 °C for 10 min, after which the bottles were stored at −18 °C pending residue collection. After thawing, the contents of each bottle were rinsed into a centrifuge tube using demineralized water and centrifuged at 11,000 × g for 30 min. The pellets were resuspended in demineralized water and centrifuged again. The residues were transferred to a 50 ml plastic bottle, freeze-dried, and residual DM determined (freeze-drying). Subsequently, samples were pooled by substrate and fermentation time and reground in a porcelain mortar. The supernatants from the centrifugation procedure were pooled

by bottle and brought up to a volume of 250 ml with demineralized water. A sub-sample of 10 ml was taken and 0.5 ml of phosphoric acid (85%) was added, after which the sample was stored at −18 °C pending volatile fatty acid (VFA) analysis.

Chemical analyses

Substrates were analysed for DM, ash, nitrogen (N), and sugar composition. Fermentation residues were only analysed for sugar composition. Dry matter was determined by drying to a constant weight at 103 °C following ISO method 6496, ash by combustion at 550 °C (ISO method 5984), and N by the Kjeldahl method using $CuSO_4$ as the catalyst, following ISO method 5983. Neutral sugar composition was determined by high pressure liquid chromatography (HPLC; Dionex PA-10 column and PA-10 guard column) with Pulsed Amperiometric Detection, using allose as an internal standard, similar to the method described by Lebet *et al.* (1997). The samples were pre-treated with 12 M sulphuric acid (1 h, 30 °C) followed by hydrolysis with 1 M sulphuric acid for 3 h at 100 °C. After hydrolysis, uronic acids were determined by a colorimetric *m*-hydroxydiphenyl assay using a spectrophotometer at 520 nm (Blumenkrantz and Asboe-Hansen, 1973). VFA in fermentation liquids were analysed using gas chromatography (Packard 419; CE Instruments, Milan, Italy; glass column filled with chromasorb 101, carrier gas N_2 saturated with methanoic acid, 190 °C with isocaproic acid as the internal standard). Soluble carbohydrates in the fermentation liquid were determined as neutral sugars by a phenol-sulphuric acid assay, which was adapted from Dubois *et al.* (1956).

Calculations and statistics

Gas production

To smooth the gas production profiles, gas volume was regressed against the gas pressure yielding a volume-pressure relationship for each individual bottle. This relationship was used to recalculate the gas volume produced for each pressure, which were added per measurement time to give the cumulative gas production profiles. Gas production profiles were corrected for gas production in the blank bottles.

For each bottle fermented for 167 h, gas production profiles (ml g^{-1} of total sugars in material, TS) were fitted to mono-, di- and tri-phasic models as described in Equation 1 (adapted from Groot *et al.*, 1996). For each phase, the maximal fractional rate of substrate degradation (R_M) was calculated, using the C and B value according to Groot *et al.* (1996). The statistical comparison between the mono-, di-, and tri-phasic models was done with an F-test as described by Motulsky and Ransnas (1987).

$$Y = \sum_{i=1}^{n} \frac{A_i}{1+(C_i/t)^{B_i}} \qquad \text{(Eq. 1)}$$

Y Total gas production (ml g^{-1} TS in material);
A_i Asymptotic gas production for phase i;
B_i Switching characteristic factor for phase i;
C_i Half-time for asymptotic gas production for phase i;
t Time (h).

Parameter fittings and other results for the 167 h fermentation time were analysed with Model 1 using the GLM procedure of SAS (1989). Differences between individual substrates were analysed by a multiple comparison test (Tukey), using the MEANS statement of SAS (1989).

$$Y = \mu + Substrate_i + \varepsilon_{ij} \qquad \text{(Model 1)}$$

Y	Result;
μ	Mean;
$Substrate_i$	Effect for substrate *i*;
ε_{ij}	Error.

Results

Composition of cell walls and fractions

Van Laar *et al.* (1999; Chapter 3) reported a yield of 13.6% cell walls, for the same batch of soya beans and similar extraction procedures as used in this experiment. Although the yield has not been determined in this experiment it is expected to be similar to this value. Table 1 shows the composition of the soya bean CW and the different polysaccharide fractions extracted from the CW. Contrary to expectations, the glucose content of Extract 1 was very high, and decreased for the subsequent extracts. As was expected, the glucose content increased for Residues 1 to 4.

Cell wall fractionation and solubility

The fractionation of the cell walls (CW) into the different residues and extracts, as well as the solubility of the substrates is shown in Table 2. Because the total recovery of cell wall polysaccharides in the fractionation could not be determined, the percentage of CW in each fraction was calculated based on an assumed total recovery of 100%. Solubility of the extracts in water was calculated from the residuals of the 0 h incubations, after correction for material originating from the inoculum. Solubility of Extract 2 could not be determined because there was merely material to analyse the 167 h fermentation. Sugar solubility of the extracts increased from 13.1% to 90.7% for Extracts 1 through 4. For CW and residues the solubility was lower than 10% with exception of Residue 3.

Figure 1 shows the amount of soluble carbohydrates (mg g^{-1} TS in starting material), for the fermentation up to 48 hours for Extracts 1, 3, and 4 and Residue 3. Because the solubility (Table 2) of the other substrates was fairly low, the amount of soluble carbohydrates was not determined. The amount of soluble carbohydrates for Extract 1 and Residue 3 was very low. For Extract 4, the amount of soluble carbohydrates was high at 0 h of incubation, after which it decreased to 20 to 30 mg g^{-1} TS at 16 h of incubation. For Extract 3, the amount of carbohydrates present at 0 h of incubation was lower than was expected from its solubility (Table 2). However, at 4 h of incubation the soluble carbohydrates for Extract 3 were higher than at 0 h, which was in better agreement with its solubility.

Gas production results

Gas production profiles (ml g^{-1} TS) for CW, extracts and residues are shown in Figure 2. Gas

Table 1: Dry matter content (DM, g kg^{-1}), organic matter content (OM), nitrogen content (N), total sugar content (TS, g kg^{-1} DM) and sugar composition (g kg^{-1} total sugar) of the soya cell wall (CW), extracts and residues.

	DM	OM	N	TS	Arabinose	Galactose	Glucose	Xylose	UA[1]
CW	865.2	969.6	16.9	784	159	364	183	65	174
Residue 1	870.7	984.3	19.8	770	167	371	190	65	152
Residue 2	857.7	969.6	14.9	792	164	373	202	63	145
Residue 3	860.9	966.5	2.0	914	125	303	340	62	115
Residue 4	882.7	980.3	2.8	933	42	80	741	18	65
Extract 1	868.8	961.9	34.7	625	164	345	164	62	201
Extract 2	870.5	963.2	30.9	730	172	332	155	60	224
Extract 3	866.8	944.1	16.7	768	202	451	42	70	182
Extract 4	853.8	836.9	0.4	832	183	434	10	87	139

[1] Uronic Acids.

Table 2: Proportion of cell wall consisting of extract or residue (%) on a dry matter (DM) and total sugar (TS) basis, and DM and TS solubility (%) of the substrates.

	% CW DM[1]	% CW TS[1]	DM solubility[2]	TS solubility[2]
CW	-	-	6.2	11.9
Residue 1	62.6	68.4	6.4	4.6
Residue 2	60.8	66.6	10.4	9.7
Residue 3	28.9	34.0	31.5	36.4
Residue 4	10.3	13.0	13.2	6.1
Extract 1	37.4	31.6	30.1	13.1
Extract 2	1.8	1.7	-	-
Extract 3	31.9	32.7	74.6	75.7
Extract 4	18.6	20.9	86.1	90.7

[1] Calculated with the total recovery of fractionation set to 100%.

[2] Calculated using the residue of the 0 h incubation.

production profiles indicate that fermentability of the residues increased for the first two extraction steps (CW and Residues 1 and 2), and was similar for Residues 2 and 3 (extraction step 3), whereas the fermentability of Residue 4 was decreased (Figure 2A). However, the fermentability of the extracts increased with each subsequent extraction step (Figure 2B).

For the first extraction step (Figure 2C) the extracted material (Extract 1) was fermented with a lower rate (slope of curve) than the CW it was extracted from. However, the lag time of pectin fermentation was shorter for the fermentation of Extract 1. The fermentation of the first residue (Residue 1) had a shorter lag time than the fermentation of CW material. A similar pattern was observed for the second extraction step (Figure 2D). For the third extraction step, the profiles were reversed, the extracted material (Extract 3) was rapidly fermented (steep slope and short lag time), whereas the residue after extraction (Residue 3) was less rapidly fermented than the

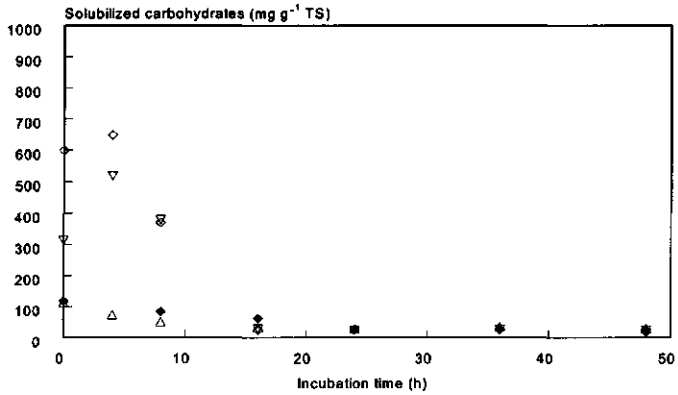

Figure 1: Amount of carbohydrate (mg) in solution per gram of total sugar (TS) in starting material for Extracts 1 (Δ), 3 (∇), and 4 (◇) and Residue 3 (◆), during the time course of incubation.

material before extraction (Residue 2). For extraction step 4, these differences were even more apparent.

Sugar degradation profiles

Degradation profiles of the individual sugars from the insoluble material for CW, Extract 1 and Residues 1 through 4 are shown in Figure 3. The degradation profiles for Extracts 3 and 4 are not shown, because these extracts were largely soluble. For all substrates the degradation of sugars at 167 h was complete, so it appeared that all cell wall polysaccharides had been degraded. The ranking of the individual sugars in the sugar degradation profiles was similar, with a rapid disappearance of galactose and arabinose, an intermediate disappearance of xylose and uronic acids and a slow disappearance of glucose.

Curve fit data

The fitted parameters of the gas production profiles for the mono- and di-phasic models, and the maximal rates of substrate degradation, are shown in Table 3. The di-phasic model improved ($P < 0.05$) the curve fits for all bottles. Adding a third phase to the model did improve the fit for most bottles, but not for all. Furthermore, adding a third phase increased the variation of the parameter estimates. Therefore, fitting the tri-phasic model was considered inappropriate for the work described here.

For the mono-phasic curve the asymptotic gas production (A) ranged from 345 to 504 ml g^{-1} TS, the half-time of gas production ranged from 18.7 to 85.6 h and the maximal rate of substrate degradation ranged from 1.1 to 10.5% h^{-1}. The sequential extracts had a decreasing half-time of gas production, whereas the maximal rate of substrate degradation increased. The half-time of gas production decreased for CW to Residue 2, whereas the half-time of gas production in-

creased again for Residues 3 and 4. The maximal rate of substrate degradation decreased going from CW to Residue 4.

For the di-phasic gas production curve of the CW fraction and Residues 1 to 4, the gas production in the first phase was 68, 75, 71, 60 and 13% respectively of the total gas production. These values corresponded reasonably well with the proportion of pectin-related sugars

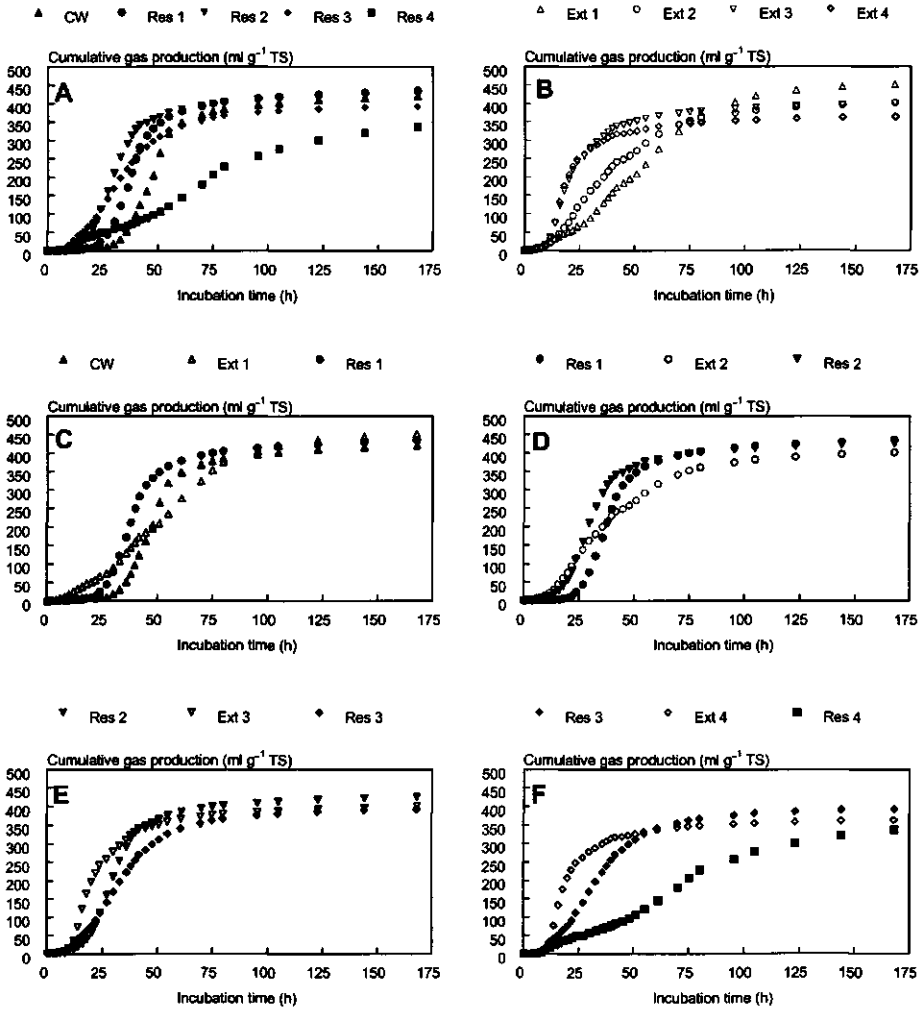

Figure 2: Gas production profiles (ml g^{-1} total sugar, TS) for the incubation of the substrates grouped by either (A) residues and (B) extracts, or by extraction step 1 through 4 (C, D, E, and F, respectively).

(arabinose, galactose and uronic acids) present in these materials (70, 69, 68, 54, and 19% w/w TS, respectively). For the Extracts 1 to 4, the proportion of gas, which was described by the first phase, was 80, 53, 35, and 55%, respectively. Except for Extract 1, these values are not in agreement with the proportion of pectin-related sugars in these substrates (71, 73, 83, and 76% w/w TS, respectively).

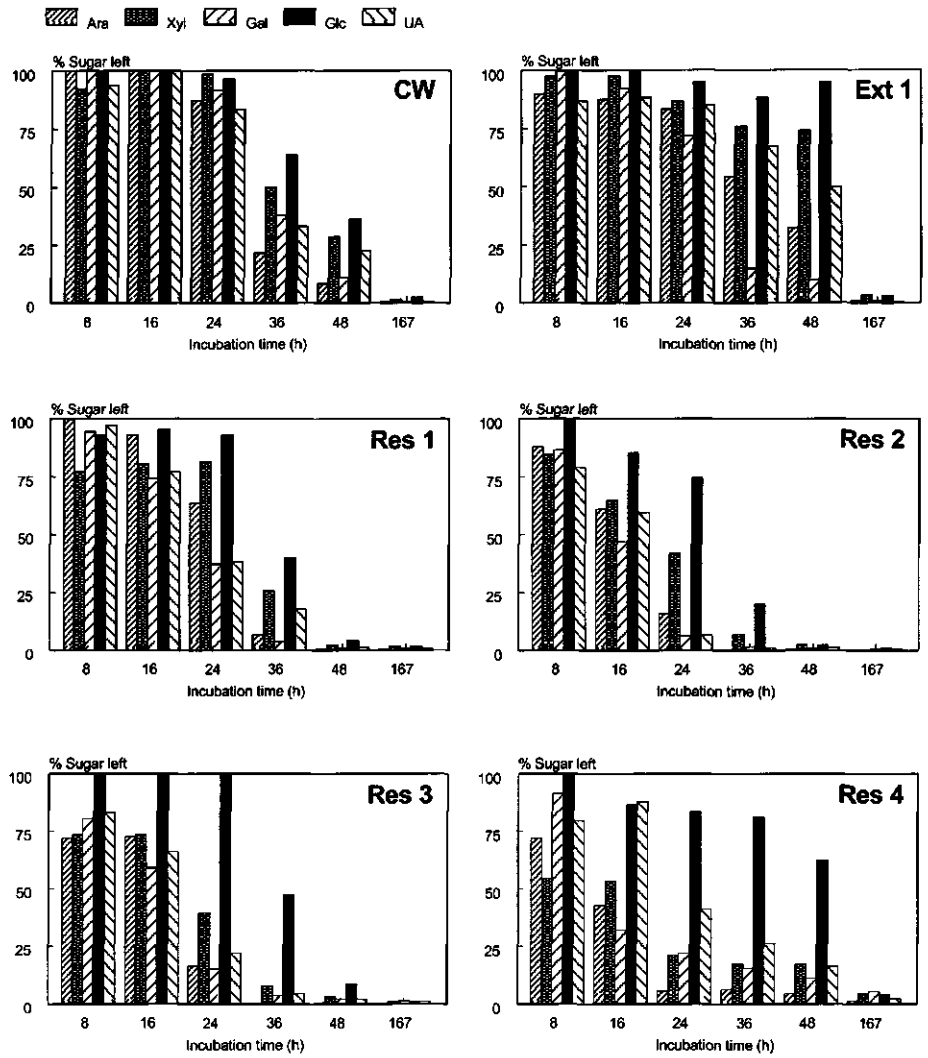

Figure 3: Degradation profiles for the individual sugars from the insoluble material of CW (CW), Extract 1 (Ext 1), and Residues 1 through 4 (Res 1 to Res 4) for incubation with a pig faeces inoculum (Ara: Arabinose; Xyl: Xylose; Gal: Galactose; Glc: Glucose; UA: Uronic Acids).

Table 3: Asymptotic gas production (A; ml g^{-1} TS), switching characteristic (B), half-time (C, h), and maximal rate of substrate degradation (R_M, % h^{-1}) for the nine substrates, when fitted with the mono-phasic or the di-phasic model.

	Mono-phasic				Di-phasic							
	A	B	C	R_M	A_1	B_1	C_1	A_2	B_2	C_2	R_{M1}	R_{M2}
CW	404^{c}	7.3^{a}	47.0^{c}	10.5^{a}	339^{ab}	9.5^{a}	46.5^{a}	158^{ab}	1.6^{b}	183.9^{a}	14.6^{bc}	0.8^{b}
Res 1	417^{bc}	5.9^{b}	37.9^{d}	10.0^{ab}	333^{ab}	7.8^{ab}	36.7^{ab}	108^{b}	2.7^{b}	63.5^{ab}	14.5^{bc}	2.2^{b}
Res 2	412^{c}	4.4^{c}	29.7^{de}	8.8^{bc}	378^{abc}	6.5^{bc}	28.8^{bc}	153^{ab}	2.1^{b}	39.3^{ab}	14.6^{bc}	2.6^{b}
Res 3	396^{c}	2.9^{de}	32.8^{d}	4.6^{d}	240^{abc}	3.3^{d}	32.2^{bc}	159^{ab}	4.8^{b}	34.9^{b}	5.8^{d}	8.2^{b}
Res 4	447^{b}	1.9^{f}	85.6^{a}	1.1^{f}	48^{d}	4.1^{cd}	14.1^{d}	298^{a}	3.7^{b}	73.8^{ab}	17.0^{b}	2.8^{b}
Ext 1	504^{a}	2.4^{ef}	56.0^{b}	2.2^{ef}	387^{a}	2.2^{d}	46.9^{a}	92^{b}	16.6^{a}	62.2^{ab}	2.4^{d}	21.5^{a}
Ext 2	413^{c}	2.4^{ef}	37.0^{d}	3.3^{ed}	217^{bc}	2.9^{d}	21.6^{cd}	189^{ab}	6.7^{b}	54.9^{ab}	8.0^{cd}	7.8^{b}
Ext 3	386^{c}	3.0^{de}	21.0^{ef}	7.4^{c}	140^{cd}	8.3^{ab}	15.6^{d}	255^{ab}	2.7^{b}	27.7^{b}	36.8^{a}	5.1^{b}
Ext 4	345^{d}	3.4^{d}	18.7^{f}	10.0^{ab}	199^{bcd}	7.5^{ab}	15.6^{d}	161^{ab}	2.7^{b}	30.4^{b}	32.4^{a}	4.6^{b}
SEM[1]	6.6	0.1	1.9	0.3	33	0.5	2.5	35	1.2	30.3	1.5	1.7

Table 4: Percentage of residue after fermentation, amount of VFA produced (acetic acid equivalents (AAE, mmol g^{-1} OM), VFA production pattern (%), ratio of acetic acid to propionic acid ratio (A/P), acetic acid equivalents yield (AAEY, AAE produced per DM fermented (mmol g^{-1})), and gas production yield (gas Y, gas per DM fermented, ml g^{-1}) for cell walls (CW) and sequential extracts and residues.

	Residue	AAE	% HAc	% HPr	% HBu	A/P	AAEY	Gas Y
CW	10.6^{a}	9.8^{b}	58.8^{c}	26.1^{g}	15.1^{a}	2.3^{a}	10.7^{a}	369^{a}
Residue 1	11.5^{a}	10.1^{b}	51.6^{e}	36.1^{bc}	12.3^{b}	1.4^{d}	11.3^{a}	379^{a}
Residue 2	8.3^{a}	10.3^{b}	51.3^{e}	38.3^{ab}	10.4^{c}	1.3^{d}	10.9^{a}	368^{a}
Residue 3	8.0^{a}	10.8^{ab}	52.8^{ed}	39.2^{a}	8.0^{d}	1.3^{d}	11.5^{a}	389^{a}
Residue 4	11.0^{a}	10.0^{b}	54.1^{d}	38.0^{ab}	7.8^{d}	1.4^{d}	10.9^{a}	349^{a}
Extract 1	7.9^{a}	7.8^{c}	52.3^{cd}	34.8^{cd}	12.9^{b}	1.5^{d}	8.2^{a}	307^{a}
Extract 2	8.1^{a}	10.3^{b}	58.3^{c}	32.7^{de}	9.0^{d}	1.8^{c}	10.8^{a}	329^{a}
Extract 3	22.1^{a}	10.8^{ab}	62.1^{b}	31.6^{ef}	6.4^{e}	2.0^{b}	17.5^{a}	497^{a}
Extract 4	13.8^{a}	11.4^{a}	65.9^{a}	29.4^{f}	4.7^{f}	2.2^{a}	11.1^{a}	351^{a}
SEM[1]	5.9	0.2	0.4	0.5	0.3	0.0	2.6	68

[1] Standard Error of the Mean.

[abcdef] Means with different superscripts within one column differ significantly ($P < 0.05$).

Percentage of residue and VFA production patterns

The percentage of DM remaining in the bottles was around 10% for all substrates (except for Extract 3 (22.1%), Table 4). The VFA production during fermentation from 0 to 167 h, had increasing proportions of acetic and propionic acid, whereas the proportion of butyric acid decreased, for Residues 1 through 4 (data not shown). For the VFA production during fermentation from 0 h to 167 h, for Extracts 1 through 4, the proportion of acetic acid increased, whereas the proportion of propionic and butyric acid decreased (data not shown).

When comparing the VFA production patterns at 167 h of fermentation for CW and the sequential Residues 1 to 3, the proportion of propionic acid increased, whereas the proportion of acetic and butyric acid decreased. For Residue 4, the proportion of propionic acid rose slightly, compared to Residue 3. For the sequential Extracts 1 to 4, there was a decrease in proportions of propionic and butyric acids, whereas the proportion of acetic acid rose as witnessed by an increasing acetic/propionic ratio.

Numerically there were great differences in the yield of volatile fatty acids (AAEY, mmol of acetic acid equivalents produced per gram fermented DM) and the yield of gas (ml of gas produced per gram fermented DM). However, none of these differences were significant. The absence of a significant difference is probably due to the relatively large variation on the determination of the DM disappearance.

Discussion

Cell wall fractionation and composition

The fractionation of soya bean cell wall into different polysaccharide fractions has been described in detail by Huisman *et al.* (1998), where it is reported that the first two extracts are composed of mainly pectin, the third extract of approximately 50% pectin and the fourth of mainly hemicellulose. In this experiment, the total content of pectin-related sugars (arabinose, galactose, and uronic acids) accounted for approximately 70% of the CW, however, these sugars are not all associated with the pectin molecule. In the present fractionation, 31.6% of the cell wall sugars was extracted in the first extraction step. Pectins were also extracted in the subsequent extraction steps, as was witnessed from the high arabinose, galactose and uronic acid content in Extracts 2, 3 and 4. The first extraction step uses CDTA to extract pectic material by complexing Ca^{2+} ions (Rihouey *et al.*, 1995), thereby solubilizing the pectic polysaccharides held in the cell wall by ionic cross-links (Huisman *et al.*, 1998). Further extractions with 0.05 M, 1 M and 4 M alkali (extraction steps 2, 3, and 4) solubilize the pectins bound by ester linkages and the hemicellulose fraction (Huisman *et al.*, 1998).

The glucose content of sequential residues increased, whereas the arabinose, galactose and uronic acid content decreased. This is consistent with the removal of pectin and also hemicellulose from the cell wall leaving a residue with an increasing proportion of cellulose. The most puzzling result of the extraction procedure was the high glucose content in Extracts 1 and 2 (164 and 155 g kg^{-1} TS, respectively), which was unexpected, because Huisman *et al.* (1998) found only 1% glucose in their CDTA extract from soya bean meal CW. Another puzzling result was the decrease in glucose content in the sequential extracts, whereas it had been expected to start at low concentrations in Extract 1 and then increase, due to an increased solubilization of xyloglucan containing hemicellulose (Huisman *et al.*, 1998). The origin of the glucose in Extracts 1 and 2 is, therefore, unclear. Eriksson *et al.* (1997), when extracting pectins from rapeseed, found a glucose level of 3.5% when using 0.05 M CDTA at pH 6.5. However, when using 0.02 M CDTA at pH 5, a glucose level of 14.2% was found. The extraction conditions used in this experiment are closer to the former conditions, yet slight errors in concentration or pH could have led to a more glucose-rich extract. Eriksson *et al.* (1997) indicate that some of this

glucose could originate from glycoproteins, however, they used water-insoluble material for their extraction which still contained proteins, and may have had a higher glycoprotein content than the extracted cell wall material used in the present experiment. Another source of glucose could be from contamination with insoluble cell wall material. Microscopic evaluation of Extract 1 did reveal the presence of a small amount of cell wall fragments. However, this could only have accounted for a very small proportion of the glucose present. A last, possibility for the origin of the glucose is starch, which might not have been properly removed during cell wall isolation, and is extracted during fractionation. Although the starch content of soya beans is very low, already 1% starch in the soya bean cotyledon (when it is not removed and all extracted in the first sequential extraction) could cause a starch content of larger than 20% in the first extraction. It is assumed that the glucose in Extracts 1 and 2 was not structurally linked to the pectin molecule, but may partly originate from glycoproteins, insoluble cell wall material, and starch.

Extract 1 contained more nitrogen than could have been extracted from the cell wall. This can be attributed to the presence of CDTA, which was not removed during dialysis (Mort *et al.*, 1991), because it contains 2 N atoms. Based on the increase in N content and the recoveries of Extract 1 and Residue 1, the contamination of Extract 1 with CDTA was estimated to be around 25%. Therefore, the presence of CDTA was likely to have been the major cause for the low total sugar content of Extract 1.

Cell wall and polysaccharide solubility

The apparent solubility of CW material and Residues 1, 2, and 4 ranged from 4.6% to 11.6%, though these materials were most likely insoluble, and the solubility calculated here was probably due to errors in DM collection and in errors correcting for the DM of the inoculum. The solubility of extracts increased for Extracts 1, 3 and 4. The solubility of Extract 1 was fairly low which was probably caused by the contamination with CDTA, which was not removed during dialysis (Mort *et al.*, 1991), but forms insoluble complexes with Ca^{2+} and pectins (Eriksson *et al.*, 1997; Gooneratne *et al.*, 1994).

Hatfield and Weimer (1995) found a water-soluble arabinogalactan from larch wood, which was virtually non-degradable by rumen microbes. Thus, it is possible that polymers were solubilized from the CW matrix, but were not fermentable. However, Figure 2 showed that all soluble sugars disappeared during fermentation of the extracts. Therefore, the polymers present in the soya bean cotyledon CW were all fermentable after they had been solubilized.

Fermentation of CW and CW fractions

The removal of polysaccharides from the CW matrix increased fermentability (shorter half-time of gas production, Table 3) as shown by an increased fermentability for Residues 1, 2 and 3 compared to original CW material. Carpita and Gibeaut (1993) have postulated that the association of pectin with hemicellulose and cellulose determines the porosity of the plant cell wall. Therefore, removing the pectin from the CW matrix would increase the porosity of the remaining matrix. This causes an increased accessibility by microbial enzymes, which increases fermentability. However, the fermentability of Residue 4 was decreased compared to CW and the other residues. Residue 4 was composed of mainly glucose, which was most likely present in a crystalline cellulose structure, as it was fairly resistant to degradation (Hatfield, 1989).

For the first two extraction steps (Extracts 1 and 2), the gas production profiles of the extracts had a longer or similar half-time (*C*-value) and a lower rate of maximal substrate degradation than the CW material and Residues 1 and 2 (Table 3). This would indicate a relatively slow fermentation of isolated pectins, which is contrary to results reported elsewhere, where isolated pectins (Hatfield and Weimer, 1995; Van Soest *et al.*, 1991) are found to ferment very rapidly. In fermentation experiments with whole CW, the pectins are generally fermented more rapidly than other CW polysaccharides (Chesson and Monro, 1982; Van Laar *et al.*, 1999; Chapter 3). Therefore, pectin is considered to be the most fermentable complex carbohydrate in the rumen (Van Soest *et al.*, 1991).

The low rate of fermentation of Extracts 1 and 2 (Table 3), may have been a result of:

1. Toxicity of CTDA in the extract for micro-organisms;
2. The microbes were not adapted to fermentation of pectins (maize diet);
3. The decreased solubility due to complexing of pectins with CDTA and Ca^{2+}; or
4. A true decreased fermentability of the extracted polysaccharides.

Ad 1. Theoretically, CDTA could only form insoluble complexes with metal ions. Furthermore, Oderinde *et al.* (1990) added CDTA to a yeast culture, which fermented sugar cane molasses and found an increased ethanol production due to the complexing of CDTA with metal ions, which would otherwise reach toxic levels. Furthermore, CDTA has been used as a detoxicant, against metal accumulation in *Drosophila melanogaster* (Massie *et al.*, 1983) and mice (Domingo *et al.*, 1988). It seems, therefore, that CDTA itself has toxic effect on cells, and may not be toxic for micro-organisms.

Ad 2. The microbes from the pig faeces could be less adapted to fermentation of soya pectin, because the pigs were fed a maize diet. However, preliminary research (Van Laar, unpublished data) showed that when pigs were fed a maize diet the *in vitro* fermentation of soya bean meal with faeces as inoculum, was not depressed, but even tended to be more rapid as compared with pigs that had been fed soya bean cell walls. Therefore, it is unlikely, that Extract 1 was fermented relatively slowly due to a decreased pectinolytic capacity of the faecal microbes.

Ad 3 and 4. The low solubility of Extract 1 was probably caused by CDTA, yet in the CW matrix the pectins are insoluble, due to complexing with Ca^{2+}. It is, therefore, possible that a combined effect of a decreased solubility and a truly decreased fermentation, due to the nature of pectin, led to the decreased fermentability of Extract 1. If the latter is indeed the case, this would shed new light on the fermentation of extracted pectins from cell walls, because usually pectins are assumed to be rapidly degraded (Van Soest *et al.*, 1991), and were degraded first from the intact soya cell wall (Figure 3A). In this experiment, the fermentation of extracted pectins did start earlier than cell wall degradation, but the rate of degradation was lower than for degradation of the whole cell wall.

Sugar degradation profile and curve fit parameters

The degradation pattern of the CW sugars (Figure 3A) was similar to previous experiments (Van Laar *et al.*, 1999; Chapter 3), although the disappearance of galactose was slightly slower than expected. For Residues 1 to 4 (Figures 3C, D, E, and F), the ranking of sugar degradation is similar to that of CW, with galactose, arabinose and uronic acids degrading fairly rapidly, whereas xylose and glucose were degraded more slowly. This was as expected, because the

residues were CW material from which pectic polysaccharides had been removed. However, for the sequential residues, the degradation of glucose became slower, related to the increased cellulose content (increased glucose).

For the CW and residues, the amount of gas produced in the first phase of gas production (Table 3) was in good agreement with the amount of pectin related sugars present in the material. Furthermore, sugar degradation profiles (Figure 3) showed that the pectin-related sugars (arabinose, galactose) were rapidly degraded. Therefore, it could be argued that, the first gas production phase represented the degradation of pectins. The maximal rates of degradation of the first gas production phase were all fairly high (>14% h^{-1}, except for Residue 3). Therefore, pectin, if present within the cell wall matrix, is degraded fairly rapidly. This seems contrary to the slow fermentation of extracted pectins (Extract 1).

For the extracts, only for Extract 1 the division of gas into two phases agreed with a division into pectin and non-pectin sugars. Extract 1 should have consisted solely of pectin, but the high glucose content in Extract 1 suggested that this was not the case. However, the sugar degradation profile showed that the glucose was not degraded until after 48 h of fermentation. Therefore, the first phase in gas production was related to the fermentation of pectin, whereas the second phase was related to the fermentation of glucose (the possible origin of this glucose has been discussed already). The maximal rate of substrate degradation for the first phase, however, was rather low (2.2% h^{-1}), confirming the low rate of fermentation for extracted pectins in this experiment.

Extent of fermentation and VFA production profile

The low residual DM after fermentation of the residues was in agreement with the unlignified nature of the material (Chesson, 1993). For the extracts, the high total degradability was in agreement with results of Gaillard (1962) who found a total degradability of generally higher than 90% for polysaccharides extracted from various CW materials.

For residues, the VFA production profiles at 167 h reflected the fermentability of the material. More rapidly fermentable residues had a higher propionic acid production, as would be expected from Van Houtert (1993) and Hungate (1966). For the sequential extracts this relationship seemed to be reversed, with the more rapidly fermentable extracts (Extracts 3 and 4) producing relatively more acetic, instead of propionic acid. However, this is most likely due to the composition of the extracted materials. For the sequential Extracts 1 through 4, the glucose content decreased, whereas the pectin related sugars increased. Because fermentation of pectin leads to a high acetic acid production (Marounek *et al.*, 1985), it is likely that this was the cause for the acetogenic fermentation of Extracts 3 and 4.

Soya cotyledon cell wall composition and fermentation

The cell wall of soya bean cotyledons is relatively rapidly fermentable compared to e.g. the (unlignified) cell walls of maize (Van Laar, unpublished data). This must be explained from both anatomical and chemical structure of the cell wall. The anatomical structure of cotyledon cell walls has been described (Van Laar *et al.*, 1999; Chapter 3) as being a thin cell wall, which at the 0.5 mm sieve width used for grinding, is present as relatively small particles. This structure allows for the preferential removal of pectin-related sugars. The rapid fermentation is further increased by the architecture of the soya cell wall, with its relatively 'open' cell wall matrix

(Hatfield, 1993). The 'basis' of this matrix would consist of cellulose micro-fibres, which are relatively resistant to degradation, between which hemicellulose and pectins fill the gaps (McCann and Roberts, 1991). Pectin would function as the determinant of the pore-size (Carpita and Gibeaut, 1993) of the cell wall. This would explain the results of this experiment, which showed an increased fermentation of residues after removal of pectin. Removal of pectin from the wall would lead to a larger porosity of the CW, which could result in an enhanced fermentability. Spagnuolo *et al.* (1997) speculated that for the degradation of sugar beet pulp, using purified enzymes, pectin forms a barrier to the degradation of the other polysaccharides.

Soya pectin is composed of a galacturonic acid backbone with side chains of arabinan, galactan and arabinogalactans. The backbone is further substituted with methyl-esters and xylose, rhamnose, fucose and mannose residues (Huisman *et al.*, 1998). The sugar degradation pattern has shown that the pectin molecule is being degraded from the 'outside' inwards. The arabinose and galactose containing side-chains are degraded most rapidly, whereas the galacturonic acid in the backbone is degraded at a lower rate. This is probably because the substitutions (Huisman *et al.*, 1998) on the backbone have to be removed before the backbone itself can be fermented.

Conclusions

Pectin in the soya cell wall is believed to fill the gaps between the cellulose and hemicellulose polymers. Therefore, pectin may determine the pore-size of the cell wall material, and function as a barrier for the degradation of the other polysaccharides. The preferential degradation of pectin from the cell wall may not be caused by a rapid degradability of the pectin molecule itself, but may be due to the position of pectin in the cell wall, which causes it to be removed first, in order to be able to degrade the other polysaccharides. Pectin-rich cell walls are generally highly degradable. This is probably related to the relatively 'open' structure of cell walls containing pectin, making the whole cell wall matrix more readily accessible. The pectin molecule itself is degraded from the outside inwards. The arabinose and galactose side-chains are degraded first, after which degradation of the galacturonic backbone can start.

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Chapter 6

Fermentation characteristics of polysaccharide fractions extracted from the cell walls of maize endosperm*

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Fermentation characteristics of polysaccharide fractions extracted from the cell walls of maize endosperm

Abstract

Cell walls (CW) were extracted from maize endosperm and separated into different polysaccharide fractions by sequential extraction with solutions of: saturated BaOH, demineralized water, 1 M KOH, and 4 M KOH. Solubilized polysaccharides were collected after each extraction. Residues were collected, following the extractions with demineralized water, 1 M and 4 M KOH. The original CW material, extracts, and residues were analysed for their fermentation characteristics using an *in vitro* cumulative gas production technique. The fermentation of the alkali treated residues was faster than for the original CW, except for the 4 M KOH residue, which had a similar rate of degradation to the original CW material. The polysaccharides solubilized from the cell wall (extracts) were all rapidly fermented, more rapidly than both the CW and residues. A division of the gas production profile into two phases using curve fitting was in good agreement with a division of the cell wall fermentation, into the fermentation of arabinoxylans and cellulose. Therefore, the likelihood of preferential degradation of arabinoxylans from the maize cell wall was discussed. The VFA production pattern was fairly well explained by fermentation rate and composition of the substrates. The results are discussed in relation to the structure of the maize endosperm cell wall.

Key words: fermentation, cell walls, gas production, maize, polysaccharides

Introduction

Improving the utilization of cell walls in farm animals can have a tremendous economic impact (Akin, 1993). Therefore, the cell wall fraction of feedstuffs and its utilization has been under investigation for many of years (Crampton and Maynard, 1938). Much of this work was conducted using whole plants, which were composed of a mixture of different cell wall types. However, cell wall types within a plant can differ in their composition and fermentation characteristics (Chesson, 1993). To understand cell wall fermentation at the level of the individual cell wall, cell wall fermentation has to be analysed using cell walls of a single type. Furthermore, much of the early work was conducted with lignified cell wall material. Much less attention has been paid to the fermentation of the unlignified primary cell wall of concentrate feedstuffs (such as maize), which are relevant to the nutrition of pigs.

The primary cell wall of maize is composed of cellulose, much arabinoxylan and little pectin. Hatfield (1993) describes a model for the primary cell wall of grasses (maize is a grass), in which cellulose, xyloglucans and arabinoxylans are integrated in a very tightly packed network. Little is known about the effect of the individual polysaccharides, or removal of these polysaccharides on cell wall fermentability. Bourquin *et al.* (1992) found that material reconstituted from the separated polysaccharides was more rapidly fermentable than the original unfractionated cell walls. Therefore, the tight interactions of the cell wall polysaccharides may decrease cell wall

fermentability (Hatfield, 1993). Knowledge of the cell wall, and its polysaccharides, can be obtained by fractionation of the cell wall into different polysaccharide fractions, as has been done by Gruppen *et al.* (1992) and Verbruggen *et al.* (1995). Others have fractionated cell wall material into its polysaccharides and investigated their fermentability (Bourquin *et al.*, 1992; Gaillard, 1962; Hatfield and Weimer, 1995). The integration of knowledge on cell wall structure and fermentability of the cell wall polysaccharides should lead to a better understanding of the fermentation of intact cell walls.

A better understanding of the cell wall polysaccharides in feedstuffs for monogastrics and their fermentation characteristics may lead to designing new to tools (e.g. enzymes) to improve the utilization of cell wall material. This experiment focuses on the fermentation characteristics of polysaccharides fractionated from the endosperm cell wall of maize, using an inoculum prepared from pig faeces.

Materials and methods

Cell wall preparation

An industrially prepared maize kernel fraction, predominantly composed of endosperm tissue, was used to extract cell wall (CW) material as described by Huisman *et al.* (1998). In brief, the material (2000 g) was sequentially extracted, using demineralized water (3 h, room temperature), a solution of 10 g l^{-1} sodium dodecyl sulphate and 1.5 g l^{-1} dithiothreitol (3 h, room temperature), and using a maleic acid buffer containing 2 mg l^{-1} bacterial α-amylase (Boehringer Mannheim 161 764; pH 6.5 for 16 - 20 h at 30 °C). Before the α-amylase extraction, the material was wet-sieved over a 40 μm sieve to remove starch granules. After each extraction step, the material was centrifuged at 11,000 × g for 30 min, the supernatant discarded, and the pellet subjected to the subsequent extraction step.

Cell wall fractionation

To fractionate the maize cell wall into polysaccharide fractions, the cell wall was sequentially extracted using: a saturated BaOH solution with 10 g l^{-1} $NaBH_4$ (16 h, room temperature; Extract 1), demineralized water (pH 7, 16 h, room temperature; Extract 2), a 1 M KOH solution with 10 g l^{-1} $NaBH_4$ (16 h, 4 °C; Extract 3), and a 4 M KOH solution with 10 g l^{-1} $NaBH_4$ (16 h, room temperature; Extract 4). After each extraction, the solubilized polymers (expected to be mainly arabinoxylans) were separated from the insoluble residue by centrifugation (12,300 × g; 45 min), and a sub-sample of the pellet was taken (Residues 2, 3 and 4). After the BaOH extraction the residue was not washed, but water-soluble polysaccharides, which were insoluble in saturated BaOH, were extracted using a separate extraction with demineralized water instead. Therefore, Residue 1 (originally after BaOH extraction) is lacking, and Residue 2 is the residue after extraction with demineralized water. All residues and extracts were neutralized with HAc and dialysed against demineralized water. Extract 1 was first dialysed against a 0.2 M NH_4Ac buffer (pH 5) at 4 °C, before extensive dialysis against demineralized water. All extraction procedures were designed to extract mainly the arabinoxylans from the maize cell wall.

Assessment of fermentability

The eight substrates (CW, Residues 2, 3, and 4, and Extracts 1, 2, 3, and 4) were assessed for their fermentability according to the *in vitro* cumulative gas production technique, described by Theodorou *et al.* (1994), using an automated pressure evaluation system (Davies *et al.*, 1995). Approximately 0.5 g dry matter (DM) of each substrate was weighed into 100 ml bottles (Schott, Germany; cat no 2180524) containing 82 ml of a semi-defined medium, which should support growth of most fermentative micro-organisms (Lowe *et al.*, 1985). Bottles were inoculated with 5 ml of an inoculum prepared from the mixed faeces of four pigs fed a predominantly maize diet, containing no added antibiotics or copper. The faeces was diluted 1:5 with saline (9 g l^{-1} NaCl). Four bottles containing the same substrate (except Extract 4; three bottles) were incubated for 144 h. An extra four bottles, containing only medium and inoculum, were used as a blank.

Fermentation was stopped by autoclaving the bottles at 110 °C for 10 min, after which the bottles were stored at −18 °C pending residue collection. After thawing, the contents of each bottle were rinsed into a centrifuge tube using demineralized water, and centrifuged at 11,000 × g for 30 min. The pellets were then resuspended in demineralized water and centrifuged again. The residues were transferred to a 50 ml plastic bottle, freeze-dried, and the residual DM was determined (freeze-drying). The supernatants from the centrifugation procedure were pooled by bottle and brought up to a volume of 250 ml with demineralized water. A sub-sample of 10 ml was taken and 0.5 ml of phosphoric acid (85%) was added, after which the sample was stored at −18 °C pending volatile fatty acid (VFA) analysis.

Chemical analyses

Substrates were analysed for DM, ash, and sugar composition. Dry matter was determined by drying to a constant weight at 103 °C following ISO method 6496 and ash by combustion at 550 °C (ISO method 5984). Neutral sugar composition was determined by high pressure liquid chromatography (HPLC; Dionex PA-10 column and PA-10 guard column) with pulsed amperiometric detection, using allose as an internal standard, similar to Lebet *et al.* (1997). The samples were pre-treated with 12 M sulphuric acid (1 h, 30 °C) followed by hydrolysis with 1 M sulphuric acid for 3 h at 100 °C. After hydrolysis, uronic acids were determined by a colorimetric *m*-hydroxydiphenyl assay using a spectrophotometer at 520 nm (Blumenkrantz and Asboe-Hansen, 1973).

VFA in fermentation liquids were analysed using gas chromatography (Packard 419, (CE Instruments, Milan, Italy), glass column filled with chromasorb 101, carrier gas N_2 saturated with methanoic acid, 190 °C with isocaproic acid as the internal standard). The total VFA production, for the three main VFA (acetic, propionic, and butyric acids), is expressed as acetic acid equivalents (AAE) which was calculated by adding: the production of acetic acid, 1.5 times the production of propionic acid, and two times the production of butyric acid.

Calculations and statistics

Gas production

To smooth the gas production profiles, the gas volume was regressed against the gas pressure yielding a linear volume pressure relationship for each individual bottle. This relationship was

used to recalculate the gas volume produced for each pressure, which were added per measurement time to give the cumulative gas production profiles. Gas production profiles were corrected for gas production in the blank bottles.

For each bottle, gas production profiles (ml g^{-1} of total sugars in starting material, TS) were fitted to the mono- and di-phasic modified Michaelis-Menten equation as described by Groot *et al.* (1996). For each phase, the maximal fractional rate of substrate degradation (R_M) was calculated, using the equations described by Groot *et al.* (1996). The statistical comparison between the mono- and di-phasic models was done with an *F*-test as described by Motulsky and Ransnas (1987).

Parameter fittings and other results were analysed with Model 1 using the GLM procedure of SAS (1989). Differences between individual substrates were analysed by a multiple comparison test (Tukey), using the MEANS statement of SAS (1989).

$$Y = \mu + Substrate_i + \varepsilon_{ij} \qquad \text{(Model 1)}$$

Y	Result;
μ	Mean;
$Substrate_i$	Effect for substrate i;
ε_{ij}	Error.

Results

Cell wall extraction, fractionation, recovery and composition

The recovery of cell wall material from the maize endosperm was 2.1% on a DM basis. The recovery for the fractionation of the cell wall, and the proportion of the cell wall within each fraction, is shown in Table 1. The proportion of the cell wall within each fraction was calculated based on a total recovery of the fractionation of 100%.

The composition of CW, residues and extracts is shown in Table 2. The crude protein content of the CW was 101 g kg^{-1}, the other substrates were not analysed for crude protein content. The total sugar content of Residues 2 and 4 was rather low, though this coincided with a low organic matter content, possibly caused by inorganic material originating from residual extraction chemicals. For CW and the sequential residues, the glucose content (cellulose) increased, whereas the arabinose and xylose content (arabinoxylans) decreased. The arabinose:xylose ratio is indicative for the substitution of arabinose on the xylose backbone of arabinoxylans. For the CW and Residues 2, 3, and 4 the arabinose:xylose ratios were 0.75, 0.52, 0.67, and 0.61, respectively. For the Extracts 1, 2, 3, and 4, the arabinose and xylose contents decreased, whereas the glucose content increased. The arabinose:xylose ratios for the sequential Extracts 1 to 4 were 0.97, 0.69, 0.93, and 0.68, respectively.

Gas production profiles

The gas production profiles for the CW, residues and extracts are shown in Figure 1. The gas production profiles of extracts were faster than gas production profiles of CW and residues. For the CW and residues, the gas production profile of CW was the slowest, whereas the gas

Table 1: Proportion of which the CW is composed of each fraction (%), based on a total recovery of 100% and the true total recovery (%), on both a dry matter (DM) and a total sugar (TS) basis.

	% of CW DM basis	% of CW TS basis
CW	100	100
Residue 2	64.6	59.8
Residue 3	51.4	45.2
Residue 4	41.7	32.5
Extract 1	13.8	13.7
Extract 2	21.7	26.5
Extract 3	13.1	14.7
Extract 4	8.5	9.5
Recovery	86.2	80.9

Table 2: Dry matter (DM, g kg^{-1} substrate), organic matter (OM), total sugar content (TS, g kg^{-1} DM) and sugar composition (g kg^{-1} TS) of cell wall (CW), cell wall residues (Residues 2, 3 and 4) and extracted polysaccharides (Extracts 1, 2, 3 and 4).

	DM	OM	TS	Arabinose	Galactose	Glucose	Xylose	UA[1]
CW	930.2	987.1	688	236	50	287	314	80
Residue 2	950.8	639.0	498	161	34	470	312	69
Residue 3	947.0	848.0	657	126	27	539	187	58
Resisue 4	961.6	654.7	533	105	23	584	171	46
Extract 1	902.6	963.5	641	434	19	6	446	94
Extract 2	918.0	959.9	790	327	63	48	475	78
Extract 3	916.8	959.1	750	272	48	254	293	108
Extract 4	920.1	959.3	766	194	51	334	282	96

[1] Uronic Acids.

production profile of Residues 2 and 3 was faster. The gas production profile of Residue 4 was slower compared to Residues 2 and 3. The gas production profiles of the extracts were all rapid, with some differences in the final gas production volume.

Curve fit data

The parameters for fitting of the cumulative gas production profiles of the mono- and di-phasic model are shown in Table 3. The reduction in sums of squares when using the di-phasic model compared to the mono-phasic model, was significant ($P < 0.05$; F-test) for all bottles. From visual inspection of the curves and the significant decrease in sums of squares for the di-phasic model, it was concluded that all gas production curves were essentially di-phasic.

The mono-phasic curves provide the simplest comparison of the fermentation characteristics. Although the differences in asymptotic gas production between the substrates were rather large, only the extremes differed significantly. Thus, most substrates had a similar asymptotic gas production. The maximal rate of substrate fermentation, using the mono-phasic model, was very

high for Extracts 1, 2 and 3 (70.6, 71.3 and 47.5% h^{-1}, respectively), whereas Extract 4, and the CW and residues all had a similar maximal rate (around 10% h^{-1}).

The di-phasic curves divide the production of gas into two phases. For the CW and Residues 2, 3, and 4, the percentage of gas produced in the first phase was 58, 40, 36, and 29%, respectively. These values correspond well with the sum of arabinose and xylose present in these materials (55, 47, 31, and 28%, respectively (on TS basis)). For the CW and residues the maximal rate of substrate degradation (R_M) is generally slightly lower for the second phase of gas production. For the extracts the proportion of gas produced in the first phase was 74, 47, 80, and 61%. These values do not agree with the sum of arabinose and xylose present in these materials

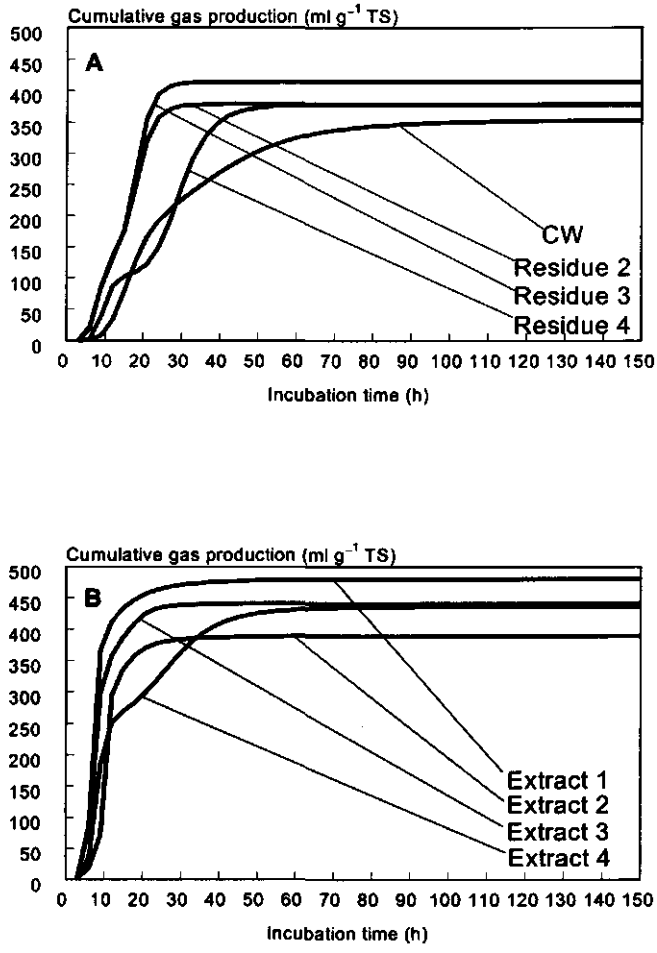

Figure 1: Gas production profiles (ml g^{-1} total sugar, TS) for the different polysaccharide fractions of the maize cell wall (CW): (A) CW and residues and (B) extracts.

Table 3: Asymptotic gas production (A, ml g^{-1} total sugars), switching characteristic (B), half-time (C, h), and maximal rate of substrate degradation (R_M, % h^{-1}) for the different polysaccharide fractions of the maize cell wall (CW), when fitted with a mono-phasic or a di-phasic model.

	Mono-phasic				Di-phasic							
	A	B	C	R_M	A_1	B_1	C_1	A_2	B_2	C_2	R_{M1}	R_{M2}
CW	342^{d}	2.7^{c}	22.9^{b}	6.2^{c}	206^{c}	5.1^{b}	16.7^{a}	148^{d}	4.1^{cd}	42.4^{a}	18.8^{c}	5.5^{d}
Residue 2	409^{abc}	3.1^{c}	15.2^{c}	11.0^{c}	151^{cd}	5.5^{b}	8.5^{cd}	228^{abc}	8.9^{a}	18.6^{c}	40.1^{c}	33.9^{a}
Residue 3	461^{a}	3.4^{c}	16.0^{c}	11.7^{c}	150^{cd}	5.1^{b}	8.5^{cd}	265^{a}	9.6^{a}	18.4^{c}	36.3^{c}	37.2^{a}
Residue 4	405^{abcd}	2.9^{c}	27.2^{a}	5.5^{c}	107^{d}	6.4^{b}	11.8^{b}	262^{ab}	6.4^{b}	31.1^{b}	40.0^{c}	13.8^{bcd}
Extract 1	445^{ab}	7.5^{b}	7.2^{e}	70.6^{a}	356^{a}	9.8^{b}	6.9^{d}	125^{de}	2.8^{d}	12.6^{d}	101.1^{b}	11.8^{cd}
Extract 2	366^{cd}	10.1^{a}	10.2^{d}	71.3^{a}	183^{c}	25.5^{a}	10.1^{bc}	207^{bc}	3.7^{cd}	11.3^{d}	212.2^{a}	18.7^{bc}
Extract 3	410^{abc}	5.7^{b}	7.5^{e}	47.5^{b}	353^{a}	7.0^{b}	7.1^{d}	88^{e}	5.8^{bc}	16.1^{c}	64.9^{bc}	22.4^{b}
Extract 4	391^{bcd}	2.8^{c}	10.4^{d}	13.9^{c}	264^{b}	6.6^{b}	8.0^{cd}	172^{cd}	4.6^{cd}	28.2^{b}	54.1^{bc}	9.7^{cd}
SEM[1]	13	0.4	0.2	3.3	12	1.4	0.6	12	0.5	0.7	11.8	2.1

[1] Standard Error of the Mean.

[abcde] Means with different superscripts within a column differ significantly ($P < 0.05$).

Table 4: Remaining DM (% DM), acetic acid equivalents produced (AAE mmol g^{-1} total sugar), proportion of acetic, propionic and buteric acid production (HAc, HPr, HBu, %), acetic to propionic ratio (A/P), acetic acid equivalent yield (AAEY, mmol g^{-1} dry matter digested) and gas yield (gas Y, ml g^{-1} dry matter digested), for the fermentation of cell walls (CW) and sequential residues.

	Rem. DM	AAE	HAc	HPr	HBu	A/P	AAEY	gas Y
CW	20.7^{d}	9.1^{a}	61.5^{a}	30.3^{b}	8.1^{b}	2.0^{a}	7.9^{a}	825^{c}
Residue 2	46.8^{a}	9.6^{a}	58.0^{abc}	34.4^{b}	7.6^{b}	1.7^{a}	8.8^{a}	926^{abc}
Residue 3	31.4^{c}	9.5^{a}	59.1^{ab}	33.6^{b}	7.3^{b}	1.8^{a}	8.9^{a}	1046^{a}
Residue 4	39.3^{b}	9.4^{a}	58.1^{ab}	32.0^{b}	9.8^{a}	1.8^{a}	7.9^{a}	838^{bc}
Extract 1	9.0^{e}	13.0^{a}	53.8^{c}	41.6^{a}	4.6^{c}	1.3^{b}	8.8^{a}	920^{abc}
Extract 2	8.1^{e}	10.1^{a}	55.8^{bc}	41.2^{a}	2.9^{d}	1.4^{b}	8.5^{a}	928^{abc}
Extract 3	7.2^{e}	11.7^{a}	59.8^{ab}	35.1^{b}	5.1^{c}	1.7^{a}	9.7^{a}	981^{ab}
Extract 4	8.3^{e}	9.4^{a}	59.6^{ab}	35.1^{b}	5.2^{c}	1.7^{a}	8.1^{a}	992^{a}
SEM[1]	1.3	1.5	0.9	1.1	0.3	0.1	1.1	32

[1] Standard Error of the Mean.

[abcde] Means with different superscripts within a column differ significantly ($P < 0.05$).

(88, 80, 57, and 48%), and no other obvious relationships with sugar composition were found. For all substrates the maximal rate of substrate degradation (R_M) was generally slightly lower for the second gas production phase compared with the first.

Residue and VFA production profiles

The remaining dry matter and VFA production profiles, after fermentation for 144 h, are shown in Table 4. For Residues 2, 3, and 4 the remaining dry matter was very high. This remaining dry

matter was probably composed of non-fermentable inorganic matter, because the ash content of Residues 2, 3 and 4 was very high (Table 2). For extracts, the remaining dry matter was lower than 10%. There was no significant difference in the amount of acetic acid equivalents produced per gram of total sugars, indicating a similar extent of carbohydrate degradation of the different substrates. For CW and residues the VFA production profiles were similar, although there was a slight decrease in acetic acid and a slight increase in propionic acid for CW and sequential residues. For the sequential extracts the proportion of acetic acid increased, whereas the proportion of propionic acid decreased.

Discussion

Cell wall extraction and fractionation

The goal of the cell wall isolation was to obtain a substrate containing mainly primary maize cell walls. The recovery of cell wall material (2.1%) was in the same order as the recovery of cell wall material from wheat flour and sorghum flour of 1.2% and 5.3%, respectively (Gruppen *et al.*, 1989; Verbruggen *et al.*, 1993). The fractionation of the maize cell wall into different polysaccharide fractions had a recovery of 80.9% on a sugar basis. Loss of material in these extensive polysaccharide fractionation procedures, using many centrifuging steps, is unavoidable.

The fractionation of wheat flour cell walls using BaOH yields a fairly pure arabinoxylan fraction (Bergmans *et al.*, 1996). This also seems to be the case for maize endosperm, because the first two sequential extracts were mainly composed of arabinose and xylose, with a very low glucose content. The high glucose content of Extracts 3 and 4 was probably caused by solubilization of (1-3, 1-4)-β-glucans, as has also been shown for wheat flour and sorghum flour (Gruppen *et al.*, 1992; Verbruggen *et al.*, 1995). Therefore, these extracts were composed of a mixture of polysaccharides. For the sequential residues, the arabinose and xylose content decreased, and the glucose content increased, due to the removal of arabinoxylans from the cell wall matrix, leaving a cellulose-rich residue.

Phenolic acids (ferulic and *p*-coumaric acid) in the maize cell walls were not determined. Huisman *et al.* (1999) reported levels of 0.1% and 1.6% for esterified *p*-coumaric and ferulic acid in maize cell wall. However, they used whole maize kernels instead of maize endosperm, which led to a much higher cell wall yield (8.7%) probably caused by the maize bran, which had a high phenolic acid content. The esterified ferulic and *p*-coumaric acid content in cell walls extracted from wheat and sorghum flour (endosperm) was below 1% (Gruppen *et al.*, 1989; Verbruggen *et al.*, 1993). Phenolic acids may cause a depression in cell wall degradability (Chesson *et al.*, 1983; Hartley, 1972). However, this effect is thought to be a result of cross-linking of phenolic acids to lignin, whereas the presence of esterified phenolic acids alone would be of limited importance (Jung, 1989). Although lignin content could not be determined due to a shortage of material, the lignin content of these primary cell walls of maize endosperm is thought to be negligible, similar to that of primary cell walls from sorghum flour (Verbruggen *et al.*, 1995). It is expected that the degradability of the maize cell wall fraction was not affected by the presence of lignin of phenolic acids, but was mainly related to its polysaccharide composition.

A low concentration of uronic acids was present in the CW material. Harris *et al.* (1997) using maize seedlings, reported that only about half of the uronic acids were present as galacturonic acid (pectins), whereas the other half was present as glucuronic acid (bound to xylose). Because, young tissues such as maize seedlings are expected to consist mainly of primary cell wall material, the uronic acids in the primary cell walls of maize endosperm are expected to have a similar composition. Therefore, the impact of pectins (galacturonic acid, only 50% of the uronic acid present) on maize cell wall fermentation is considered to be minimal.

The water solubility of polysaccharides is an important factor in fermentability, because water-soluble polysaccharides are usually deemed to be rapidly and extensively fermented (Hall *et al.*, 1998). Due to a shortage of starting material, the water solubility of the cell walls and cell wall fractions was not determined. However, CW and residues were most likely insoluble, because they were extensively extracted with either water or aqueous solutions. Although the actual water solubility of extracts was not determined, visual inspection indicated that the extract appeared to dissolve completely in the medium used for fermentation. Therefore, the extracts were considered to be completely soluble.

Gas production profiles

Fermentation of Residue 2 was more rapid than fermentation of the original cell wall material. This was most probably due to the destruction of the cell wall matrix by extraction of arabinoxylans with BaOH. Alkali treatment of cell walls has been reported to improve over all cell wall degradation (Chesson, 1981; Miron *et al.*, 1997; Rai and Mudgal, 1996). Therefore, it was not surprising that the alkali-extracted residues were fermented more rapidly.

Arabinoxylans in the cell walls of grasses (such as maize) are thought to form a large integrated network throughout the cell wall by hydrogen-bonding to cellulose (Carpita, 1996) and cross-linking with esterified di-ferulic acid bridges (Jung and Deetz, 1993). These alkali labile bonds (hydrogen bonds) are broken by alkali treatment and arabinoxylans are solubilized. Therefore, the integral structure of the cell wall matrix would be broken down by BaOH extraction. Theoretically, this would increase cell wall accessibility for microbial enzymes, so that fermentability of Residue 2 would logically be increased compared with the original CW material. The fermentability of Residue 3 did not increase compared to that of Residue 2, indicating that further extraction of arabinoxylans from Residue 2 did not lead to any further increase in cell wall accessibility. The fermentability of Residue 4 was decreased compared to Residue 3. Residue 4 was mainly composed of glucose, most likely originating from cellulose (Verbruggen *et al.*, 1995). Cellulose is known to be a polysaccharide which is not very rapidly degraded (Hatfield, 1989). However, the fermentability of the original cell wall was similar or slightly lower (not significantly), compared with the fermentability of Extract 4. Therefore, the interactions between the polysaccharides in the maize cell wall are believed to be very 'tight', as is also shown for grass cell walls by Hatfield (1993). This tight packing of cell wall polysaccharides, with their cross-links via hydrogen bonding and ferulic acid bridges, was apparently equally, or more, resistant to fermentation as the cellulose polymers from Residue 4.

The solubilized arabinoxylan fractions were all rapidly fermentable, although there did appear to be some differences in asymptotic gas production. This rapid fermentation of the extracts was expected for soluble carbohydrate fractions (Hall *et al.*, 1998). However, Hatfield and Weimer

(1995) reported a water-soluble, yet unfermentable arabinogalactan, which originated from larchwood. Thus, solubility does not always guarantee fermentability.

Curve fit data

The mono-phasic curves support the conclusions already drawn from the gas production profiles. For CW and residues, the differences in asymptotic gas production were fairly large, although only the extremes (461 *vs* 342 ml g^{-1} TS) differed significantly. Also, for the extracts there were large differences in asymptotic gas production, but only the two extremes (445 *vs* 366 ml g^{-1} TS) were significantly different.

For the di-phasic curves of CW and residues, the proportion of gas produced in the first phase was similar to the proportion of arabinose and xylose in the total cell wall sugars content of the material. Therefore, it could be concluded that the first phase of gas production describes the fermentation of the arabinoxylans, whereas the second phase concurs with the fermentation of cellulose. This would mean there was a preferential degradation of arabinoxylans in the early fermentation. To confirm this conclusion, it would be necessary to analyse the sugar composition data of the residual DM at different time points during fermentation. However, these data were not collected in this experiment.

For purified parenchyma cell walls of grasses, it has been found that uronic acids and xylose disappear more rapidly than other cell wall sugars (Grabber and Jung, 1991a). Preferential degradation of pectic cell wall polysaccharides has been shown for soya bean cotyledon cell walls (Van Laar *et al.*, 1999; Chapter 3). However, in other experiments it was concluded that cell wall sugars of maize had similar rates of degradation (for kernels: Van Laar *et al.*, 2000a; Chapter 2; for endosperm: Van Laar *et al.*, 2000e; Chapter 7). Chesson *et al.* (1986) and Gordon *et al.* (1983), working with grasses, also found a similar composition of fermentation residues and starting materials, indicating no preferential degradation of polysaccharides. With the current data it is not possible to ascertain whether there is a preferential degradation of arabinoxylans from the primary maize cell wall.

Gas production profiles of Extracts 1, 2, and 3 were also fitted to the di-phasic model, yet the shape of the curve was fairly smooth, indicating a relatively homogeneous fermentation. Furthermore, the proportion of gas produced in the first phase did not agree with a division of the carbohydrates into arabinoxylans and glucose (cellulose or β-glucans). Therefore, the division of gas into two phases for Extracts 1, 2, and 3 was considered to be inappropriate for separating substrate characteristics. Extract 4 had the most pronounced di-phasic curve of the extracts. This does suggest a relationship between the gas production phases and arabinoxylan and β-glucan fermentation. However, the proportion of gas produced in the first phase did not match the arabinoxylan content. Therefore, the biological meaning of the di-phasic profile for the Extract 4 is still unclear.

VFA production profiles

The VFA profiles are determined by the interaction of substrate composition with the microflora fermenting the material. Cellulosic materials are generally thought to produce more acetic acid (Hungate, 1966), whereas rapid fermentation is generally accompanied by a more propionic acid directed fermentation (Van Houtert, 1993). The slightly higher proportion of propionic acid for

Residues 2, 3 and 4, compared with CW, is probably a result of the more rapid fermentation, and not of a different composition. This, because the residues had a higher cellulose (glucose) content than the CW, which would normally increase acetic acid production, which was not the case here.

The differences in VFA production profile for the extracts were related to composition of the substrate. The extracts low in glucose (Extracts 1 and 2) had a high proportion of propionic acid whereas the extracts with a higher glucose content (Extracts 3 and 4) had a high proportion of acetic acid. The glucose in Extracts 3 and 4, was probably more related to β-glucans than cellulose. Therefore, these β-glucans also appear to have an acetic acid directed fermentation pattern.

Conclusions

Maize endosperm cell wall structure and fermentation

The cell wall of grasses, e.g. maize, is a relatively 'compact' cell wall compared to the cell wall of legumes (Hatfield, 1993). The cell wall is composed of micro-fibrils of cellulose embedded in a matrix of tightly packed arabinoxylans (Carpita, 1996). The rapid fermentation of extracted cell wall polysaccharides, compared with intact cell walls, shows the importance of interactions between polysaccharides in the cell wall in determining fermentability. As shown for the residues, breaking this interaction by alkali extraction, led to a more rapidly fermentable substrate. The importance of the interactions in the cell wall is supported by results of Bourquin *et al.* (1992) who found that cell wall polysaccharide mixtures in the same composition as the cell walls were more rapidly fermented than the original cell wall material. As pectin is postulated to determine the pore-size in legumes (Carpita and Gibeaut, 1993) the tight network of arabinoxylans in maize might function similarly. As pore-size could influence fermentation of cell walls (Chesson *et al.*, 1997) the tight packing could possibly impede cell wall fermentation.

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Chapter 7

Fermentation of the endosperm cell walls of monocotyledon and dicotyledon plant species: The relationship between cell wall characteristics and fermentability*

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Fermentation of the endosperm cell walls of monocotyledon and dicotyledon plant species: The relationship between cell wall characteristics and fermentability

Abstract

Cell walls from the endosperm of four monocotyledon (maize, wheat, rye, and rice) and four dicotyledon (soya bean, lupin, faba bean, and pea) seeds were studied, so as to relate cell wall composition and structure with fermentation characteristics. Cell wall material was isolated from the endosperm of the mono- and dicotyledons. The fermentation characteristics of isolated cell walls from mono- and dicotyledons were analysed in two separate *in vitro* gas production experiments. At 0, 12, 24, 36, 48, and 144 hours of fermentation, fermentation was stopped in selected bottles to analyse VFA production (144 h only) and sugar degradation patterns. The relationship between cell wall characteristics (composition, particle size) and fermentation characteristics (half-time of gas production and maximal rate of substrate degradation) was analysed using linear regression. For the monocotyledon cell walls, the rate of substrate degradation was decreased by increasing particle size of the cell walls, a clear effect of cell wall composition on fermentation characteristics could not be determined, though this might have been obscured by the differences in particle size. During fermentation of the monocotyledon cell wall, arabinoxylans (arabinose and xylose) and cellulose (glucose) appeared to be degraded simultaneously. For the dicotyledon cell walls, an increase in total sugar content, decreased the half-time of gas production, though total sugar content was probably confounded with the crude protein content. During fermentation of the dicotyledon cell wall, pectins or pectin-related sugars (galactose, arabinose, uronic acids), appeared to be degraded faster than cellulose, whereas for the monocotyledon cell walls, arabinoxylans and cellulose were degraded simultaneously. The differences in cell wall fermentation and sugar degradation pattern between monocotyledon and dicotyledon cell walls are discussed in relation to differences in cell wall architecture.

Key words: cell walls, monocotyledons, dicotyledons, fermentation, sugar composition

Introduction

In the past, most cell wall research has focused on fermentation of whole plant material. However, whole plant material contains a mixture of primary and secondary cell walls, which clearly differ in their fermentation characteristics (Chesson, 1993). Therefore, to relate the composition of an individual cell wall type to its fermentation characteristics, it is important to study isolated cell walls of a single cell wall type. Also, earlier cell wall research has mainly focused on secondary cell wall fermentation in ruminants, whereas the fermentation of primary cell walls, predominantly present in the endosperm of seeds commonly used in pig nutrition (e.g., maize and soya beans) has received much less attention. Feed evaluation for monogastrics would benefit from a better understanding of the factors influencing the fermentation characteristics of seed endosperm cell walls.

Current feed evaluation for cell walls is mostly based on 'crude' fractions such as neutral detergent fibre and acid detergent fibre (Van Soest *et al.*, 1991). These methods provide a crude separation into different polysaccharide fractions, but do not give information on differences in polysaccharide composition within those fractions. Analysis of the composite sugars gives a more detailed view of the composition of the polysaccharides in the cell wall. However, this is complicated by the fact that in different cell wall types the same sugar may be present in totally different polysaccharides, within a completely different cell wall architecture. This is especially the case for arabinose, which in monocotyledonous cell walls is present mainly in the arabinoxylan fraction, whereas in dicotyledonous cell walls it is present mainly in the pectin fraction. This illustrates the importance of knowing the general cell wall architecture of a cell wall when interpreting sugar composition data.

Monocotyledon and dicotyledon cell walls are different in their composition and the structure of their primary cell walls. Generally, monocotyledon cell walls contain mainly hemicellulose and cellulose, with only little pectin, whereas dicotyledon cell walls contain mainly pectin and cellulose and little hemicellulose (Bailey *et al.*, 1976). Furthermore, these polysaccharides have a different arrangement within the cell wall, resulting from differences in cell wall architecture. The monocotyledon cell wall is composed of a tightly packed network of both arabinoxylans and cellulose, whereas the dicotyledon cell wall has a very open hydrated cell wall structure (Hatfield, 1993). These differences in cell wall structure are likely to have an effect on the fermentation of these cell walls. Whereas, differences in cell wall structure may influence cell wall fermentation, differences in sugar composition within a certain cell wall type might also be important.

A study of differences in the composition of polysaccharides could be done using structurally similar cell walls, which differ in composition of their polysaccharides. However, surface area to volume ratio (or particle size/cell wall thickness) which influences fermentation rate (Fisher *et al.*, 1989; Weimer, 1996) would have to be similar, to be able to compare differences in cell wall composition. This experiment was designed to study the relationship between the sugar composition of the primary cell wall and their fermentation by gastro-intestinal microbes of pigs, and to study differences in the fermentation characteristics of monocotyledons and dicotyledons.

Materials and methods

Feedstuffs

Four species from two plant groups (monocotyledonous and dicotyledonous plants) were chosen to be analysed for fermentation characteristics of their endosperm cell walls. For the monocotyledonous feedstuffs, cell walls were obtained from maize endosperm (*Zea mays*) (Meneba Meel Weert b.v., The Netherlands), wheat flour (*Triticum aestivum*) (Meneba), rye flour (*Secale cereale*) (Vibe Glitsø, Danish Institute of Animal Science (Glitsø and Bach Knudsen, 1999), and dehulled polished rice (*Oryza sativa*) (food grade). For the dicotyledonous feedstuffs, cell walls were obtained from the cotyledons of Argentinean soya beans (*Glycine max*) (Schouten Giessen b.v., The Netherlands), lupins (*Lupinus albus*), faba beans (*Vicia faba*) (white hulled variety), and peas (*Pisum sativum*) (Goelema, 1999).

Cell wall extraction

The dicotyledonous materials were soaked overnight in iced water. After soaking, they were manually separated into hulls and cotyledons. Cell walls (CW) were isolated from the cotyledons as described by Huisman *et al.* (1998). Briefly, cotyledons were ground over a 0.5 mm sieve, and extracted with petroleum ether 40-60 in a Soxlet extractor to remove fat. Defatted endosperm was sequentially extracted using demineralized water (3 h, room temperature) and a solution of 10 g l^{-1} sodium dodecyl sulphate (SDS) and 1.5 g l^{-1} dithiothreitol (3 h, room temperature). After gelatinization (pH 5, 85 °C, 1 h), starch was removed using a maleic acid buffer containing 2 mg l^{-1} porcine α-amylase (Merck 16312) (pH 6.5 for 16 - 20 h at 30 °C). After each extraction step, the material was centrifuged at 11,000 × g for 30 min, the supernatant discarded, and the pellet subjected to the subsequent extraction step. The final residue was freeze-dried and left to air-equilibrate for a minimum of 3 h.

For the monocotyledonous feedstuffs, the endosperm fractions (flours) were subjected to the same cell wall extractions procedure as for the dicotyledonous feedstuffs, but without soaking or grinding, except for rice which was ground over a 0.5 mm sieve. Furthermore, the materials were wet-sieved over a 0.45 μm sieve width, to remove most of the starch granules prior to gelatinization. Thus the extraction procedures yielded eight cell wall fractions, four from monocotyledonous feedstuffs (maize, wheat, rye, rice) and four from dicotyledonous feedstuffs (soya beans, lupins, faba beans, peas).

Experimental procedures

The monocotyledonous and dicotyledonous cell wall substrates were assessed for their fermentability in two separate gas production runs, using the *in vitro* cumulative gas production technique described by Theodorou *et al.* (1994). Approximately 0.5 g substrate dry matter (DM) was weighed into 100 ml serum bottles. To these bottles, 82 ml of semi-defined medium, supporting growth of most micro-organisms from the rumen (Lowe *et al.*, 1985), was added. Bottles were inoculated with 5 ml of an inoculum which had been prepared from faeces of pigs by diluting 1:5 with saline (9 g l^{-1} NaCl), mixing in a blender for 1 min., and straining through a double layer of cheesecloth. The maize based diet, fed to the pigs, contained no added antibiotics or copper. Bottles were incubated in an incubator at 39 °C. Sufficient replicates were used so that bottles containing substrate could be removed after 0, 12, 24, 36, 48, and 144 hours of incubation. For 144 h of incubation, four bottles were used for every substrate, and four bottles were used as a blank, which contained only medium and inoculum. For all other combinations of fermentation time and substrate, the number of bottles used was determined based on an estimated total residue of approximately 1 g DM (minimum of two, maximum of eight bottles depending on substrate and fermentation time). For each bottle, gas production was measured by recording pressure and volume of gas produced, at regular intervals during fermentation.

Fermentation was stopped by autoclaving the bottles at 110 °C for 10 min, after which the bottles were stored at −18 °C pending residue collection. After thawing, the contents of each bottle were rinsed into a centrifuge tube using demineralized water and centrifuged at 11,000 × g for 30 min. The pellets were resuspended in demineralized water and centrifuged again. Subsequently pellets (residues) were transferred to a 50 ml plastic bottle, freeze-dried, and residual DM determined (freeze-drying). Subsequently, samples were pooled by substrate and

fermentation time and reground with a porcelain pestle and mortar. The supernatants from the centrifugation procedure were pooled by bottle and brought up to a volume of 250 ml with demineralized water. A sub-sample of 10 ml was taken and 0.5 ml of phosphoric acid (85%) was added, after which the sample was stored at −18 °C pending volatile fatty acid (VFA) analysis.

Chemical analyses, particle size analyses and contamination analyses

Substrates were analysed for DM, ash, nitrogen (N), fat, and sugar composition. Fermentation residues were only analysed for sugar composition. Dry matter was determined by drying to a constant weight at 103 °C (ISO, 1983), ash by combustion at 550 °C (ISO, 1978), N was determined using the Kjeldahl method with $CuSO_4$ as the catalyst (ISO, 1979), and fat was determined using a soxleth extraction with petroleum-ether (ISO, 1996). Neutral sugar composition was analysed by high-pressure liquid chromatography (HPLC; Dionex PA-10 column and PA-10 guard column) with pulsed amperiometric detection, using allose as an internal standard, similar to Lebet *et al.* (1997). The samples were pre-treated with 12 M sulphuric acid (1 h, 30 °C) followed by hydrolysis with 1 M sulphuric acid for 3 h at 100 °C. After hydrolysis, uronic acids were determined by a colorimetric *m*-hydroxydiphenyl assay using a spectrophotometer at 520 nm (Blumenkrantz and Asboe-Hansen, 1973). VFA in fermentation liquids were analysed using gas chromatography (Packard 419, CE Instruments, Milan, Italy; glass column filled with chromasorb 101, carrier gas N_2 saturated with methanoic acid, at 190 °C with iso-caproic acid as the internal standard).

To analyse the particle size distribution, the cell wall materials were analysed with a Coulter laser LS 130 particle size analyser (Keetels, 1995), using the Fraunhofer optical mode. This method assumes that all particles are spherical, which for cell wall material is not the case. The Coulter counter measures both the number of particles, and the volume and surface area of specific particle size classes. For this experiment only the ratio of volume (μm^3) to surface area (μm^2), the d3/2 value, was used as a measure of particle size.

Earlier experiments indicated that cell wall extraction using a detergent such as sodium dodecyl sulphate (SDS) can contaminate the cell wall sample with residuals of the detergent used. This could possibly affect the rate of fermentation (Van Laar, *unpublished results*). By analysing the CW material with pyrolysis gas chromatography mass spectrometry (P-GC-MS), which is usually used for analyses of the lignin composition (Lapierre, 1993), the relative amount of contamination (relative to other samples) could be quantified. However, the exact chemical structure and the absolute concentration of the contaminant could not be determined.

Calculations and statistics

Gas production

To smooth the gas production profiles, the gas volume was regressed against the gas pressure yielding a linear volume pressure relationship for each individual bottle. This relationship was used to recalculate the gas volume produced for each pressure, which were added per measurement time to give the cumulative gas production profiles. Gas production profiles were corrected for gas production in the blank bottles.

For each bottle fermented for 144 h, gas production profiles (ml g^{-1} of total sugars in material,

TS) were fitted to a mono- and di-phasic model as described by Groot *et al.* (1996). For each phase, the maximal fractional rate of substrate degradation (R_M) was calculated, using the C (half-time of gas production) and B (switching characteristic of curve) value according to Groot *et al.* (1996). The statistical comparison between the mono- and di-phasic models was done with an F-test as described by Motulsky and Ransnas (1987).

Parameter fittings and other results within each separate gas production run of monocotyledons and dicotyledons were analysed with Model 1 using the GLM procedure of SAS (1989). Differences between individual substrates were analysed by a multiple comparison test (Tukey), using the MEANS statement of SAS (1989).

$$Y = \mu + Substrate_i + \varepsilon_{ij} \qquad \text{(Model 1)}$$

Y	Result;
μ	Mean;
$Substrate_i$	Effect for substrate i;
ε_{ij}	Error.

Cell wall characteristics and fermentability

The relationship of particle size, contamination, and sugar composition, with the maximal rate of substrate degradation and the half-time of gas production for the fermentation of the cell wall materials was analysed by linear regression using the REG procedure of SAS (1989) (Model 2). This analysis was performed using the means of the maximal rate of substrate degradation and half-time of gas production for each cell wall fraction, yet separately from mono- and dicotyledons. However, differences in particle size could be confounded with contamination or sugar composition. Therefore, this simple linear regression must be seen as a tentative approach to investigating the factors governing the fermentation of different feedstuff cell walls.

$$Y_{ij} = \beta_0 + \beta_1 x_i + \varepsilon_{ij} \qquad \text{(Model 2)}$$

Y	R_M, C (maximal rate of substrate degradation, half-time of gas production);
β_0	Intercept;
β_1	Slope;
x_i	Characteristic (e.g. particle size, sugar composition);
ε_{ij}	Error term.

Results

Cell wall composition and extraction

The composition of the cell walls from the mono- and dicotyledonous feedstuffs is given in Table 1. The protein content of both monocotyledon and dicotyledon cell walls varied from 25 to 109 g kg^{-1} DM. In terms of sugars, the cell walls of the monocotyledons contained large amounts of arabinose, xylose and glucose, except for rice cell walls, which were mainly glucose (cellulose).

Table 1: Dry matter (DM, g kg^{-1}), organic matter (OM), crude protein (CP), crude fat (CF), and total sugar (TS) content (g kg^{-1} DM), and sugar composition (g kg^{-1} TS) for both the dicotyledonous and monocotyledonous cell wall materials.

	DM	OM	CP	CF	TS	Ara[1]	Gal	Glc	Xyl	UA
	Monocotyledonous cell walls									
Maize	909.7	989.1	93.7	11.0	742	254	52	281	315	77
Wheat	890.0	1000	25.0	0	899	244	15	256	408	26
Rye	891.7	998.2	25.2	0	873	207	19	364	284	21
Rice	898.8	995.4	70.6	1.6	761	36	7	880	36	32
	Dicotyledonous cell walls									
Soya	921.3	964.0	109.3	0.9	752	184	356	164	65	172
Lupin	909.1	985.6	53.5	1.7	796	128	646	93	23	86
Faba	925.0	953.0	28.1	3.7	819	426	56	202	48	220
Pea	928.4	960.7	30.4	2.2	793	464	59	182	44	201

[1] Respective sugars are Arabinose, Galactose, Glucose, Xylose, and Uronic Acids.

For the cell walls of the dicotyledons, lupins contained a lot of galactose, as did soya, whereas faba beans and peas contained large amounts of arabinose.

Table 2 shows the yield of cell wall material from the original material and the average particle size, expressed as the d3/2 value. For the monocotyledons, the cell wall yield was around 2% or lower. For the dicotyledons the cell wall yield differed from 3.8% for peas to 32% for lupins. For the monocotyledons only maize and rice cell wall had a measurable contamination with extraction residue, and was twice as high for rice compared to maize. For the dicotyledons the relative contamination (relative to lowest contamination) with extraction residue was 14.2, 10.5, 1.0, and 1.25 for soya, lupin, faba bean and pea cell wall materials, respectively.

Table 2: Yield of cell wall (CW) material from the original material and particle size expressed as volume over area (d3/2 value, $\mu m^3\ \mu m^{-2}$) for both the monocotyledonous and dicotyledonous cell wall materials.

	CW yield	d3/2 value
	Monocotyledonous cell walls	
Maize	2.1[2]	26.6
Wheat	0.7	16.6
Rye	1.5	15.1
Rice	1.2	4.2
	Dicotyledonous cell walls	
Soya	10.4[1]	9.3
Lupin	32.0[1]	8.6
Faba	4.1[1]	12.1
Pea	3.8[1]	11.4

[1] Yield calculated on an air dry matter basis.

[2] Value from earlier experiment (Van Laar *et al.*, 2000d; Chapter 6).

Gas production results

Gas production profiles

Figure 1 shows the gas production profiles for all substrates divided into mono- and dicotyledons. For the monocotyledons, there were marked differences in gas production profiles, both in lag time and total gas production. Most prominent is the rapid and extensive degradation of the rice cell wall, and the relatively long lag time for the degradation of maize cell walls. For the dicotyledons, the asymptotic gas production was similar, though for soya bean cell walls there seemed to be an increased lag phase, compared to the other three cell walls, which had similar gas production profiles.

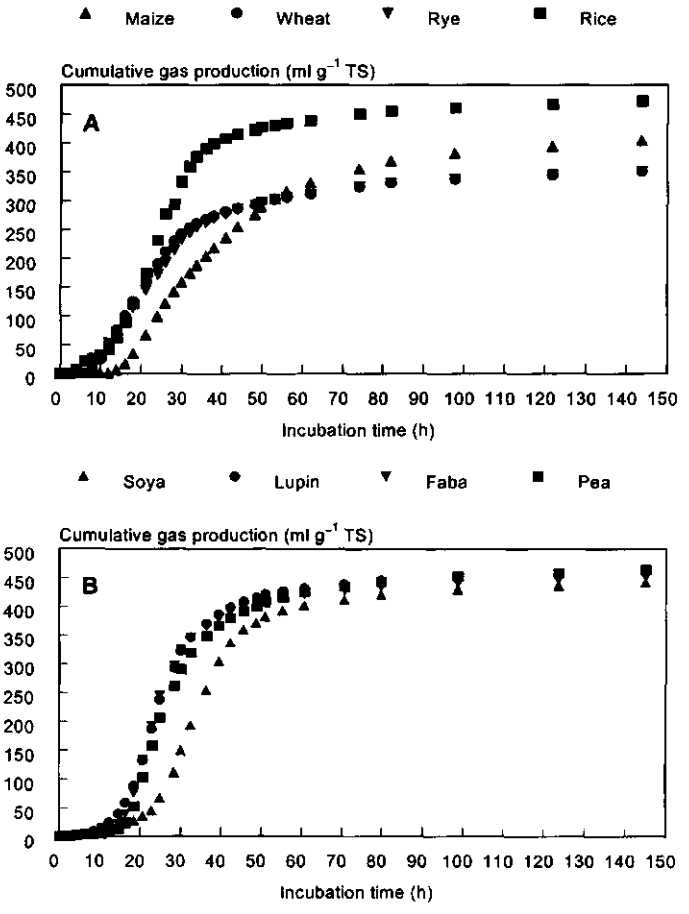

Figure 1: Gas production patterns for the fermentation of (A) monocotyledon and (B) dicotyledon cell walls.

Table 3: Asymptotic gas production (A; ml g^{-1} TS), switching characteristic (B), half-time (C; h), and maximal rate of substrate degradation (R_M, % h^{-1}) for the fermentation of the monocotyledonous and dicotyledonous cell wall materials, when fitted with the mono-phasic or the di-phasic model.

	Mono-phasic				Di-phasic							
	A	B	C	R_M	A_1	B_1	C_1	A_2	B_2	C_2	R_{M1}	R_{M2}
	Monocotyledonous cell walls											
Maize	400^{b}	3.0^{b}	35.7^{a}	4.4^{c}	108^{b}	7.5^{a}	22.4^{ab}	295^{a}	3.4^{a}	44.0^{ab}	22.3^{a}	4.2^{a}
Wheat	337^{c}	2.7^{bc}	22.0^{b}	6.5^{b}	304^{a}	3.3^{c}	20.1^{b}	51^{c}	4.0^{a}	76.2^{a}	8.8^{c}	2.9^{a}
Rye	343^{c}	2.6^{c}	23.3^{b}	5.8^{b}	315^{a}	2.8^{c}	22.3^{ab}	38^{c}	10.9^{a}	72.1^{a}	6.6^{c}	25.4^{a}
Rice	461^{a}	3.7^{a}	23.5^{b}	8.7^{a}	315^{a}	5.5^{b}	23.9^{a}	171^{b}	1.4^{a}	29.8^{b}	14.3^{b}	2.9^{a}
SEM[1]	12.0	0.1	0.6	0.2	14.7	0.4	0.8	10.1	3.1	8.6	1.1	11.1
	Dicotyledonous cell walls											
Soya	430^{a}	5.2^{a}	33.9^{a}	9.5^{b}	329^{a}	7.2^{a}	33.6^{a}	129^{b}	1.7^{c}	47.6^{a}	14.3^{a}	1.9^{b}
Lupin	446^{a}	4.4^{b}	24.8^{bc}	10.3^{b}	248^{a}	7.4^{a}	23.6^{b}	215^{a}	2.6^{bc}	30.2^{b}	21.3^{a}	4.5^{a}
Faba	429^{a}	5.0^{ab}	24.4^{c}	12.3^{a}	289^{a}	7.7^{a}	22.6^{b}	161^{ab}	3.0^{ab}	36.1^{b}	23.3^{a}	4.4^{a}
Pea	441^{a}	4.5^{b}	26.8^{b}	10.0^{b}	271^{a}	7.5^{a}	23.5^{b}	189^{ab}	3.4^{a}	38.8^{ab}	21.5^{a}	4.8^{a}
SEM	6.4	0.1	0.6	0.5	19.6	0.5	0.6	18.5	0.2	2.3	2.1	0.4

[abc] Means with different superscripts within one column differ significantly ($P < 0.05$).

[1] Standard Error of the Mean.

Gas production curve fitting

The fitted parameters and the maximal rates of substrate degradation are shown in Table 3. For the mono-phasic curve fit of the monocotyledons, the rice cell walls had the highest asymptotic gas production and highest maximal rate of substrate degradation. Maize cell walls had the lowest maximal rate of substrate degradation and had a very high half-time of gas production. The asymptotic gas production was lower and much more variable for the monocotyledons compared with the dicotyledons. For the dicotyledons, the asymptotic gas production was similar for all the cell walls, though there were some differences in maximal rate of substrate degradation. Soya cell walls had a very high half-time of gas production compared to the other three dicotyledons. The half-times of gas production for mono- and dicotyledons were comparable, whereas the maximal rate of substrate degradation was slightly higher for the dicotyledons.

Fitting a di-phasic curve through the gas production profiles significantly improved the curve fit for all bottles. The division of gas production into two phases could be related to fermentation of specific sugars within each substrate. For the monocotyledons, the proportion of gas produced in the first phase for maize (26.8%), wheat (85.5%), rye (89.2%), and rice (64.8%) was not similar to the proportion of arabinoxylan (arabinose and xylose) of the total cell wall sugars (56.9%, 65.2%, 57.1%, and 7.2%, respectively). In the case of the dicotyledons, the proportion of gas produced in the first phase for soya (71.8%), and faba beans (59.5%) was fairly similar to the proportion of pectin-related sugars (arabinose, galactose and uronic acids) (71.2% and 64.8%, for soya and faba beans, respectively). However, the proportion of gas produced in the first phase for

lupin (53.6%) and pea (58.9%) did not agree with the proportion of pectin-related sugars (86% and 72.4%, respectively).

Fermentation characteristics

The percentage of residue and the VFA production profile after fermentation for 144 h is shown in Table 4. For monocotyledons, wheat and rye had more residue than maize and rice. Although differences in the amount of VFA produced were not statistically significant, the higher residue was reflected in the lower amount of VFA produced. Fermentation of rice cell wall led to a different pattern of VFA production, with less acetic acid and more propionic acid, as reflected in the lower A/P ratio, compared to the other monocotyledons. For dicotyledons, the percentage of residue was fairly similar for the different feedstuffs. However, the amount of VFA produced was slightly lower for soya and lupin cell walls. For lupins, the production of acetic acid was very low, whereas the propionic acid production was very high, reflected in a low A/P ratio.

Sugar degradation patterns

Figure 2 shows the sugar degradation pattern of arabinose, xylose and glucose, for the four monocotyledonous substrates. The other sugars were present in low amounts, which made the analysis less reliable, and could have introduced large errors to the degradation pattern, and have, therefore, been omitted. For maize and wheat, the degradation of arabinose, xylose and glucose was similar. For rye, the degradation of glucose seemed more rapid, whereas for rice the degradation of glucose was slower than for arabinose and xylose.

Table 4: Percentage of residue after 144 h fermentation, amount of VFA produced (acetic acid equivalents; AAE, mmol g^{-1} total sugars), VFA production pattern (%), acetic acid to propionic acid ratio (A/P), acetic acid equivalents yield (AAEY, AAE produced per DM fermented (mmol g^{-1})) gas production yield (gas produced per DM fermented, ml g^{-1}) for the fermentation of the dicotyledonous and monocotyledonous cell wall materials.

	Residue	AAE	HAc	HPr	HBu	A/P	AAEY	Gas Y
	Monocotyledonous cell walls							
Maize	19.9^{b}	10.8^{a}	59.0^{a}	28.5^{c}	12.5^{a}	2.1^{a}	10.1^{a}	375^{b}
Wheat	27.8^{a}	8.3^{a}	59.8^{a}	30.2^{b}	10.0^{c}	2.0^{a}	10.4^{a}	437^{a}
Rye	34.0^{a}	8.5^{a}	60.0^{a}	28.9^{bc}	11.1^{bc}	2.1^{a}	11.2^{a}	464^{a}
Rice	14.9^{b}	10.2^{a}	55.6^{b}	33.0^{a}	11.4^{ab}	1.7^{b}	9.1^{a}	423^{ab}
SEM[1]	1.6	0.6	0.5	0.4	0.3	0.04	0.7	13.4
	Dicotyledonous cell walls							
Soya	17.1^{a}	8.0^{b}	54.3^{c}	33.6^{b}	12.1^{a}	1.6^{b}	7.2^{b}	391^{b}
Lupin	12.8^{b}	7.8^{b}	47.8^{d}	43.4^{a}	8.8^{b}	1.1^{c}	7.1^{b}	420^{ab}
Faba	14.4^{ab}	10.4^{ab}	60.2^{a}	31.4^{b}	8.4^{b}	1.9^{a}	9.8^{ab}	428^{a}
Pea	15.7^{ab}	12.1^{a}	57.8^{b}	32.8^{b}	9.4^{b}	1.8^{ab}	11.3^{a}	433^{a}
SEM	0.9	0.7	0.5	0.6	0.5	0.04	0.7	6.7

[abcd] Means with different superscripts within one column differ significantly ($P < 0.05$).

[1] Standard Error of the Mean.

Figure 3 shows the sugar degradation pattern of arabinose, xylose, galactose, glucose, and uronic acids for the four dicotyledonous cell wall substrates. The sugar degradation pattern was fairly similar for the four substrates, with arabinose and galactose having the most rapid, uronic acids and xylose an intermediate, and glucose the slowest degradation rate.

Cell wall characteristics and fermentation characteristics

The half-time of gas production (C) and maximal rate of substrate degradation (R_M) were regressed on volume to surface area ratio (d3/2, particle size), relative contamination, proportion of arabinoxylans, proportion of pectin, and content of: crude protein, total sugar, arabinose, xylose, galactose, glucose, and uronic acids. For the monocotyledons, three linear relationships (20 analysed) were significant ($P < 0.05$), these were:

C (h) = 18.9 + 0.31 × galactose content (g kg^{-1} TS) ($r^2 = 0.86$)

C (h) = 16.15 + 0.25 × uronic acid content (g kg^{-1} TS) ($r^2 = 0.93$)

R_M (% h^{-1}) = 9.34 − 0.19 × d3/2 value ($\mu m^3\ \mu m^{-2}$) ($r^2 = 0.91$)

The relationship between arabinose content and the maximal rate of substrate degradation was nearly significant ($P = 0.1002$):

R_M (% h^{-1}) = 9.32 − 0.016 × arabinose content (g kg^{-1}) ($r^2 = 0.71$)

For the dicotyledons, only one significant ($P < 0.05$) linear relationship was found:

C (h) = 146.8 − 0.151 × total sugar content (g kg^{-1} TS) ($r^2 = 0.86$)

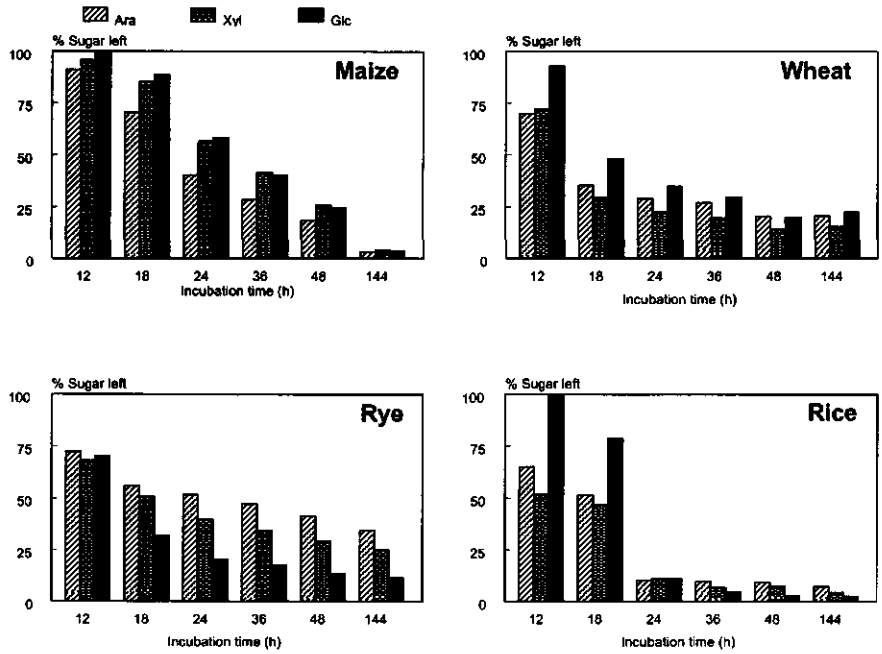

Figure 2: Sugar degradation patterns for the monocotyledon cell walls: maize, wheat, rye, and rice (Ara: Arabinose; Xyl: Xylose; Glc: Glucose).

The relationship between crude protein content and half-time of gas production was nearly significant ($P = 0.095$):

$$C\,(\text{h}) = 21.64 + 0.105 \times \text{crude protein content (g kg}^{-1}) \qquad (r^2 = 0.73)$$

Discussion

Cell wall composition and characteristics

The monocotyledon (wheat, rye and rice) crude protein content was low as would be expected for primary cell walls. However, maize cell walls had a higher crude protein content, though not higher than expected from previous results (Van Dijk, 1996; Van Laar *et al.*, 2000d; Chapter 6). For the dicotyledons, the crude protein content of lupin, faba bean and pea cell walls was around 4% as was expected for the cotyledon cell walls of dicotyledons (Selvendran, 1983). The crude protein content of the soya cotyledon cell walls was higher than expected (Huisman *et al.*, 1998; Van Laar *et al.*, 1999; Chapter 3), though similar levels have been found previously (Van Laar *et al.*, 2000c; Chapter 5).

The sugar composition of the monocotyledon cell walls (rich in arabinose, xylose, and glucose) is characteristic for the monocotyledon primary cell wall, which is mainly composed of

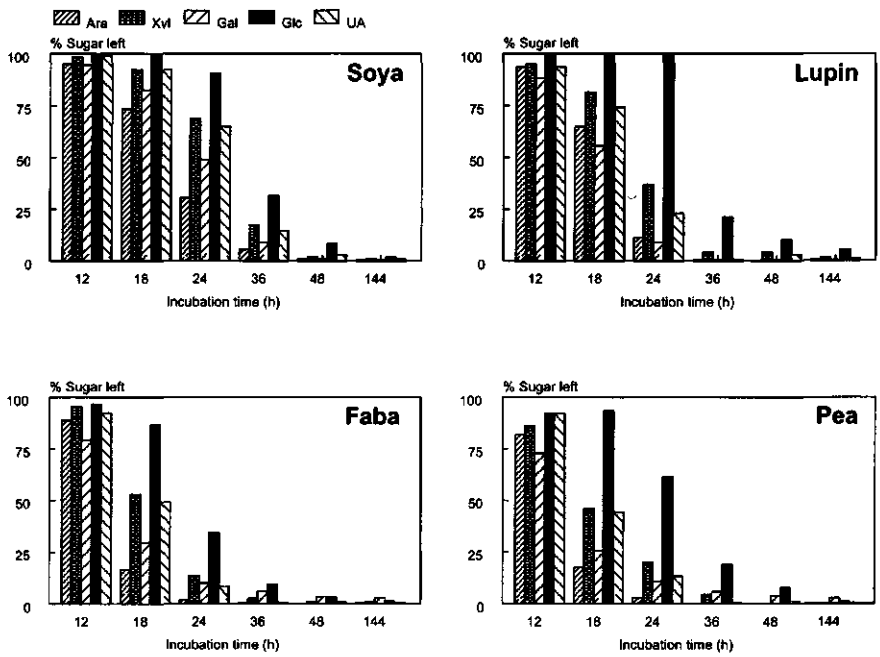

Figure 3: Sugar degradation patterns for the dicotyledon cell walls: soya bean, lupins, faba beans, and peas (Ara: Arabinose; Xyl: Xylose; Gal: Galactose; Glc: Glucose; UA: Uronic Acids).

arabinoxylans and cellulose (maize: Harris *et al.*, 1997; wheat: Gruppen *et al.*, 1992). However, the rice cell wall is mainly composed of glucose, which is probably present mostly as cellulose and some xyloglucan (Selvendran, 1983). The dicotyledon cell walls were either rich in arabinose or in galactose, whereas the levels of glucose and uronic acids were comparable (except for lupin cell walls). The arabinose and galactose in the dicotyledon cell wall are believed to be associated mainly with the pectin fraction by covalent linkage of arabinose and galactose containing polymers to a galacturonic acid backbone (for soya beans and lupins: Van de Vis, 1994). However for lupins the galactose content is very high, which suggests that not all galactose can be covalently linked to a galacturonic acid backbone.

For the monocotyledons, the average particle size (d3/2 value) varied considerably. Only the sieve widths for grinding of rice (0.5 mm) and rye flour (0.25 mm; Glitsø and Bach Knudsen, 1999) were know, whereas the wheat flour and maize endosperm were obtained in a ground state and were not reground. Although the sieve width used for rice was larger than for rye endosperm, the cell wall particles were smaller for rice than for rye (Table 2). For the cell walls from the dicotyledons, particle sizes were fairly similar, probably because the intact cotyledons had all been ground over a sieve width of 0.5 mm, and were similar in characteristics that influence particle size during grinding (all cotyledons).

Cell wall characteristics and fermentation

Linear regression between cell wall and fermentation characteristics

Studying the effects of cell wall characteristics on the maximal rate of substrate degradation (R_M, % h^{-1}) and half-time of gas production (C, h) using linear regression, is a tentative approach to uncover the principles governing fermentation kinetics of a cell wall. For the monocotyledons, there was an increase in the half-time of gas production with increasing amounts of uronic acids and galactose. Possibly the micro-organisms fermenting the cell wall needed more time to be able to adapt their enzyme systems to uronic acids and galactose (pectin) in the cell wall. The maximal rate of substrate degradation was mainly influenced by the difference in particle size of the different monocotyledon cell walls. Particle size, more correctly, surface area to volume ratio, has been known to be an important factor in cell wall fermentation (Chesson *et al.*, 1997; Fisher *et al.*, 1989; Weimer *et al.*, 1990). Other significant relationships between cell wall characteristics and maximal rate of fermentation were not found, though these may have been concealed by the effect of particle size.

For the dicotyledon cell walls, the only significant relationship was between total sugar content and half-time of gas production. A higher total sugar content of the cell wall led to a shorter half-time of gas production. However, this relationship could be confounded with the nearly significant positive relationship between crude protein content and half-time of gas production (more crude protein, longer half-time). For the dicotyledons, there was no significant linear relationship between any of the individual sugars, and fermentation characteristics.

For both monocotyledons and dicotyledons, there was no significant linear relationship between contamination of the cell wall material with detergent residue, and the half-time of gas production nor with the maximal rate of substrate degradation. Therefore, the effect of the presence of a contaminant on the cell wall fermentation characteristics is considered to be

negligible at the concentrations found in the present experiment.

VFA production profiles

For the monocotyledons, the VFA production profiles were similar for the fermentation of maize, wheat, and rye cell walls (Table 4). Therefore, it would appear that the similar composition (arabinoxylans and glucose) of these three monocotyledons led to similar VFA production profiles. The high propionic acid production for the fermentation of the rice cell walls is most probably a reflection of the rapid fermentation, which can stimulate propionic acid production (Van Houtert, 1993). This is contrary to the high glucose (cellulose) content of the rice cell wall, which would be expected to lead to an acetogenic fermentation (Hungate, 1966; Van Houtert, 1993).

For the dicotyledons, the differences in VFA production profiles between the substrates were much larger than for the monocotyledons. The VFA production profiles for faba bean and pea cell wall fermentation were fairly similar, with the highest relative acetic acid production, which may have reflected the high pectin content (Marounek *et al.*, 1985). This would also be the case for the fermentation of soya bean cell walls, though this fermentation had a slightly lower proportion of acetic acid, and a higher proportion of butyric acid. The faba bean and pea cell walls are rich in arabinose, whereas the soya bean cell wall contains more galactose than arabinose. The high arabinose content of the pectin could possibly stimulate acetic acid production, which could be an explanation for the low proportion of acetic acid for the fermentation of lupin cell walls, which are mainly composed of galactose.

Sugar degradation patterns

The sugar degradation pattern (disappearance from the insoluble cell wall fraction) for the monocotyledons, was different for the four cell walls (Figure 2). For maize and wheat cell walls, the degradation of arabinose and xylose was similar to the degradation of glucose. This is as would be expected from the degradation of monocotyledon cell walls from previous experiments with maize cell walls (Van Laar *et al.*, 2000a; Chapter 2). Probably this is caused by the structure of the monocotyledon cell wall, which is a very tightly associated cellulose and hemicellulose (arabinoxylan) network (Carpita, 1996; Hatfield, 1993). Due to this tight association, arabinoxylans and glucose are degraded at similar rates, and one cannot be degraded without degradation of the other. For rye cell walls the degradation of glucose was more rapid than the degradation of arabinose and xylose. Glitsø *et al.* (1998) found a more complete faecal digestion of non-cellulosic cell wall glucose than for cellulose and arabinoxylans, when rye endosperm was fed to pigs. This more rapidly fermented non-cellulosic cell wall glucose was believed to be mainly β-glucan. For rice, glucose seemed to disappear more slowly from the cell wall than arabinose and xylose. However, the content of arabinose and xylose in the rice cell wall was very low, and, therefore, hard to determine accurately. For rice cell walls, it is hard to conclude whether the degradation rates of arabinose, xylose and glucose were different.

The sugar degradation patterns for dicotyledons were fairly similar with respect to the sequence with which cell wall sugars disappeared from the cell wall (Figure 3). The relatively rapid degradation of arabinose and galactose, the slightly slower degradation of uronic acids and xylose, and the slow degradation of glucose, has also been found in previous experiments using

soya bean cell walls (Van Laar *et al.*, 1999; Chapter 3). The cause of this sequence of degradation is believed to be a combination of the structures of dicotyledon cell walls and pectin. The pectin in most dicotyledons is composed of a galacturonic backbone, with branches of arabinose and galactose (Huisman *et al.*, 1998; Van de Vis, 1994). Because the pectin molecule would be degraded from the outside inward the arabinose- and galactose containing side-chains would be removed prior to degradation of the backbone. The pectin-rich cell wall of dicotyledons are composed of an relatively 'open' structure, in which a cellulose-xyloglucan network forms the backbone of the cell wall, and the space between this network is filled with pectin (Hatfield, 1993; Carpita and Gibeaut, 1993; McCann and Roberts, 1991). Probably because of this 'open' structure, pectin can and must be degraded first, before degradation of the cellulose, hemicellulose network, thus causing the differences in degradation of the different sugars within one cell wall.

Di-phasic curve fit

Fitting two phases through the gas production data can be used to obtain more information, when these phases can be correlated with the fermentation of a specific fraction from the substrate (Beuvink and Kogut, 1993). However, for the monocotyledons the division of the gas production into two phases did not agree with a division of the cell wall into arabinoxylans and cellulose. From the sugar degradation pattern it could be concluded that the cell wall of the monocotyledons is degraded as a whole, without preferential degradation of a specific polysaccharide. Rye may have been an exception, where some β-glucans may have been preferentially degraded, though these could not be separated form the degradation of the other glucose (cellulose). Therefore, the size and characteristics of the two phases for a di-phasic fit do not yield extra information on the fermentation of the monocotyledon cell wall.

For the dicotyledon cell wall, a division of the gas production into two phases agreed well with a division of the cell wall into a pectin (arabinose, galactose, uronic acids) and a hemicellulose/cellulose fraction, for the soya and faba bean cell walls. For soya bean cell walls, this has also been shown in previous experiments (Van Laar *et al.*, 2000b; Chapter 4). The lack of agreement between the gas production phases and amount of pectin (arabinose, galactose, uronic acids) for lupin cell walls, might be explained by the assumption that a large proportion of galactose is not structurally related to the pectin molecule, and, therefore, would not be fermented in a single phase. The lack of agreement between the gas production phases and amount of pectin (arabinose, galactose, uronic acids) for pea cell walls remains unclear, although it is also possible that for pea cell walls not all arabinose is part of the pectin molecule.

Conclusions

For the monocotyledon cell walls an increase in particle size decreased the maximal rate of substrate degradation, which seemed to conceal possible compositional effects on fermentation rate. For the dicotyledon cell wall the amount of total sugar and protein seemed to influence the half-time of gas production. No clear effects of differences in sugar composition on cell wall fermentation could be found. This may be because of the small number of feedstuffs used, with

too many confounding parameters (unequal particle size for the monocotyledons, unequal protein content for the dicotyledons).

For the monocotyledons, the disappearance of the individual sugars from the cell wall during fermentation was similar for cell walls, which did not contain β-glucans. Therefore, arabinoxylans and cellulose from the monocotyledon primary cell walls were broken down simultaneously. For the primary cell wall of dicotyledons, the sugars associated with the pectin molecule (arabinose, galactose, and uronic acids) were broken down more rapidly than glucose (cellulose). The cause of this discrepancy in the mode of breakdown of monocotyledon and dicotyledon cell walls is probably related to the structural arrangement and functional properties of the polysaccharides within the cell wall.

Acknowledgements

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Chapter 8

General discussion

General discussion

Introduction

This thesis has focused on the *in vivo* and *in vitro* fermentability of primary cell walls from the endosperm of soya beans and maize. Both feedstuffs are widely used as ingredients in animal diets, for both ruminants and monogastric animals. Although knowledge on the overall digestibility of the different nutrients in various animal species is available (CVB, 1999), detailed knowledge on the susceptibility to fermentation, of their fibre fractions (cell wall) is lacking. Generally, for feedstuffs used in animal nutrition, the analysis of fibre digestibility is based on the digestibility of fractions such as neutral and acid detergent fibre (NDF, ADF) (Van Soest *et al.*, 1991). Analysis of these fractions in feed and faecal material, give an indication of the total amount of cell wall, and their digestibility. However, these fractions do not yield information on the composition of the fibre fraction, nor on their structure in relation to their functionality (such as fermentability, amount and pattern of volatile fatty acid production etc.). To understand more about the relationships between cell wall structure (composition) and function (fermentability), this thesis investigates the fermentation characteristics of the primary endosperm cell walls of soya beans and maize, as influenced by cell wall composition and structure. Maize and soya were used because of their importance in the farm animal feed industry, but also as model feedstuffs (representatives) for two groups of plants, namely monocotyledons (maize) and dicotyledons (soya), which differ substantially in their cell wall composition (Bailey *et al.*, 1976; Harris *et al.*, 1997).

Animal, microbial, and plant characteristics all influence the extent and rate of cell wall fermentation in the gastrointestinal tract of animals (Buxton and Redfearn, 1997; Varga and Kolver, 1997). The main animal characteristics are animal species, anatomy of the intestines, and passage rates. The microbial characteristics are mainly related to microbial activity, adaptation of microbes to a certain substrate and no doubt other factors as well. Although animal characteristics are important for cell wall fermentation, this aspect was beyond the scope of this thesis, and will not be discussed.

Source of micro-flora and cell wall fermentation

The effects of microbial characteristics were briefly investigated in the experiment described in Chapter 2, which deals with the fermentation of whole soya bean meal and maize, and their cell wall fractions, using either sheep rumen fluid or pig faeces (*in vitro*). The gas production profiles of original feedstuffs (maize kernels and soya beans) were fairly similar for both sheep rumen fluid and pig faeces when used as inoculum. For the fermentation of the cell wall fractions, the half-time of gas production was substantially higher for the pig faeces, compared to the sheep rumen fluid inoculum. This was not caused by a lower density of the bacterial population, because the amount of microbial material added with both inocula was comparable. Therefore,

Table 1: Cell wall characteristics (sequence of decreasing impact on fermentation characteristics), and their proposed mode of action, by which they influence cell wall accessibility to microbial enzymes.

Plant / cell wall characteristic	Mode of action
Plant anatomy	Inclusion of material
Plant tissue type(s)	Inclusion of material
Particle size	Inclusion of material / Surface to volume ratio
Cell wall thickness	Surface to volume ratio
Cell wall composition/architecture	
Lignin	Porosity / shielding
Polysaccharides	Porosity / Cell wall structure and architecture
Monosaccharides	Fermentability of polysaccharides

it was concluded that the pig faeces inoculum used was less adapted to fermenting cell wall material, than the sheep rumen fluid inoculum. These results were the basis for the next series of experiments, in which cell wall fermentation of soya beans was investigated in the rumen of a cow (*in situ*) and using sheep rumen fluid (*in vitro*) (Chapters 3 and 4), or using pig faeces (*in vitro*) (Chapters 5, 6, and 7). For the latter experiments, it was decided to use pig faeces, because of the lower adaptation to fibre fermentation for pig faeces (Chapter 2), and because fermentation of the endosperm fibre fraction of soya beans and maize is relatively more important for monogastric animals such as the pig, than for ruminants.

The principles of how cell wall and plant characteristics influence fermentation are thought to be similar for both sheep rumen fluid and pig faeces inocula. For physical characteristics, such as plant anatomy and particle size (Table 1), this will certainly be true. However, it is not certain whether there are differences in the effect of cell wall composition on fermentation, when using sheep rumen fluid or pig faeces as an inoculum. The only pure cell wall type, that has been fermented using both sheep rumen fluid and pig faeces, is the cell wall of the soya bean cotyledon (Chapters 3, 4, 5, and 7). The rate of disappearance, for the different sugars of the soya bean cotyledon cell wall when using sheep rumen fluid (Chapter 3), was the most rapid for galactose, which disappeared slightly more rapidly than arabinose. When the soya bean cotyledon cell wall was fermented using pig faeces (Chapters 5 and 7), arabinose disappeared most rapidly, although only slightly more so, than galactose. However, for the pectin fractions (Chapter 5), galactose was again the most rapidly disappearing cell wall sugar. Therefore, it would seem that the micro-organisms in pig faeces were slightly less effective, compared to sheep rumen fluid, in removing galactose from the intact cell wall, but not from the isolated polysaccharide fractions. The sequence by which the other cell wall sugars from the soya bean cotyledon cell wall were degraded, was similar for both sheep rumen fluid and pig faeces inocula.

Sequence of impact of plant and cell wall characteristics on cell wall fermentability

Table 1 shows the plant and cell wall characteristics that are thought to be relevant to cell wall

fermentation. These are ordered in such a way (sequence of impact) that the characteristics at the top have the greatest impact on cell wall fermentation of a whole plant, whereas the factors below have a lesser impact. The table also shows their proposed mode of action, or how the cell wall characteristics are thought to influence cell wall fermentation.

Interactions in sequence of impact

Before discussing the relationships between the individual plant cell wall characteristics and their relation to fermentability, it is important to realize that the sequence of impact should not be considered as absolute. Interactions between the different cell wall components may occur. For example, lignin content and particle size can interact. Although small particles are fermented more rapidly than large particles, small particles with a higher lignin content could be fermented more slowly than larger particles with a low lignin content. An interaction of lignin and particle size is one very obvious possibility, but other, less obvious interactions could also exist.

Plant anatomy

A comprehensive description of plant anatomy and its relevance to the fermentability of different tissues, has been given by Wilson (1993). Wilson (1993) also discussed differences in plant tissues and anatomy. When feeding animals, the first factor to be considered is the anatomy of the plant, i.e. the tissues that are present (primary or secondary cell walls, lignification, etc.), and how these tissues are organized. Microbes need to physically reach the cell wall to be able to ferment it. When whole plants are ingested, structures such as the epidermis and cuticula (waxy layer on the epidermis) can act as a barrier for the material underneath. Mastication is, therefore, an important step in the fermentation of the cell walls underneath the epidermis (Tamminga, 1993). Although plant anatomy is a very important factor for cell wall fermentation, this thesis focuses on the fermentation of cell walls from seeds, that are composed of fewer and different tissues than whole plants.

In Chapters 2, 3, and 4, the presence of different tissues was ascertained by analysing the cell wall material using light microscopy. The soya bean meal cell wall fraction consisted of cell walls from the hull and the cotyledons, whereas the maize cell wall fraction consisted of cell walls from the bran and endosperm. For soya bean meal, cell walls from the hull had a markedly different composition and fermentation rate, than cell walls from the cotyledons. When two or more tissues with a different fermentation rate and a different composition are present in the same material, the degradation pattern of the cell wall sugars can never reflect the degradation of individual cell wall types (Chesson, 1993). Therefore, when studying the relationship between cell wall composition and fermentation, purified cell walls of a single type have to be used (Chapters 3 and 4).

In the case of soya bean meal, the tissues are present as anatomically separate structures, as the cotyledon and hull of the soya bean are not structurally linked, and are easily separated. Microscopic examination revealed that this was probably also the case for the maize grain, although the bran and the endosperm of maize have a tighter association than the hull and cotyledon of the soya bean. Furthermore, there seemed to be much more variation in tissue structures, for maize grain than for soya beans. Unlike intact plants, when soya beans or maize are ground, the separate tissues do not influence each other in accessibility for micro-organisms,

and, therefore, fermentability, because they have no structural link.

Particle size / cell wall thickness

The size of the cell wall particle, combined with the thickness of the cell walls, determine the surface area to volume ratio of the cell wall material. The surface area to volume ratio is an important factor in cell wall fermentation (Fisher *et al.* 1989; Weimer, 1996), because the surface area available for fermentation, relative to the volume, determines the rate at which the material will be fermented. Both particle size and cell wall thickness determine this ratio, and are important in determining the rate of fermentation of cell walls from ground materials. The final extent of fermentation in a prolonged fermentation should not be influenced by the surface area to volume ratio. However, in animals where, due to the passage of material through the intestines, fermentation time is limited, the extent of fermentation can indeed be influenced, because larger particles take longer to be completely fermented than passage time allows.

In the experiments described in this thesis, cell wall material was subjected to prolonged fermentation, so the maximum extent of fermentation was not influenced by particle size or cell wall thickness. From Chapters 3 and 4, it was concluded that particle size could have a major influence on the rate of fermentation of cell walls. In Chapter 7, where different cell walls of monocotyledons and dicotyledons were compared, the particle size was measured. The original intention was to have a similar particle size for all cell wall materials, or at least within the groups of monocotyledons and dicotyledons, so that differences in fermentation characteristics would be caused only by differences in cell wall composition. For the dicotyledon cell wall materials, the particle sizes were similar, and no effect of particle size was found. However, for the monocotyledons the particle sizes of the cell wall materials were different, and large particles were fermented at a slower rate. Therefore, to study the relationship between cell wall composition and fermentation characteristics, the particle size distribution of the materials should be similar, when comparing the fermentation of different cell walls.

From the results of Chapter 3, an effect of cell wall thickness on the preferential fermentation of specific polysaccharides (pectins) from the cell wall was hypothesized. For the relatively thin cell walls of the soya cotyledon, preferential degradation of pectins occurs from the cell wall. With thicker cell walls it could well be that in a thin top layer of the cell wall specific polysaccharides are preferentially degraded, but that the remaining polysaccharides (e.g., cellulose) form a physical barrier for microbial enzymes to reach the lower layers of cell wall material. Preferential degradation of polysaccharides should cause a change in cell wall composition. However, because of the thick cell wall this change in sugar composition is so small that it cannot be accurately measured. Thus thicker cell walls were found to be degraded layer by layer, as hypothesized for lignified cell walls by Chesson (1993), although preferential degradation may have occurred in a thin top layer. If cell walls are thin enough, preferential fermentation of pectins from the cell wall can be measured, as it was measured for soya bean cotyledons.

Cell wall composition and architecture

Table 1 shows several characteristics of cell wall composition, which have an impact on cell wall fermentation. These are: lignin content, polysaccharides present, and composition of the polysaccharides. The impact of these cell wall composition aspects will be discussed in the

following sections.

Lignification

The most important characteristic of cell wall composition influencing cell wall fermentation is the presence of lignin (Jung and Allen, 1995), and to a lesser extent phenolic compounds (Jung and Deetz, 1993). Although, this thesis focuses on the fermentation characteristics of unlignified primary cell walls, the relationship between lignin content and cell wall fermentation will be discussed briefly. Generally, a decrease in both extent and rate of cell wall fermentation has been reported for an increasing lignin content (Jung and Deetz, 1993). This effect is mostly ascribed to both cross-linking of the polysaccharides in the cell wall matrix, and physical shielding of polysaccharides from fermentation. For lucerne, this effect seems to be present when comparing older tissues that have a higher lignin content and a lower fermentability, with younger tissues. However, when tissues of similar maturity were compared, the relationship between lignin content and fermentability was apparently absent. Therefore, other factors such as lignin composition (e.g. guanicyl, syringyl lignin) could be important in regulating fermentability within stage of maturity (H.G. Jung, University of Minnesota, personal communication; Jung and Deetz, 1993). The presence of phenolics is generally accompanied by a decrease in fermentability. Phenolics can form the bridge between polysaccharides and lignin, and are closely related to the depression of cell wall fermentability caused by lignin. Furthermore, phenolic dimers can form cross-links between two adjacent polysaccharides, increasing cell wall rigidity. These cross-links are deemed to have an effect on the rate of cell wall degradation, but the total extent of cell wall fermentation might not be influenced (H.G. Jung, University of Minnesota, personal communication).

Lignification has a major impact on cell wall fermentation, and is probably the chemical characteristic with the greatest influence on cell wall fermentability. However, not all tissues in plants are lignified, and especially the tissues from seed endosperm contain very little lignin. Therefore, the fermentation characteristics of these cell walls must be governed by other compositional factors.

Cell wall polysaccharides

This thesis is mainly focusing on the relationship between cell wall composition and cell wall fermentation for primary cell walls of soya bean and maize endosperm. The design of the experiments was to investigate the fermentability of: *i*) whole soya bean and maize grain cell walls (Chapters 2, 3, and 4); *ii*) extracted (fractionated) polysaccharides of soya bean and maize endosperm cell walls (Chapters 5 and 6); and *iii*) to compare the fermentation of similar cell walls with the fermentation of soya bean and maize endosperm cell walls (Chapter 7). The combination of the results from the analysis of whole, fractionated, and comparable cell walls, gives a complete view of soya and maize cell wall fermentation.

i) Cell wall composition The soya bean cotyledon cell wall is basically unlignified and composed of polysaccharides (90%), protein (4 - 10%), and fat (1%). The polysaccharides are composed mainly of cellulose (20%), some hemicellulose (10 - 15%), and a lot of pectin (60 - 70%). The maize endosperm cell wall, like that of soya bean, is composed of polysaccharides (90%),

protein (10%), and fat (1%). The polysaccharides are composed of mainly cellulose (25 - 30%), much hemicellulose (arabinoxylan, 50 - 60%), and only little pectin (5 - 10%). For soya bean cotyledon cell walls (Chapters 3 and 4), it was concluded that during fermentation of the cell wall the sugars, galactose, arabinose and to a smaller extent uronic acids and xylose were removed more rapidly than glucose. Therefore, there appeared to be a preferential degradation of especially these pectin-related sugars, compared to cellulose (glucose). No preferential degradation of cell wall polysaccharides was found for the maize endosperm cell wall (Chapters 6 and 7). The main cell wall sugars, arabinose, xylose and glucose, were all degraded at approximately the same rate. This was a remarkable difference in mode of polysaccharide breakdown between the endosperm cell walls of soya beans and maize grain.

The soya bean cotyledon and maize endosperm cell walls were analysed in different gas production experiments and, therefore, cannot be compared statistically. However, when comparing the gas production profiles for maize and soya bean cell walls (Chapters 5, 6 and 7), the fermentation of soya bean cell walls seems to be faster (steeper curves), though starting slower (lag time), than the fermentation of maize cell walls. From the curve fit parameters, the later start of fermentation for soya bean cell walls was not apparent from the half-time of gas production. However, the maximal rate of substrate degradation seemed to be slower for maize cell walls (4.4 - 6.2% h^{-1}) than for soya bean cell walls (9.5 - 10.5% h^{-1}). Furthermore, the extent of fermentation (100 – dry matter in residue) seemed to be slightly higher for soya bean cell walls (82.9 - 89.4%) than for maize cell walls (79.3 - 80.1%). The earlier start of fermentation for maize cell walls may have been caused by adaptation of the microbes from the pig large intestines to maize cell walls. The pig diets were based on maize, whereas no soya bean cell walls were present. Despite the possible adaptation of the inoculum to fermenting maize cell walls, the rate and extent of fermentation still seemed to be lower for maize, than for soya bean cell walls.

ii) Cell wall fractionation and fermentability The extraction of the soya bean and maize endosperm cell wall, using sequential steps with different chemicals, resulted in: polysaccharide fractions which could be solubilized by extraction, and residues of insoluble polysaccharides remaining after each extraction step. For soya bean cell wall, removal of pectin from the cell wall resulted in a shorter half-time of gas production for the residue. The extracted pectin fractions seemed to have a low rate of fermentation, although residues of the chemicals used in the extraction procedures, may have been responsible for this. When following the removal of pectin, hemicellulose was removed from the cell wall, the cellulose-rich residue was less fermentable than that of the original soya bean cell wall. The extracted hemicelluloses, on the other hand, were highly fermentable. For the maize cell walls, the first extraction removed arabinoxylans, which were highly fermentable. As for soya beans, the residues after removal of the initial amounts of arabinoxylans were more fermentable than of the original cell walls. After further removal of polysaccharides, the cellulose-rich residue decreased in fermentability to a level similar to or slightly better fermentable than the original maize cell wall. Thus, for both soya bean and maize, initial removal of polysaccharides from the cell walls increased cell wall fermentability, whereas further removal of polysaccharides decreased the cell wall fermentability (cellulose-rich residue). This decrease was more pronounced for soya bean than for maize cell walls.

The sequence of disappearance of the different sugars was similar for both extracted polysaccharides and insoluble residues. Galactose and arabinose were degraded most rapidly, uronic acids and xylose were degraded at an intermediate rate, and glucose was degraded at the lowest rate. No sugar degradation patterns were analysed for maize cell wall polysaccharide degradation.

iii) Comparison of cell walls The comparison of the fermentability of the cell walls from different sources (Chapter 7) did not yield a clear relationship between cell wall composition and cell wall fermentability. Maize cell walls were compared with other monocotyledons (wheat, rye, and rice) cell walls, whereas the cell walls of soya beans were compared with cell walls from other dicotyledon species (lupins, faba beans, and peas). For the monocotyledon cell walls, a larger particle size decreased the rate of degradation. The slightly higher galactose and uronic acid content of maize cell walls compared to the other monocotyledons, although still low (around 5%), seemed to increase half-time of gas production. For soya bean cell walls, a higher total sugar content seemed to decrease half time of gas production (more rapidly fermentable). However, this effect may have been confounded with the protein content of the dicotyledon cell walls and may suggest an effect of cell wall proteins on fermentation characteristics. No significant effects of any specific cell wall sugar on fermentability were detected.

Cell wall architecture/composition and fermentation

The fermentation of soya bean and maize cell walls, as described in the previous sections, has to be interpreted considering both cell wall composition and architecture. Hatfield (1993) proposed a model for the architecture of the pectin-rich primary cell walls of legumes (Chapter 1, Figure 4A), which may be applicable for the primary cell walls of soya beans cotyledons (soya beans are legumes). In this model, a cellulose-xyloglucan network is embedded in a pectin matrix, which form an open and hydrated structure. Because of this open structure, preferential degradation of polysaccharides would be possible, as can be seen from the preferential removal of pectin-related sugars during fermentation. The pectin molecule itself is degraded from the outside inwards, with the arabinose and galactose side chains being degraded first, after which the pectin backbone of uronic acids is degraded. The decreased fermentability of the extracted pectins suggests that the rapid removal of pectins, during fermentation of the soya bean cell wall, is not because the pectin molecule itself is rapidly fermentable. However, it could be that, because the pectin in the soya cell wall embeds all other polysaccharide, pectin has to be removed first to be able to reach the other cell wall polysaccharides. Furthermore, pectin may influence pore-size (Carpita and Gibeaut, 1993) of the cell wall, which could also have an effect on fermentability (Chesson *et al.*, 1997). The increase in fermentability of the soya cell walls, after removal of the pectins, could then be explained by an increased pore-size, leading to a more open cell wall structure, giving microbes and microbial enzymes a better access to the other cell wall polysaccharides. The decrease in fermentability of the cellulose-rich residue after removal of both pectins and hemicellulose is not surprising, because cellulose from cell walls is a poorly degradable polysaccharide (Weimer, 1996).

Hatfield (1993) (Chapter 1, Figure 4B), also proposed a model for the arabinoxylan-rich primary cell walls of grasses, which may be applicable for the primary cell walls of maize

endosperm (maize is a grass). Unlike the open structure of the legume cell wall, this model shows the cellulose micro-fibrils embedded in another network of arabinoxylan, which is very closely associated with the cellulose network. This suggests a cell wall structure with very tightly associated polysaccharides, from which preferential degradation of polysaccharides cannot occur. Therefore, all sugars from the maize cell wall are degraded at similar rates. In this case, the increased fermentability after removal of arabinoxylans (Chapter 6) would probably be due to destruction of the close association between arabinoxylans, which were solublized, and the other cell wall polysaccharides. The arabinoxylans, which had been solublized from the cell wall, were all highly fermentable, so the poor degradation of arabinoxylans, when present within the cell wall, must be attributed to the tight interactions with the other cell wall polysaccharides.

The difference in cell wall architecture between soya bean (legume) and maize (grass) primary cell walls, as shown in the two models of Hatfield (1993), probably provides an explanation for the more rapid fermentation of the soya cell walls compared to the maize cell walls. The open cell wall structure of soya beans is more rapidly degraded than the very tightly closed cell wall structure of maize. Generally, pectin-rich cell walls are reported to be more fermentable than cell walls low in pectin (Hatfield, 1993). It might be that the presence of pectin in a cell wall indicates a more 'open' structure of the cell wall. This is consistent with the higher degradation rates and higher uronic acids and galactose content of the dicotyledon plants compared to the monocotyledon plants (Chapter 7). It is likely that the more open cell wall structure is responsible for the higher fermentability of pectin-rich cell walls, and not that (water-insoluble) pectin itself is rapidly fermentable (Chapter 5). In terms of fermentability, this would mean that there is an optimal level for the pectin content of cell walls. Up to this level an increase in pectin content creates a more 'open' cell wall structure and increases cell wall fermentability. When this optimal level is reached, further increases in pectin would not increase the 'openness' of the cell wall, and fermentability would remain constant, or possibly even decrease, because of the slow fermentability of the pectin molecule itself (Chapter 5).

Fermentation products

The main products of cell wall fermentation are micro-organisms, gas, and volatile fatty acids. The volatile fatty acids are an important energy source in both ruminants and pigs (France and Siddons, 1993; Mason, 1983). The pattern of production of the three main volatile fatty acids (proportions of acetic, propionic and butyric acids) is influenced by the interaction of micro-organisms (microbial species) and substrate (composition). The composition of the substrate and rate of degradation, which is also influenced by composition, influence the proportions of acetic and propionic acid produced. Therefore, the A/P-ratio (acetic acid/propionic acid) can be used as an indicator for the fermentation process. Generally, rapidly fermentable substrates have a relatively higher propionic acid production, whereas more slowly fermentable and cellulose-rich substrates have an acetic acid directed fermentation. This is caused by differences in intracellular conditions of the microbes (pH, electron donor/acceptor-ratio), which favour the production pathway of propionic acid when fermenting rapidly-fermentable materials (Van Houtert, 1993). The fermentation of pectin is generally accompanied by an acetic acid directed fermentation

(Marounek *et al.*, 1985).

The A/P-ratio for the fermentation of soya bean cell walls in the different experiments, varied from 1.4 to 2.3, though the value of 2.3 was found in only one experiment (Chapter 5). For the other experiments, the A/P-ratio for soya bean cell wall fermentation varied from 1.4 to 1.6. During fermentation, the A/P-ratio for soya bean cell wall fermentation dropped from 2.4 to 1.6 (Chapter 4). The relatively high acetic acid production at the beginning, was probably a result of fermentation of cell wall pectins. For the relatively pectin-rich soya bean cell walls, an A/P-ratio of 1.4 to 1.6 would seem rather low, compared with A/P- ratios of higher than 3.0, found for the fermentation of pectin itself (Marounek *et al.*, 1985). It is possibly that the rapid fermentation of the soya bean cotyledon cell wall resulted in propionic acid production, keeping the A/P-ratio low, despite its high pectin content. The A/P ratio for the fermentation of maize cell walls for the different experiments varied from 1.8 to 2.1. The higher acetic acid production compared to that of soya bean cell walls was probably related to the slightly slower fermentation rate and higher cellulose content of the maize cell walls. In most experiments, the production pattern of volatile fatty acids could be explained by the combination of cell wall composition (more pectin or cellulose; more acetic acid) and fermentation rate (higher fermentation rate, more propionic acid). Therefore, the production pattern of volatile fatty acids is a useful addition to the analysis of fermentation rate and extent, and provides extra insight into the processes of fermentation.

Gas production curve fitting related to cell wall composition and fermentation

When gas production profiles are fitted with a multi-phasic model, the different phases can be considered to represent fermentation of different components of the substrate (Beuvink and Kogut, 1993). This was illustrated in Chapter 4, where the gas production profile for the fermentation of the cell wall fraction of the whole bean (CCW: combined cell walls) could be separated into two phases, representing the fermentation of cotyledon and hull cell walls, respectively. Furthermore, the gas production profile for the cell wall fraction of whole beans could be calculated, fairly accurately, from the gas production profiles of the separated endosperm and cotyledon cell walls. This shows the possibilities of multi-phasic curve fitting, and adding gas production curves, in analysing the fermentability different cell wall types in fractions with cell walls from multiple tissues.

The approach of multi-phasic curve fitting was not only applied to study different cell wall types and gas production phases, but also to analyse the relationship between composition of individual cell wall types and gas production phases. In several of the experiments, the division of gas into two phases seemed to agree well with a division of the cell wall into different polysaccharide fractions. In Chapters 4 and 5, the percentage of gas produced in the first phase for the fermentation of soya bean cotyledon cell walls, agreed well with the percentage of pectin-related sugars (arabinose, galactose, uronic acids) in insoluble cell wall material. As these sugars were fermented more rapidly than other cell wall sugars, it can be argued that the first phase of gas production represents pectin fermentation. This was expected to be also the case in Chapter 7, however, only for two of the four dicotyledon substrates (soya and faba beans), the proportion of gas produced in the first phase was similar to the proportion of pectin-related sugars in the cell

wall. For all substrates, the pectin-related sugars were degraded more rapidly than the other cell wall sugars. The interpretation of sugar degradation results is difficult, because it is not determined to what group of polysaccharides (pectin, hemicellulose, or cellulose) a cell wall sugar belongs to. This could explain why the multi-phasic gas production fitting cannot always be explained by composition of the dicotyledon cell wall (Chapter 7: peas and lupins).

For the maize endosperm cell wall (Chapter 6), the proportion of gas produced in the first phase corresponded with the proportion of arabinoxylan (arabinose and xylose) in the cell wall. However, no sugar degradation patterns were analysed, which made it impossible to verify whether the gas production in the first phase really described arabinoxylan fermentation. In Chapter 7, the division of gas production into two phases did not correspond with a division of the monocotyledon cell wall into arabinoxylan and cellulose. However, this was expected, because the arabinoxylans and cellulose were degraded simultaneously and therefore could not be related to two different gas production phases. In this case, the di-phasic gas production profile is either caused by another unknown cell wall characteristic, or possibly by a change in microbial activity.

In the experiments described, the relationship between gas production phases and cell wall components were consistent enough to conclude that they were related. However, this conclusion is based on a very crude comparison between proportions of gas produced in each phase, cell wall composition and sugar degradation patterns, between which still substantial differences exist. Therefore, at this moment, this technique is only applicable when there are considerable differences in cell wall fractions. These fractions can then roughly be divided into rapidly and slowly fermentable components. Furthermore, one should always carefully consider whether fitting of multiple phases is biologically relevant. Especially, gas production curves with more than two phases may have a significantly better fit, but adding an additional phase may have no biological relevance. Therefore in this thesis, the analysis of the experiments was conducted using no more than two phases.

Cell wall extraction and fractionation

In this thesis, all experiments were based on the extraction procedure for isolating cell wall material from feedstuffs. With this procedure, cell walls are isolated by sequential extraction of the materials with water, to remove water-soluble material; a sodium dodecyl sulphate and dithiothreitol solution to remove protein; and with an α-amylase solution to remove starch. Soya bean materials were also extracted with petroleum-ether, prior to these extractions, to remove fat. After extraction, the remaining materials, i.e. cell walls, were freeze-dried. It was expected that this extraction method would not alter the cell walls that have been isolated. However, in Chapter 7, a contamination of the cell wall material was discovered, which was most likely caused by the use of sodium dodecyl sulphate to remove the protein from the cell wall. In this experiment, no significant effect of the contamination on cell wall fermentation was found, and the effect of the contamination was considered to be negligible. However, from the present experiments, it cannot be concluded that the contamination of the cell wall with a compound, related to sodium dodecyl sulphate, has an effect. For future cell wall fermentation research, it is important to know

whether cell wall extraction procedures using sodium dodecyl sulphate, or in fact any chemical used, have an effect on the fermentation of the isolated cell walls. This more so, because sodium dodecyl sulphate is the detergent used in the isolation of neutral detergent fibre (NDF), and has been previously used to isolate cell walls and analyse these for their fermentability (Wilman *et al.*, 1996). However, Hall *et al.* (1998) and Schofield and Pell (1995) used ammonium sulphate to remove ionically-bound neutral detergent from neutral detergent residues when analysing cell wall fermentation. In this thesis, cell wall isolates have not been treated with ammonium sulphate.

Not only the isolation of the cell wall, but also the fractionation of the cell wall into different polysaccharides, involves the use of different chemicals (Chapters 5 and 6), which can cause contamination of the substrates. For the fractionation of the pectin from the cell wall, the contamination with CDTA, may have had an influence on fermentability of the pectin fraction, as has been discussed (Chapter 5). In fractionation experiments, the main question is to what extent the fermentation characteristics of the individual polysaccharide agree with the fermentation characteristics of that polysaccharide within the cell wall matrix. It has been argued that the fermentation of isolated polysaccharides bears no relation to the fermentation of the polysaccharide in the cell wall (Chesson, 1993). In Chapter 4, the gas production curve of the reconstituted cell wall fraction of the whole bean could be estimated by adding the gas production curve of the cotyledon to the hull cell wall fractions. Therefore, an attempt was made to calculate the gas production profiles of the intact cell wall of soya bean cotyledon and maize endosperm from the fitted gas production curves of the sequential extracts and the final residue. The results of this calculation are shown in Figure 1. For both maize endosperm and soya bean cotyledons, the gas production profiles calculated from the extracts and final residue do not match the gas production profile of the original cell wall. This confirms the statement by Chesson (1993), that the fermentation of an isolated polysaccharide is not related to the fermentation of that polysaccharide within the cell wall. Therefore, the results of Chapters 5 and 6, regarding the fermentability of isolated polysaccharides, have to be interpreted with care.

In Chapters 5 and 6, residues of cell wall material from which a polysaccharide fraction had been removed were also analysed for fermentability. These residues are insoluble and fermentation is, therefore, not influenced by a greater accessibility due to solubility. Comparing the fermentation of the original cell wall and the sequential residues may give insight into the functions of the removed polysaccharide fractions, within a cell wall. However, this does depend on the amount of damage caused to the cell wall structure of the residue, after removal of a certain polysaccharide fraction. Therefore, it is necessary to know what kind of damage is caused by the fractionation process (e.g. only solubilizing a cell wall fraction, breaking hydrogen bonds, or breaking covalent linkages). Thus, the interpretation of the fermentation of fractionated cell wall polysaccharides is fraught with considerable methodological questions, to which most answers are as yet unknown.

Future research

One of the principal questions, regarding fermentation of unlignified cell walls, is whether cell

walls with a different cell wall composition have a different rate and extent of fermentation. The ultimate answer to this problem would be to ferment cell walls with a different composition, but with exactly the same particle size and cell wall thickness. This would be best achieved by grinding cell wall material to a very small size, so that it only consists of particles smaller than one cell wall. Only then, a definite answer can be given concerning the effect of carbohydrate composition on fermentation of unlignified cell walls. The results of this thesis would suggest

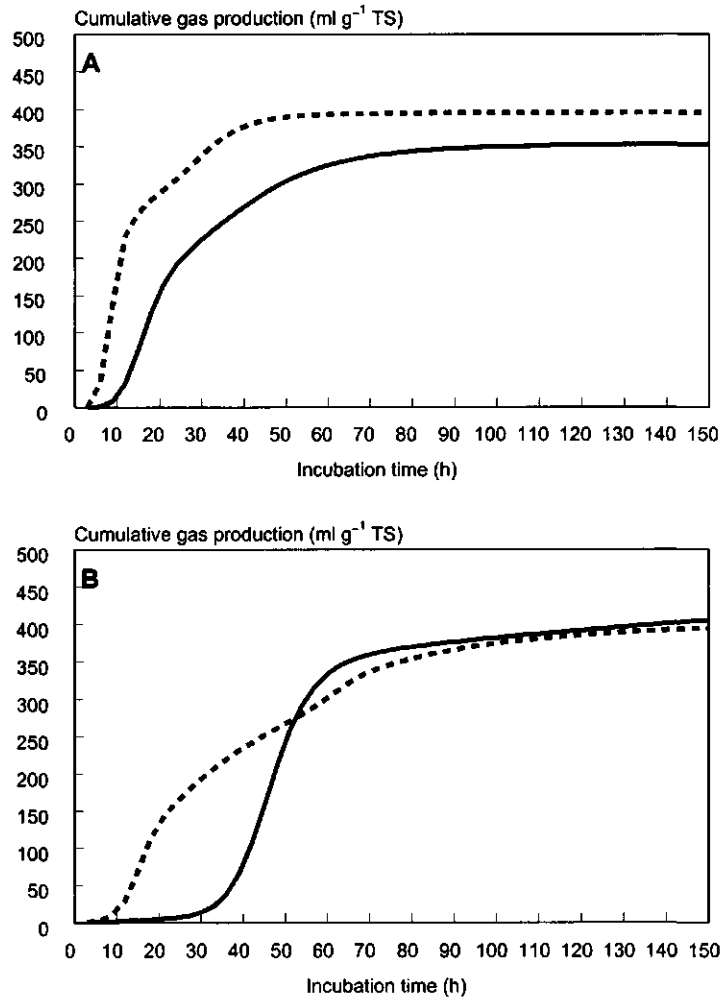

Figure 1: Gas production profiles for the fermentation of (A) maize and (B) soya bean cell walls from Chapters 5 and 6, either measured (solid line) or calculated (dashed line) from the solubilized sequential fractions and the insoluble final residue.

that there are differences between plant groups, such as monocotyledons and dicotyledons. However, it was not possible to conclude, that there were effects of cell wall carbohydrate composition on differences within mono- and dicotyledons.

The cell wall extraction procedure, as used in this thesis, results in contamination of the cell wall material with chemicals used for removal of protein. The effect of this contamination on cell wall fermentation characteristics is unknown. Removal of protein from a feedstuff could also possibly be done using enzymes, instead of sodium dodecyl sulphate. Therefore, it is necessary to compare the fermentation characteristics and possible sources of contamination for cell wall fractions, which have been extracted using either chemical-based or enzyme-based extraction procedures.

Little is known about the effects of extraction procedures of cell walls on cell wall fermentability. It is generally felt that the fermentability of a polysaccharide, which has been removed from the cell wall, bears no relation to the fermentation of that polysaccharide within the cell wall. The effect of removal of a specific polysaccharide fraction from the cell wall on the fermentability of the residue has hardly been investigated. However, the effect may depend on, the type of bonding by which the polysaccharide is anchored within the cell wall matrix (ionic interactions, hydrogen bond, or covalent bonds). More research is needed to reach a consensus on the validity of using cell wall fractionation techniques in studies aiming to explain cell wall fermentation.

Gas production curve fitting can be a useful tool to help untangle the complex relationship between substrates and fermentation characteristics. At present, this technique is sufficiently developed to separate relatively simple substrates into fractions with different fermentation characteristics, as has been shown in this thesis. However, more research into the use of multi-phasic curve fitting to explain gas production profiles of more complex substrates could lead to developing a powerful tool for analysing fermentation characteristics of feedstuffs.

Integrated approach

Integrating the different techniques for cell wall composition and fermentation analyses could be a large step forward in cell wall fermentation research. This thesis has made a beginning with integrating results from techniques such as light microscopy (Chapter 4), particle size analyses (Chapter 4), solid state nuclear magnetic resonance (NMR) (Chapter 4), cell wall fractionation (Chapters 5 and 6), sugar composition analysis (whole thesis), and gas production (whole thesis). This approach integrated different cell wall characteristics, which influence cell wall fermentation. The experiments were conducted with individual cell wall types, from finely ground cell wall materials. The analysis of the interaction of the different tissues present within whole plant materials requires additional information on plant anatomy, and its relationship with cell wall fermentation. Possible techniques to study this relationship are: image analyses (Tidwell *et al.*, 1989) of whole plant tissues and spatial modelling of plant anatomy (Travis *et al.*, 1993; 1997). An extra technique, which could provide data on accessibility of the cell wall to microbial enzymes, is the measurement of porosity, as described by Chesson *et al.* (1997). A possible scheme for a 'totally integrated' approach to analysing the factors important in cell wall fermentability is shown in Table 2.

Table 2: Cell wall characteristics important in cell wall fermentation and the techniques to analyse them.

Plant cell wall characteristic	Analysis				
Plant anatomy				Image analysis and Spatial modelling	
Particle size	Light microscopy	Particle size			
Cell wall thickness					
Cell wall composition/architecture					
Lignin				Lignin	
Polysaccharides	NMR	Porosity	Crystallinity		Fractionation
Monosaccharides				Sugar composition	

Conclusions

The main conclusions to be drawn from this thesis are:

- Pig faeces based inocula are less effective in fermenting cell walls, than inocula based on sheep rumen fluid, this reflects the increased adaptation of the rumen micro-flora of sheep to cell wall fermentation.
- Both particle size and cell wall thickness are important characteristics, which determine the rate of cell wall fermentation.
- The thin primary cell walls of the soya bean cotyledon permit the preferential degradation of arabinose and galactose from the cell wall. However, this is not the case for soya bean hull cell walls nor for maize endosperm cell walls, where no preferential degradation occurs.
- The difference in sugar degradation pattern between soya bean and maize endosperm cell walls (soya beans: preferential degradation; maize: no preferential degradation) is most likely due to the differences in cell wall architecture as described by Hatfield (1993).
- The higher degradation rate of the soya bean and other dicotyledon cell walls, compared with maize and other monocotyledon cell walls, is caused by the more 'open' cell wall structure of legume cell walls (dicotyledons), as described by Hatfield (1993).
- Alkali treatment of cell wall material has different consequences for the fermentability of either soya bean cotyledon or maize endosperm cell walls.
- Soya bean pectin is fermented from the outside inwards, with the arabinose and galactose side chains being degraded first.
- The more rapid fermentation of pectin-rich cell walls does not necessarily mean that pectin itself is easily fermentable, but may predominantly be the result of an 'open' cell wall structure (architecture) of pectin-rich cell walls.

Summary

Introduction

In today's animal husbandry a large proportion of the diets for farm animals comprises of seed residues, which remain following processing for production of human foods. Also, feedstuffs such as soya beans and maize are imported for use in animal diets. General knowledge on the digestibility of the nutrients (e.g., protein and fat) within these residues and feedstuffs is available through the CVB (1999). However, there is little or no detailed knowledge concerning the digestibility of the fibre fraction in particular. This knowledge is vital if better use is to be made of fibre in animal nutrition, because such use could improve welfare and health status of the animals, and possibly effect the efficiency of nutrient use.

In order to gain better insight into the structure and the related functions of cell wall polysaccharides, a project funded by the STW (Dutch Technology Foundation), Gist Brocades (now DSM Gist), and the Commodity Board for Feeding Stuffs (PDV, Productschap voor Diervoeders) was started in 1995, located at four groups from two universities. These were the Departments of Bio-Organic Chemistry and Analytical Mass Spectrometry from the Utrecht University, and the Departments of Feed Chemistry and Animal Nutrition from the Wageningen Agricultural University (nowadays Wageningen University and Research Centre). These four groups worked on the relationship between composition and function of cell wall polysaccharides from of soya beans and maize, two feedstuffs commonly used for animal nutrition in The Netherlands. Maize and soya are representative of the two plant groups: mono- and dicotyledons, respectively, and were considered as model feedstuffs for the cell walls of plants from these two groups.

The research at the Animal Nutrition Group of the Wageningen University focused on the relationship between cell wall composition and fermentation by gastrointestinal micro-organisms from farm animals. The analysis of this relationship was approached from three different angles. Firstly, the fermentation (rate and extent of fermentation, the sugar degradation pattern, and volatile fatty acid production) of soya and maize cell walls was analysed, both *in situ* and *in vitro*. Secondly, isolated cell wall polysaccharide fractions, which had been extracted from soya and maize, and subsequently soya and maize cell walls from which polysaccharide fractions had been extracted, were fermented. Lastly, the fermentation of cell walls from different plants within the same plant group (mono- and dicotyledons) were compared with the fermentation of the soya and maize cell wall so as to detect the effect of differences in carbohydrate composition on cell wall fermentation.

Fermentation of soya and maize cell walls

The fermentation of soya bean meal and maize and their respective cell wall fractions, was analysed using an *in vitro* gas production technique with an inoculum from either sheep rumen fluid or pig faeces (Chapter 2). Cell walls were isolated by sequential extraction with water, a solution of sodium dodecyl sulphate combined with dithiothreitol, and an α-amylase solution. The fermentation of the original maize and soya bean meal was similar for both sheep rumen

fluid and pig faeces. However, pig faecal micro-organisms seemed to be less suited to fermenting the cell wall fractions of soya bean meal and maize than the micro-organisms present in sheep rumen fluid. Light microscopic analysis revealed that the cell walls of both maize and soya bean meal were composed of a variety of cell wall types. Thus the sugar content of the cell wall fraction during fermentation did not represent the sugar degradation pattern of a single cell wall type. This was especially the case for the cell wall fraction of soya bean meal, which was composed of both the cell walls of the hull and the endosperm of the soya bean.

To analyse individual cell wall types, soya beans were separated into hull and cotyledon, from each of which cell wall fermentation was analysed *in vitro* (Chapters 3 and 4). The soya bean cotyledon cell wall was composed of polysaccharides (90%), protein (4 - 10%), and fat (1%), The polysaccharides were composed of cellulose (20%), little hemicellulose (10 - 15%), and a lot of pectin (60 - 70%). The polysaccharide fraction of the hull cell wall was composed mainly of cellulose (up to approximately 60%), and some hemicellulose and pectin. The fermentation of hull cell walls was markedly slower than the fermentation of cotyledon cell walls, which was mainly attributed to a larger particle size and thicker cell walls of the hulls. The sugar degradation pattern was also markedly different for cotyledon and hull cell walls. The cell wall sugars of the cotyledon cell wall were degraded at different rates, with the sugars galactose and arabinose disappearing the fastest, whereas uronic acids and xylose had an intermediate, and glucose the slowest rate of disappearance. For the hull cell wall on the other hand, all cell wall sugars were degraded at a similar rate.

To analyse the single cell wall types for maize, cell walls from a maize milling fraction rich in endosperm, were analysed for their fermentation characteristics (Chapters 6 and 7). The maize endosperm cell wall was composed of polysaccharides (90%), protein (10%), and fat (1%), of which the polysaccharides comprised mainly cellulose (25 - 30%), a lot of hemicellulose (arabinoxylan, 50 - 60%), and only a little pectin (5 - 10%). Similar to the sugar degradation pattern of soya bean hulls, all cell wall sugars were degraded at approximately equal rates. Summarized over all experiments, the cell walls of maize endosperm were less fermentable than soya bean endosperm (cotyledon) cell walls, with both a lower rate (maize: 4.4 - 6.2% h^{-1}; soya beans: 9.5 - 10.5% h^{-1}) and extent (maize: 79.3 - 80.1%; soya bean: 82.9 - 89.4%) of fermentation.

Fermentation of cell wall polysaccharides and partly fractionated cell walls

In order to study the fermentation characteristics of polysaccharide fractions from the maize endosperm and soya cotyledon cell walls, isolated cell wall fractions were subjected to several sequential extraction steps to solubilize different polysaccharide fractions (Chapters 5 and 6). For soya bean cell walls, these sequential extractions were conducted using a solution of 0.05 M CDTA (1,2-cyclohexylene-dinitrotetraacetic acid) combined with 0.05 M NH_4-oxalate (step 1), and solutions of 0.05 M NaOH (step 2), 1 M KOH (step 3), and 4 M KOH (step 4). These extractions were designed to solubilize pectins during the first two steps, pectins and hemicellulose in the third step, and hemicellulose in the fourth step, leaving a cellulose-rich residue. For maize cell walls, the sequential extractions steps were conducted using a saturated BaOH solution (step 1), demineralized water (step 2), 1 M KOH (step 3), and 4 M KOH (step 4). These extraction procedures were designed to solubilize mainly arabinoxylans from the maize cell wall.

The solubilized polysaccharides and the insoluble residues in between each extraction step were used as substrates in an *in vitro* gas production experiment using pig faeces as an inoculum.

For soya beans, the fermentation of the extracted pectins started early, but proceeded very slowly. The pectin fractions contained more glucose than was expected from previous research (Huisman *et al.*, 1998). The origin of this glucose remains unknown, and makes interpretation of the fermentation profile difficult. However, the combination of multi-phasic curve fitting and the sugar degradation pattern, made it possible to separate the fermentation of pectin-related sugars and that of glucose. The fermentability of the insoluble cell wall material after the first and second pectin removal step, was increased relative to the fermentability of the intact cell wall material. On the other hand, the fermentability of the cellulose-rich residue (after all extraction steps) was decreased, relative to the original cell wall material. The sugar degradation patterns of residues and solubilized polysaccharides were all fairly similar, in that galactose and arabinose had the highest, uronic acids and xylose an intermediate, and glucose the lowest rate of disappearance.

For the maize cell wall, the polysaccharide fractions, which had been solubilized, were all rapidly fermented. The fermentability of the insoluble cell wall residues increased for the first three extraction steps (removal of arabinoxylans), whereas the fermentability of the last cellulose-rich residue was similar to that of the intact maize cell wall. Sugar degradation patterns were not analysed in this experiment.

Comparison of different mono- and dicotyledon cell walls

To compare the fermentation characteristics of cell wall types with a similar physical structure (primary cell walls), yet with a different carbohydrate composition, cell wall fractions were isolated from the endosperm of the seeds of four monocotyledon (maize, wheat, rye, and rice) and four dicotyledon (soya bean, lupines, faba beans, and peas) plants (Chapter 7). These cell wall fractions were fermented in an *in vitro* gas production system using pig faeces as an inoculum. The gas production profiles of the monocotyledons were markedly different, with both different rates and extents of gas production. For the dicotyledons, the gas production profiles were fairly similar, with soya bean cell walls being slightly slower fermentable (longer half-time) than the other cell wall types.

For the monocotyledons, the sugar degradation patterns of maize and wheat cell walls was similar, with all sugars disappearing at the same rate. For rye cell walls, glucose disappeared more rapidly than arabinose and xylose, whereas for rice cell walls, arabinose and xylose were degraded more rapidly than glucose. For the dicotyledons, the sugar degradation patterns were similar with regard to the sequence in which the sugars disappeared, which was, as was seen before for soya cell walls, galactose and arabinose first, then uronic acids and xylose, and glucose the last.

Cell wall composition and fermentation

Maize and monocotyledon cell wall fermentation

The relationship between cell wall composition and fermentation for maize cell walls is interpreted using the model for the cell wall architecture of primary grass cell walls of Hatfield

(1993). This model describes the cell wall as a tightly packed network of arabinoxylans and cellulose/xyloglucan. Due to this tight packing of the cell wall polysaccharides, the maize cell wall is less fermentable (both extent and rate) than the soya cell wall. For the fractionation experiment, the cell walls from which arabinoxylans had been removed were fermented more rapidly than the original cell wall. This can be due to destruction of the cell wall matrix by extraction of polysaccharides, which destroys the cell wall architecture (breaking covalent linkages). Therefore, the cell wall matrix becomes more open and more rapidly fermentable. The tight association of arabinoxylans and cellulose is probably also responsible for the similar degradation rates of arabinose, xylose, and glucose for the maize cell walls. The same is true for the other monocotyledon plants, especially wheat. However, for the rye cell wall, glucose was degraded more rapidly than other sugars, which was probably due to the presence of insoluble β-glucans. For rice, the rapid disappearance of arabinose and xylose relative to glucose, was probably due to the very low levels of arabinose and xylose present in rice cell walls, which made an accurate chemical analysis more difficult.

Soya and dicotyledon cell wall fermentation

Hatfield (1993) also presented a model for the architecture of the primary legume cell wall. The legume (e.g. soya bean) primary cell wall is described as an open and hydrated cell wall in which the cellulose-xyloglucan network is embedded in pectin. Most likely this open structure of the cell wall allows for the rapid degradation of the soya cell wall, relative to the maize cell wall. Furthermore, the open structure means that the pectin matrix can be degraded before the cellulose-xyloglucan network. The pectin molecule itself is degraded from the outside inwards, with the arabinose and galactose side-chains being degraded first, and the uronic acid backbone last. In the fractionation experiment, the isolated pectin was only slowly fermentable. Therefore, it can be hypothesized that the preferential removal of pectin from the soya bean cotyledon cell wall during fermentation, is not a result of the rapid degradation of pectin itself, but because pectin is the first polysaccharide micro-organisms encounter when fermenting the soya cell wall. Therefore, pectin has to be removed before micro-organisms can reach the cellulose-xyloglucan network. The more rapid degradation of the soya cotyledon cell wall is unlikely to be due to a rapid degradation of pectin itself, but because pectin creates a more open and hydrated cell wall structure. For the fractionation experiment, the increase in fermentability of the soya cell wall, when pectin was partly removed, can be explained by better accessibility of the other cell wall polysaccharides for microbial enzymes, after pectin (in which these were embedded) has been removed. Removal of the pectins created an even more open cell wall structure. These principles would also apply to the fermentation of the other dicotyledon cell wall types, although the differences in structure and composition may cause slightly different modes of breakdown.

Conclusions

The fermentation of cell wall from feedstuffs by gastrointestinal micro-organisms is influenced by many factors, depending on characteristics from both the microbial population and the cell wall itself. Cell wall characteristics that determine fermentation are physical characteristics such as particle size and cell wall thickness, and chemical characteristics, such as lignification and polysaccharide composition and organization (cell wall architecture). These characteristics differ

between the cell walls from different plant groups, such as the mono- and dicotyledons, but also differ between the different tissues within a plant. Therefore, many different types of cell walls exist, each with possibly different fermentation characteristics.

When formulating animal diets, it should be realized that conventional methods for describing cell wall, such as neutral detergent fibre and crude fibre, do not discriminate between the different types of fibre. This research has shown that different tissue types within the fibre fraction are fermented at different rates, and that even within a cell wall type different cell wall polysaccharides are degraded differently. These differences are related to plant type, and its cell wall composition. Therefore, knowledge of which plant group a feedstuff belongs to, and its cell wall composition, are useful additional information to e.g. neutral detergent fibre data, in formulating diets for farm animals, when focusing on the fibre fraction. The research described in this thesis provides a background for improvement of the use of fibre in animal nutrition.

Samenvatting

Inleiding

In de huidige veehouderij bestaat een groot deel van de rantsoenen voor landbouwhuisdieren uit bijproducten van zaden die gebruikt zijn voor de productie van humane levensmiddelen. Voedermiddelen als soja en maïs worden ook in grote hoeveelheden geïmporteerd, om vervolgens in diervoeders verwerkt te worden. Algemene kennis over de verteerbaarheid van de verschillende nutriënten uit deze voedermiddelen is beschikbaar via het Centraal Veevoeder Bureau (CVB, 1999). Echter, gedetailleerde kennis over de verteerbaarheid van in het bijzonder de vezelfractie is afwezig. Deze kennis is nodig voor een beter gebruik van vezel in de diervoeding, wat de welzijns- en gezondheidsstatus van de dieren en de efficiëntie van het gebruik van nutriënten kan verbeteren.

Om meer inzicht te krijgen in de relatie tussen de structuur en functie van celwandpolysachariden werd er een onderzoeksproject opgezet, gefinancierd door de Stichting Technische Wetenschappen (STW), Gist Brocades (nu DSM Gist) en het Productschap voor Diervoeders (PDV). Dit project werd uitgevoerd door vier groepen verbonden aan twee universiteiten. Deze groepen waren de Vakgroepen Bio-Organische Chemie en Analytische Massa-Spectrometrie van de Universiteit Utrecht en de Leerstoelgroepen Levensmiddelenchemie en Veevoeding van de toenmalige Landbouwuniversiteit Wageningen (nu Wageningen Universiteit en Researchcentrum, WUR). Deze groepen werkten gezamenlijk aan de opheldering van de relatie tussen structuur en functie van de celwandpolysachariden uit soja en maïs, twee voedermiddelen die veel gebruikt worden in veevoeders in Nederland. Maïs en soja zijn vertegenwoordigers van twee plantgroepen, te weten de één- en tweezaadlobbigen, en werden dan ook als modelgrondstoffen voor deze plantgroepen beschouwd.

Het onderzoek bij de Leerstoelgroep Veevoeding van de Wageningen UR richtte zich vooral op de relatie tussen celwandsamenstelling en celwandfermentatie door micro-organismen uit het maagdarmkanaal van landbouwhuisdieren. De analyse van deze relatie werd van drie kanten benaderd. Ten eerste werd de fermentatie (snelheid en volledigheid van fermentatie, suikerafbraakpatroon en vluchtige vetzuurproductie) van soja- en maïscelwanden zowel *in situ* als *in vitro* geanalyseerd. Ten tweede werd de fermentatie van geïsoleerde celwandpolysacharidefracties, die geëxtraheerd waren uit de celwanden van soja en maïs, geanalyseerd. Ten derde werd de fermentatie van verschillende planten uit de groep van éénzaadlobbigen en tweezaadlobbigen vergeleken om het effect van verschillen in celwandsamenstelling op celwandfermentatie te analyseren.

Fermentatie van soja- en maïscelwanden

De fermentatie van sojaschroot, maïs en hun celwandfracties werd geanalyseerd met een *in vitro* gasproductietechniek waarbij een inoculum, gemaakt uit pensvloeistof van schapen of uit varkensmest, werd gebruikt (Hoofdstuk 2). De celwandfractie van sojaschroot en maïs werd geïsoleerd door sequentiële extractie met water, een gecombineerde natriumdodecylsulfaat- en dithiothreitoloplossing, en een α-amylase-oplossing. De fermentatie van originele sojaschroot en

maïs was gelijk bij gebruik van zowel pensvloeistof van schapen als varkensmest, als inoculum. Echter, bij gebruik van het varkensmest-inoculum werd de celwandfractie van zowel soja als maïs minder snel gefermenteerd dan bij het pensvloeistof-inoculum. Dit is een aanwijzing dat de micro-organismen in varkensmest minder goed waren aangepast aan de fermentatie van celwanden, dan de micro-organismen uit pensvloeistof. Door lichtmicroscopie toe te passen werd het duidelijk dat de celwandfracties van zowel soja als maïs uit meerdere celwandtypen bestonden. Het was daarom niet mogelijk het suikerafbraak patroon van individuele celwandtypen van soja en maïs te analyseren. Dit gold met name voor sojaschroot, waarin twee zeer gedefinieerde celwandtypen, één uit de zaadlob en één uit de zaadhuid, voorkwamen.

Om individuele celwandtypen te analyseren werden sojabonen gescheiden in zaadhuid en zaadlob, waarna van beiden de celwandfermentatie werd geanalyseerd (Hoofdstukken 3 en 4). De celwand van de zaadlob bestond uit polysachariden (90%), eiwit (4 - 10 %) en vet (1%). De polysachariden bestonden uit cellulose (20 %), een klein beetje hemicellulose (10 - 15%) en veel pectine (60 - 70%). De polysacharidefractie van de celwand uit de zaadhuid bestond voornamelijk uit cellulose (tot ongeveer 60%) met daarnaast hemicellulose en pectine. De fermentatie van de zaadhuidcelwand was aanzienlijk langzamer dan de fermentatie van de zaadlobcelwand. Dit werd vooral veroorzaakt door de grotere deeltjesgrootte en dikkere celwand van de zaadhuidcelwanden. Ook het patroon van suikerafbraak verschilde tussen zaadhuid- en zaadlobcelwanden. De celwandsuikers van de zaadlobcelwand werden met verschillende snelheden afgebroken, waarbij galactose en arabinose het snelst, uronzuren en xylose met een gemiddelde snelheid en glucose het langzaamst uit de celwand verdwenen. De suikers uit de celwand van de zaadhuid verdwenen met ongeveer gelijke snelheid.

Om voor maïs een zuiver celwandtype in handen te krijgen, werd de celwandfractie uit maïsgries, een fractie vooral bestaande uit maïsendosperm, geïsoleerd en op fermentatie karakteristieken geanalyseerd (Hoofdstukken 6 en 7). De celwand uit maïsendosperm bestond uit polysachariden (90%), eiwit (10%) en vet (1%). De polysachariden bestonden vooral uit cellulose (25 - 30%), veel hemicellulose (arabinoxylanen: 50 - 60%) en weinig pectine (5 - 10%). Zoals bij de afbraak van de sojazaadhuidcelwand, werden ook voor de maïscelwand alle suikers met ongeveer gelijke snelheid afgebroken. Samengevat over alle experimenten waren de celwanden van maïsendosperm minder fermenteerbaar dan de celwanden uit sojazaadlobben. Zowel de snelheid (maïs: 4.4 - 6.2% h^{-1}; sojabonen: 9.5 - 10.5% h^{-1}) als de totale fermentatie (maïs: 79.3 - 80.1%; sojabonen: 82.9 - 89.4%) was lager voor de maïscelwanden.

Fermentatie van celwanden en daaruit geïsoleerde fracties

Geïsoleerde celwanden uit sojazaadlobben en maïsendosperm werden sequentieel geëxtraheerd zodat verschillende polysacharidefracties in oplossing gingen, die vervolgens op hun fermentatiekarakteristieken onderzocht konden worden (Hoofdstukken 5 en 6). Sojacelwanden werden sequentieel geëxtraheerd met een gecombineerde 0.05 M CDTA (1,2-cyclohexyleen-dinitrotetra azijnzuur) en 0.05 M ammonium-oxalaat oplossing (stap 1), met 0.05 M natriumhydroxide (stap 2), 1 M kaliumhydroxide (stap 3) en met 4 M kaliumhydroxide (stap 4). Deze extracties waren opgezet om in de eerste twee stappen pectines te extraheren, in de derde stap zowel pectines als hemicellulose, en vooral hemicellulose in de vierde stap, waarbij een celluloserijk residu overblijft. De sequentiële extractie van de maïscelwand werd uitgevoerd met een verzadigde

bariumhydroxide oplossing (stap 1), gedemineraliseerd water (stap 2), 1 M kaliumhydroxide (stap 3) en 4 M kaliumhydroxide (stap 4). Deze extracties waren vooral bedoeld om arabinoxylanen in oplossing te brengen. De in oplossing gebrachte celwandpolysachariden en de onoplosbare residuen van de extractiestappen, werden als substraten gebruikt in een *in vitro* gasproductie-experiment, waarbij varkensmest als inoculum gebruikt werd.

De fermentatie van de geëxtraheerde pectines begon vroeg, maar was vervolgens erg langzaam. De pectinefractie bevatte veel glucose, meer dan verwacht uit eerder onderzoek. De exacte herkomst van deze glucose is niet bekend, wat de interpretatie van de resultaten bemoeilijkte. Echter, met de combinatie van de gegevens van het suikerafbraakpatroon en de multi-fasische niet-lineaire regressie, was het mogelijk de fermentatie van pectine-gerelateerde suikers en de fermentatie van glucose te scheiden. De residuen na de eerste en tweede extractiestap waarin pectine verwijderd werd, werden meer fermenteerbaar ten opzichte van de originele celwand. De fermenteerbaarheid van het laatste celluloserijke residu was juist weer verminderd ten opzichte van de originele celwand. Het suikerafbraakpatroon van de residuen en extracten was vrijwel gelijk, waarbij galactose en arabinose de hoogste, uronzuren en xylose een gemiddelde en glucose de laagste verdwijningssnelheid had.

De geëxtraheerde celwandpolysachariden uit maïs werden allemaal snel gefermenteerd. De fermenteerbaarheid van de residuen na de eerste drie extractiestappen (verwijdering van arabinoxylanen) nam toe. De fermenteerbaarheid van het laatste celluloserijke residu lag ongeveer op hetzelfde niveau als van de originele celwand. De suikerafbraak patronen werden niet geanalyseerd.

Vergelijking van de verschillende één- en tweezaadlobbige celwanden

Om de fermentatiekarakteristieken van verschillende, doch nauw gerelateerde celwandtypen te onderzoeken, werd de celwandfractie geïsoleerd uit het endosperm van vier éénzaadlobbigen (maïs, tarwe, rogge en rijst) en vier tweezaadlobbigen (sojabonen, lupine, veldbonen en erwten) (Hoofdstuk 7). Deze celwandfracties werden gefermenteerd met een *in vitro* gasproductietechniek, waarbij varkensmest als inoculum werd gebruikt. De gasproductieprofielen waren zeer verschillend voor de éénzaadlobbigen, voor zowel gasproductiesnelheid als totale gasproductie. Voor de tweezaadlobbigen waren de gasproductieprofielen goed vergelijkbaar, alhoewel de sojacelwand iets minder snel gefermenteerd werd (langere halfwaardetijd) dan de andere celwanden. Van de éénzaadlobbigen waren voor maïs en tarwe de afbraakpatronen van de celwandsuikers vergelijkbaar, alle suikers werden met dezelfde snelheid afgebroken. Voor de roggecelwand werd glucose sneller afgebroken dan de andere suikers, terwijl voor rijstcelwanden arabinose en xylose sneller afbraken dan glucose. Voor de tweezaadlobbigen waren de afbraakprofielen van de celwandsuikers vergelijkbaar, voor wat betreft de volgorde waarin de suikers verdwenen. Deze volgorde was, zoals al eerder aangetoond voor sojacelwanden, arabinose en galactose het eerst, dan uronzuren en xylose en glucose als laatste.

Celwandsamenstelling en fermentatie

Fermentatie van maïs- en éénzaadlobbige celwanden

De relatie tussen celwandsamenstelling en celwandfermentatie voor maïscelwanden werd ge-

interpreteerd aan de hand van het model voor de architectuur van een primaire grascelwand van Hatfield (1993). Dit model beschrijft de grascelwand (maïscelwand) als een dicht gepakt netwerk van arabinoxylanen en een cellulose-xyloglucaannetwerk. Door deze dichte pakking van celwandpolysachariden is de maïscelwand minder fermenteerbaar (zowel snelheid als volledigheid) dan de sojacelwand omdat microbiële enzymen moeilijk polysachariden kunnen losmaken uit de celwand. De snellere fermentatie van het celwandmateriaal waaruit arabinoxylanen verwijderd zijn is dan te verklaren doordat extractie de dicht gepakte celwandstructuur verstoort, waarbij covalente verbindingen tussen polysachariden verbroken worden. Hierdoor wordt de celwandmatrix meer open, en meer fermenteerbaar. De dichte pakking van arabinoxylanen en cellulose/xyloglucaan is waarschijnlijk ook verantwoordelijk voor de gelijke afbraaksnelheden van arabinose, xylose en glucose in maïscelwanden, doordat cellulose en arabinoxylanen tegelijkertijd afgebroken moeten worden omdat ze zo nauw geassocieerd zijn. Hetzelfde geldt voor andere éénzaadlobbige planten zoals tarwe. De snellere afbraak van glucose bij rogge werd waarschijnlijk veroorzaakt door de aanwezigheid van onoplosbare β-glucanen, die wel snel fermenteerbaar zijn. De snelle afbraak van arabinose en xylose in rijst werd waarschijnlijk veroorzaakt doordat deze in zeer lage gehaltes in de celwand aanwezig zijn en dus moeilijk nauwkeurig te bepalen zijn.

Fermentatie van soja- en tweezaadlobbige celwanden

Hatfield (1993) geeft ook een model voor de architectuur van de primaire celwand voor leguminosen. De primaire celwand van een leguminose (zoals soja) is een open en gehydrateerde celwand, waarbij een cellulose-xyloglucaannetwerk ligt ingebed in pectine. De open celwandstructuur is waarschijnlijk de oorzaak van de snellere fermentatie van de sojacelwand, ten opzichte van de maïscelwand. Verder zorgt de open celwandstructuur ervoor dat pectine afgebroken wordt, voordat afbraak van het cellulose-xyloglucaannetwerk plaatsvindt. Het pectinemolecule zelf wordt van buiten naar binnen toe afgebroken, waarbij de arabinose- en galactosezijketens eerst en vervolgens de hoofdketen van uronzuur wordt afgebroken. In het experiment waarbij de celwanden in polysachariden werden gefractioneerd, was de fermentatie van geïsoleerd pectine langzaam. Daarom wordt verondersteld dat de snelle verwijdering van pectine uit de celwand gedurende fermentatie, niet veroorzaakt wordt door een hogere fermenteerbaarheid van het pectinemolecule zelf, maar doordat pectine het eerste polysacharide is dat de microben tegenkomen en dus verwijderd moet worden alvorens de microben het cellulose-xyloglucaannetwerk kunnen afbreken. Hieruit volgt dat de snellere fermentatie van de sojazaadlobcelwand niet veroorzaakt wordt door een snelle fermentatie van pectine, maar doordat de aanwezigheid van pectine een open en gehydrateerde celwandstructuur veroorzaakt, waardoor de celwand makkelijker afbreekbaar wordt. Hieruit kan ook de snellere fermentatie van de sojacelwand na verwijdering van een gedeelte van de pectines verklaard worden. Verwijdering van pectine veroorzaakt een nog opener celwandstructuur, omdat de pectine waarin de polysachariden ingebed liggen verwijderd is, zodat deze beter bereikbaar zijn voor de microbiële enzymen. Deze principes gelden ook voor de fermentatie van de andere tweezaadlobbige celwandtypen, alhoewel de verschillen in celwandsamenstelling kleine veranderingen in manier van afbraak zouden kunnen veroorzaken.

Conclusies

De fermentatie van celwanden door micro-organismen uit het maagdarmkanaal wordt door vele factoren beïnvloed, waaronder de eigenschappen van zowel de microflora als de celwand zelf. Celwandeigenschappen die van belang zijn voor de fermentatie zijn vooral fysische factoren deeltjesgrootte en celwanddikte en chemische factoren als lignificatie en de polysaccharide-structuur. Deze eigenschappen verschillen tussen de celwanden van de verschillende plant-groepen als één- en tweezaadlobbigen, maar verschillen ook tussen de verschillende weefsels binnen een plant. Er bestaan dus velerlei celwandtypen, met een verschillende samenstelling en fermentatiekarakteristieken.

Bij het samenstellen van een voeder is het van belang in het oog te houden dat conventionele methodes voor het beschrijven van de celwandfractie, zoals de neutral detergent fibre en acid detergent fibre methode, geen onderscheid maken tussen de verschillende typen vezels. Dit onderzoek heeft juist laten zien dat de verschillend typen celwand met een verschillende snelheid worden afgebroken en dat zelfs binnen een celwandtype polysachariden met een verschillende snelheid worden afgebroken. Aangezien deze verschillen veroorzaakt worden door de soort plant en haar celwandsamenstelling, is kennis over de plantgroep waartoe een voedermiddel behoort, gecombineerd met celwandsamenstelling en -structuur, nuttige aanvullende informatie bij het neutral detergent fibre gehalte, wanneer men zich bij het samenstellen van diervoeders richt op de vezelfractie. Het onderzoek beschreven in dit proefschrift vormt een basis voor verbetering van de benutting van vezel in de diervoeding.

References

Akin, D.E., 1989. Histological and physical factors affecting digestibility of forages. Agron. J. 81: 17-25.

Akin, D.E., 1993. Perspectives of cell wall biodegradation. In: H.G. Jung, D.R. Buxton, R.D. Hatfield and J. Ralph (Editors), Forage Cell Wall Structure and Digestibility. American Society of Agronomy Inc., Crop Science Society of America Inc., Soil Science Society of America Inc., Madison, pp. 73-82.

Aspinall, G.O., Hunt, K. and Morrison, M., 1966. Polysaccharides of soy-beans. Part II. Fractionation of hull cell-wall polysaccharides and the structure of a xylan. J. Chem. Soc.: 1945-1949.

Bach Knudsen, K.E., 1997. Carbohydrate and lignin contents of plant materials used in animal feeding. Anim. Feed Sci. Technol. 67: 319-338.

Bailey, R.W., Monro, J.A., Pickmere, S.E. and Chesson, A., 1976. Herbage hemicellulose and its digestion by the ruminant. In: Carbohydrate Research in Plants and Animals. Miscellaneous papers 12, Landbouwhogeschool Wageningen, Wageningen, pp. 1-16.

Bakker, G.C.M., 1996. Interaction between carbohydrates and fat in pigs. PhD thesis, Wageningen Agricultural University, 193 pp.

Ben-Ghedalia, D. and Miron, J., 1984. The digestion of total and cell wall monosaccharides of alfalfa by sheep. J. Nutr. 144: 880-887.

Bergmans, M.E.F., Beldman, G., Gruppen, H. and Voragen, A.G.J., 1996. Optimization of the selective extraction of glucurono-arabinoxylans from wheat bran: Use of barium and calcium hydroxide solutions at elevated temperatures. J. Cereal Sci. 23: 235-245.

Beuvink, J.M.W. and Kogut, J., 1993. Modelling gas production kinetics of grass silages incubated with buffered rumen fluid. J. of Anim. Sci. 71: 1041-1046.

Blümmel, M., Steingaß, H. and Becker, K., 1997. The relationship between *in vitro* gas production, *in vitro* microbial biomass yield and ^{15}N incorporation and its implications for the prediction of voluntary feed intake of roughages. Brit. J. of Nutr. 77: 911-921.

Blumenkrantz, N. and Asboe-Hansen, G., 1973. New method for quantitative determination of uronic acids. Anal. Biochem. 54: 484-489.

Bourquin, L.D., Titgemeyer, E.C., Garleb, K.A. and Fahey Jr, G.C., 1992. Short-chain fatty acid production and fiber degradation by human colonic bacteria: effects of substrate and cell wall fractionation procedures. J. Nutr. 122: 1508-1520.

Brillouet, J.M. and Riochet, D., 1983. Cell wall polysaccharides and lignin in cotyledons and hulls of seeds from various lupin (*Lupinus* L.) species. J. Sci. Food Agric. 34: 861-868.

Burrit, E.A., Bittner, A.S., Street, J.C. and Anderson, M.J., 1984. Correlations of phenolic acids and xylose content of cell wall with *in vitro* dry matter digestibility of three maturing grasses. J. Dairy Sci. 67: 1209-1213.

Buxton, D.R. and Redfearn, D.D., 1997. Plant limitations to fiber digestion and utilization. J. Nutri. 127: 814S-818S.

Caine, W.R., Tamminga, S., Sauer, W.C., Verstegen, M.W.A. and Schulze, H., 1999. Bacterial

contributions to total and endogeneous recoveries of nitrogen and amino acids in ileal digesta of newly weaned piglets fed protease-treated soybean meal. Livest. Prod. Sci. 57: 147-157.

Carpita, N.C., 1996. Structure and biogenesis of the cell walls of grasses. Annu. Rev. Plant Physiol. Plant Mol. Biol. 47: 445-476.

Carpita, N.C. and Gibeaut, D.M., 1993. Structural models of primary cell walls in flowering plants: Consistency of molecular structure with the physical properties of the walls during growth. Plant J. 3: 1-30.

Cecava, M.J., Merchen, N.R., Gay, L.C. and Berger, L.L., 1990. Composition of ruminal bacteria harvested from steers as influenced by dietary energy level, feeding frequency, and isolation techniques. J. Dairy Sci. 73: 2480-2488.

Chabeauti, E., Noblet, J. and Carre, B., 1991. Digestion of plant cell walls from four different sources in growing pigs. Anim. Feed Sci. Technol. 32: 207-213.

Chesson, A., 1981. Effects of sodium hydroxide on cereal straws in relation to the enhanced degradation of structural polysaccharides by rumen microorganisms. J. Sci. Food Agric. 32: 745-758.

Chesson, A., 1993. Mechanistic model of forage cell wall degradation. In: H.G. Jung, D.R. Buxton, R.D. Hatfield and J. Ralph (Editors), Forage Cell Wall Structure and Digestibility. American Society of Agronomy Inc., Crop Science Society of America Inc., Soil Science Society of America Inc., Madison, pp. 347-376.

Chesson, A. and Monro, J.A., 1982. Legume pectic substances and their degradation in the ovine rumen. J. Sci. Food Agric. 33: 852-859.

Chesson, A., Gordon, A.H. and Lomax, J.A., 1983. Substituent groups linked by alkali-labile bonds to arabinose and xylose residues of legume, grass and cereal straw cell walls and their fate during digestion by rumen microorganisms. J. Sci. Food Agric. 34: 1330-1340.

Chesson, A., Stewart, C.S., Dalgarno, K. and King, T.P., 1986. Degradation of isolated grass mesophyll, epidermis and fibre cell walls in the rumen and by cellulolytic rumen bacteria in axenic culture. J. Appl. Bact. 60: 327-336.

Chesson, A., Gardner, P.T. and Wood, T.J., 1997. Cell wall porosity and available surface area of wheat straw and wheat grain fractions. J. Sci. Food Agric. 75: 289-295.

Cone, J.W., Van Gelder, A.H., Visscher, G.J.W. and Oudshoorn, L., 1996. Influence of rumen fluid and substrate concentration on fermentation kinetics measured with a fully automated time related gas production apparatus. Anim. Feed Sci. Technol. 61: 113-128.

Cone, J.W., Van Gelder, A.H. and Driehuis, F., 1997. Description of gas production profiles with a three-phasic model. Anim. Feed Sci. Technol. 66: 31-45.

Cone, J.W. and Van Gelder, A.H., 1999. Influence of protein fermentation on gas production profiles. Anim. Feed Sci. Technol. 76: 251-264.

Coon, C.N., Leske, K.L., Akavanichan, O. and Cheng, T.K., 1990. Effect of oligosaccharide-free soybean meal on true metabolizable energy and fiber digestion in adult roosters. Poultry Sci. 69: 787-793.

Cornu, A., Besle, J.M., Mosoni, P. and Grenet, E., 1994. Lignin-carbohydrate complexes in forages: structure and consequences in the ruminal degradation of cell-wall carbohydrates. Reprod. Nutr. Dev. 34: 385-398.

Crampton, E.W. and Maynard, L.A., 1938. The relationship of cellulose and lignin content to the

nutritive value of animal feeds. J. Nutri. 15: 383-395.

CVB, 1993. Feed requirements of farm animals and feeding value of feed, shortened table 1993. Centraal Veevoeder Bureau, Lelystad, 64 pp.

CVB, 1999. Veevoedertabel. Centraal Veevoeder Bureau, Lelystad. (in Dutch)

Davies, D.R., Theodorou, M.K., Baughan, J., Brooks, A.E. and Newbold, J.R., 1995. An automated pressure evaluation system (APES) for determining the fermentation characteristics of ruminant feeds. Ann. Zoötechn. 44 (Suppl 1): 36.

Dehority, B.A. and Johnson, R.R., 1961. Effect of particle size upon the *in vitro* cellulose digestibility of forages by rumen bacteria. J. Dairy Sci. 44: 2242-2249.

Dehority, B.A. and Orpin, C.G., 1988. Development of, and natural fluctuations in, rumen microbial populations. In: P.N. Hobson (Ed.), The Rumen Microbial Ecosystem. Elsevier Applied Science, London and New York, pp. 151-183.

DePeters, E.J., Fadel, J.G. and Arosemena, A., 1997. Digestion kinetics of neutral detergent fiber and chemical composition within some selected by-product feedstuffs. Anim. Feed Sci. Technol. 67: 127-140.

Domingo, J.L., Llobet, J.M., Colomina, M.T. and Corbella, J., 1988. The removal of zinc from the mouse by polyamincarboxylic acids (CDTA and DTPA) following semichronic zinc ingestion. Veter. Human Toxic. 30: 524-527.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, D.A. and Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Analyt. Chem. 28: 350-356.

Eastwood, M.A., 1992. The physiological effect of dietary fibre: An update. Annu. Rev. Nutr. 12: 19-35.

Engels, F.M., 1996. Developments in application of light and scanning electron microscopy techniques for cell wall degradation studies. Neth. J. Agric. Sci. 44: 357-373.

Engels, F.M. and Schuurmans, J.L.L., 1992. Relationship between structural development of cell walls and degradation of tissues in maize stems. J. Sci. Food Agric. 59: 45-41.

Englyst, H.N. and Cummings, J.H., 1984. Simplified method for the measurement of total non-starch polysaccharides by gas-liquid chromatography of constituent sugars as alditol acetates. Analyst 109: 937-942.

Ensminger, A.H., Ensminger, M.E. and Konlande, J.E., 1994. Foods and nutrition encyclopedia. 2nd Edition, CRC, Boca Raton, 2415 pp.

Eriksson, I., Andersson, R. and Aman, P., 1997. Extraction of pectic substances from dehulled rapeseed. Carboh. Res. 301: 177-185.

Fisher, D.S., Burns, J.C. and Pond, K.R., 1989. Kinetics of *in vitro* cell wall disappearance and *in vivo* digestion. Agron. J. 81: 25-33.

Fondevilla, M. and Dehority, B.A., 1994. Degradation and utilization of forage hemicellulose by rumen bacteria, singly, in co-culture or added sequentially. J. Appl. Bacteriol. 77: 541-548.

France, F. and Siddons, R.C., 1993. Volatile fatty acid production. In: J.M. Forbes and J. France (Editors), Quantitative Aspects of Ruminant Digestion and Metabolism. CAB International, Wallingford, pp. 107-121.

Fransen, C.T.M., Van Laar, H., Tamminga, S., Kamerling, J.P. and Vliegenthart, J.F.G., 1999. CP-MAS NMR analysis of carbohydrate fractions of soy bean hulls and endosperm. (*in prep.*)

Gaillard, B.D.E., 1962. The relationship between the cell-wall constituents of roughages and the

digestibility of the organic matter. J. Agric. Sci. 59: 369-373.

Gassner, G., 1973. Die starkehaltigen Leguminosensamen und ihre Mahlprodukte. In: Mikroskopische Untersuchung Pflanzlicher Lebensmittel. Gustav Fischer, Stuttgart, pp. 66-84.

Glitsø, L.V. and Bach Knudsen, K.E., 1999. Milling of whole grain rye to obtain fractions with different dietary fibre characteristics. J. Cereal Sci. 29: 89-97.

Glitsø, L.V., Brunsgaard, G., Højsgaard, S., Sandström, B. and Bach Knudsen, K.E., 1998. Intestinal degradation in pigs of rye dietary fibre with different structural characteristics. Brit. J. of Nutr. 80: 457-468.

Goelema, J.O., 1999. Processing of legume seeds: Effects of digestive behaviour in dairy cows. PhD thesis, Wageningen Agricultural University, Wageningen, The Netherlands, 221 pp.

Goelema, J.O., Spreeuwenberg, M.A.M., Hof, G., Van Der Poel, A.F.B. and Tamminga, S., 1998. Effect of pressure toasting on the rumen degradability and intestinal digestibility of whole and broken peas, lupins and faba beans and a mixture of these feedstuffs. Anim. Feed Sci. Technol. 76: 35-50.

Gooneratne, J., Needs, P.W., Ryden, P. and Selvendran, R.R., 1994. Structural features of cell wall polysaccharides from the cotyledons of mung bean *Vigna radiata*. Carboh. Res. 265: 61-77.

Gordon, A.H., Lomax, J.A. and Chesson, A., 1983. Glycosidic linkages of legume, grass and cereal straw cell walls before and after extensive degradation by rumen microorganisms. J. Sci. Food Agric. 34: 1341-1350.

Gordon, A.H., Lomax, J.A., Kalgamo, K. and Chesson, A., 1985. Preparation and composition of mesophyll, epidermis and fibre cell walls from leaves of perennial ryegrass (*Lolium perenne*) and Italian ryegrass (*Lolium multiflorum*). J. Sci. Food Agric. 36: 509-519.

Grabber, J.H. and Jung, G.A., 1991a. *In-vitro* disappearance of carbohydrates, phenolic acids, and lignin form parenchyma and sclerenchyma cell walls isolated from Cocksfoot. J. Sci. Food Agric. 57: 315-323.

Grabber, J.H. and Jung, G.A., 1991b. Isolation of parenchyma and sclerenchyma cell types from the plant parts of grasses. Crop Sci. 31: 838-842.

Groot, J.C.J., Cone, J.W., Williams, B.A., Debersaques, F.M.A. and Lantinga, E.A., 1996. Multiphasic analysis of gas production kinetics for *in vitro* fermentation of ruminant feeds. Anim. Feed Sci. Technol. 64: 77-99.

Groot, J.C.J, Williams, B.A., Oostdam, A.J., Boer, H. and Tamminga, S., 1998. The use of cumulative gas and volatile fatty acid production to predict *in vitro* fermentation kinetics of italian ryegrass leaf cell walls and contents at various time intervals. Brit. J. of Nutr. 79: 519-525.

Gruppen, H., Marseille, J.P., Voragen, A.G.J., Hamer, R.J. and Pilnik, W., 1989. Mild isolation of cell wall material from wheat flour: Composition of fractions obtained with emphasis on non-starch polysaccharides. J. Cereal Sci. 9: 247-260.

Gruppen, H., Hamer, R.J. and Voragen, A.G.J., 1992. Water-unextractable cell wall material from wehat flour. 1. Extraction of polymers with alkali. J. Cereal Sci. 16: 41-51.

Hall, M.B., Pell, A.N. and Chase, L.E., 1998. Characteristics of neutral detergent-soluble fiber fermentation by mixed ruminal microbes. Anim. Feed Sci. Technol. 70: 23-39.

Harris, P.J., Kelderman, M.R., Kendon, M.F. and Mckenzie, R.J., 1997. Monosaccharide

compositions of unlignified cell walls of monocotyledons in relation to the occurrence of wall-bound ferulic acid. Biochem. Syst. Ecol. 25: 167-179.

Hartley, R.D., 1972. p-Coumaric and ferulic acid components of cell walls of ryegrass and their relationships with lignin and digestibility. J. Sci. Food Agric. 23: 1347-1354.

Hatfield, R.D., 1989. Structural polysaccharides in forages and their degradability. Agron. J. 81: 39-46.

Hatfield, R.D., 1993. Cell wall polysaccharide interactions and degradability. In: H.G. Jung, D.R. Buxton, R.D. Hatfield and J. Ralph (Editors), Forage Cell Wall Structure and Digestibility. American Society of Agronomy Inc., Crop Science Society of America Inc., Soil Science Society of America Inc., Madison, pp. 285-313.

Hatfield, R.D. and Weimer, P.J., 1995. Degradation characteristics of isolated and *in situ* cell wall lucerne pectic polysaccharides by mixed ruminal microbes. J. Sci. Food Agric. 69: 185-196.

Houdijk, J.G.M., 1998. Effects of non-digestible oligosaccharides in young pig diets. PhD thesis, Wageningen Agricultural University, 184 pp.

Houdijk, J.G.M., Williams, B.A., Tamminga, S. and Verstegen, M.W.A., 1998. Fructo-oligosaccharides and transgalactooligosaccharides have different *in vitro* fermentation characteristics when being fermented by the porcine intestinal microflora. In: J. Houdijk, Effects of Non-digestible Oligosaccharides in Young Pig Diets. PhD thesis, Wageningen Agricultural University, pp. 81-96.

Howard, B.H., 1961. Fermentation of pectin by rumen bacteria. Proc. Nutr. Soc. 20: xxix-xxx.

Hsu, J.T. and Fahey Jr, G.C., 1990, Effects of centrifugation speed and freezing on composition of ruminal bacterial samples collected form defaunated sheep. J. Dairy Sci. 73: 149-152.

Huisman, M.M.H., Schols, H.A. and Voragen, A.G.J., 1998. Cell wall polysaccharides from soybean meal; isolation and characterization. Carbohydr. Polym. 37: 87-95.

Huisman, M.M.H., Schols, H.A. and Voragen, A.G.J., 1999. Characterisation of glucurono-arabinoxylans from maize cell walls. Carbohydr. Polym. (accepted).

Hungate, R.E., 1966. The rumen and its microbes. Academic Press Inc., New York, 533 pp.

ISO (International Organization for Standardization), 1978. Animal feeding stuffs - Determination of crude ash. ISO 5984, Genève, Switzerland.

ISO (International Organization for Standardization), 1979. Animal feeding stuffs - Determination of nitrogen content and calculation of crude protein content. ISO 5983, Genève, Switzerland.

ISO (International Organization for Standardization), 1983. Animal feeding stuffs - Determination of moisture content. ISO 6496, Genève, Switzerland.

ISO (International Organization for Standardization), 1996. Animal feeding stuffs - Determination of fat content, category B. ISO 6492, Genève, Switzerland.

Jarvis, M.C. and Apperley, D.C., 1990. Direct observation of cell wall structure in living plant tissues by solid-state ^{13}C NMR spectroscopy. Plant Physiol. 92: 61-65.

Johnson, R.R., 1963. *In vitro* rumen fermentation techniques. J. Anim. Sci. 22: 792-800.

Jung, H.G., 1989. Forage lignins and their effects on fiber digestibility. Agron. J. 81: 33-38.

Jung, H.G., 1997. Analysis of forage fiber and cell walls in ruminant nutrition. J. Nutr. 127: 810S-813S.

Jung, H.G. and Deetz, D.A., 1993. Cell wall lignification and degradability. In: H.G. Jung, D.R. Buxton, R.D. Hatfield and J. Ralph (Editors), Forage Cell Wall Structure and Digestibility. American Society of Agronomy Inc., Crop Science Society of America Inc., Soil Science Society of America Inc., Madison, pp. 315-346.

Jung, H.G. and Allen, M.S., 1995. Characteristics of plant cell walls affecting intake and digestibility of forages by ruminants. J. Anim. Sci. 73: 2774-2790.

Keetels, C.J.A.M., 1995. Retrogradation of concentrated starch systems; mechanism and consequences for product properties. PhD thesis, Wageningen Agricultural University, 165 pp.

Kennedy, P.M. and Doyle, P.T., 1993. Particle size reduction by ruminants - Effect of cell wall composition and structure. In: H.G. Jung, D.R. Buxton, R.D. Hatfield and J. Ralph (Editors), Forage Cell Wall Structure and Digestibility. American Society of Agronomy Inc., Crop Science Society of America Inc., Soil Science Society of America Inc., Madison, pp. 499-534.

Kerley, M.S., Fahey Jr, G.C., Gould, J.M. and Iannotti, E.L., 1988. Effects of lignification, cellulose crystallinity and enzyme accessible space on the digestibility of plant cell wall carbohydrates by the ruminant. Food Microstr. 7: 59-65.

Lammers-Wienhoven, S.C.W., Joigt, J., Ram, L., Van Bruchem, J., Ketelaars, J. and Tamminga, S., 1997. Effect of cell walls, dry matter and protein supply on endogenous nitrogen flow in the small intestine of sheep. J. Anim. Physiol. a. Anim. Nutr. 78: 129-140.

Lapierre, C., 1993. Application of new methods for the investigation of lignin structure. In: H.G. Jung, D.R. Buxton, R.D. Hatfield and J. Ralph (Editors), Forage Cell Wall Structure and Digestibility. American Society of Agronomy Inc., Crop Science Society of America Inc., Soil Science Society of America Inc., Madison, pp. 133-166.

Lau, M.M. and Van Soest, P.J., 1981. Titrable groups and soluble phenolic compounds as indicators of the digestibility of chemically treated roughages. Anim. Feed Sci. Technol. 6: 123-131.

Lebet, V., Arrigoni, E. and Amado, R., 1997. Neutral-sugar determination by HPAEC-PAD to characterise polysaccharide degradation by colonic bacteria. Z. Lebensm. Unters. Forsch. 205: 257-261.

Lowe, S.E., Theodorou, M.K., Trinci, A.P.J. and Hespell, R.B., 1985. Growth of anaerobic rumen fungi on defined and semi-defined media lacking rumen fluid. J. of General Microb. 131: 2225-2229.

Macrae, R., Robinson, R.K. and Sadler, M.J., 1993. Encyclopaedia of food science, food technology and nutrition. Academic Press, London, 5365 pp.

Marounek, M., Bartos, S. and Brezina, P., 1985. Factors influencing the production of volatile fatty acids from hemicellulose, pectin and starch by mixed culture of rumen microorganisms. Z. Tierphysiol. Tierernah. 53: 50-58.

Mason, V.C., 1983. Microbial digestion in the hind-gut of the pig. Int. J. Vit. Nutr. Res., Suppl. 25: 27-38.

Massie, H.R., Williams, T.R. and Aiello, V.R., 1983. Influence of dietary cadmium and chelators on the survival of *Drosophila*. Gerontol. 29: 226-232.

McCann, M.C. and Roberts, K., 1991. Architecture of the primary cell wall. In: C.W. Lloyd (Editor), The Cytoskeletal Basis of Plant Growth and Form. Academic Press, London, pp.

109-129.

Mehrez, A.Z. and Ørskov, E.R., 1977. A study of the artificial fibre bag technique for determining the digestibility of feeds in the rumen. J. Agric. Sci. Camb. 88: 645-650.

Menke, K.H. and Ehrensvard, U., 1974. Die Bestimmung der Stoffbilanz und die Berechnung der Stoichiometrie und der Energiebilanz bei der Vergarung von Pansensaft *in vitro*. Zeits. Tierph. Tierernah. Futtermittelk. 34: 24-45.

Mertens, D.R., 1993. Rate and extent of digestion. In: J.M. Forbes and J. France (Editors), Quantitative Aspects of Ruminant Digestion and Metabolism. CAB International, Wallingford, pp. 13-51.

Miron, J. and Ben-Ghedalia, D., 1995. Ozonated cotton stalks as a silage additive: digestion of total and cell wall monosaccharide constituents of lucerne based dairy cows rations. Anim. Feed Sci. Technol. 51: 91-101.

Miron, J., Ben-Ghediala, D. and Solomon, R., 1997. Digestibility by dairy cows of monosaccharide components in diets containing either ground sorghum or sorghum grain treated with sodium hydroxide. J. Dairy Sci. 80: 144-151.

Mort, A.J., Moerschbacher, B.M., Pierce, M.L. and Maness, N.O., 1991. Problems encountered during the extraction, purification, and chromatography of pectic fragments, and some solutions to them. Carbohydr. Res. 215: 219-227.

Mosterd, W., 1992. Vergelijking van *in sacco* bepaalde eiwit-, neutraal detergent fibre- en zetmeelafbraak van voedermiddelen met literatuurwaarden en additiviteit van eiwit- en zetmeelbestendigheid van krachtvoedergrondstoffen en rantsoencomponenten. MSc Thesis Animal Nutrition Department, Wageningen Agricultural University.

Motulsky, H.J. and Ransnas, L.A., 1987. Fitting curves to data using nonlinear regression: A practical and nonmathematical review. Faseb J. 1: 365-374.

NEN, 1988. Cereals, pulses and animal feeding stuffs - Determination of crude fibre content by the abbreviated method. Method 5417. Standards of the Netherlands Normalization Institute, Delft, The Netherlands.

Nevins, D.J., 1993. Analysis of forage cell wall polysaccharides. In: H.G. Jung, D.R. Buxton, R.D. Hatfield and J. Ralph (Editors), Forage Cell Wall Structure and Digestibility. American Society of Agronomy Inc., Crop Science Society of America Inc., Soil Science Society of America Inc., Madison, pp. 105-132.

Newman, R.H., Davies, L.M. and Harris, P.J., 1996. Solid state ^{13}C nuclear magnetic resonance characterization of cellulose in the cell walls of *Arabidopsis thaliana* leaves. Plant Physiol. 111: 475-485.

Oderinde, R.A., Okogun, J.I. and Esuoso, K.A., 1990. The influence of CDTA and DPTA on ethanol production from sugar cane molasses using *Saccharomyces cerevisiae*. Die Nahrung 34: 171-175.

Olubobokun, J.A., Craig, W.M. and Nipper, W.A., 1988, Characteristics of protozoal and bacterial fractions from microorganisms associated with ruminal fluid or particles. J. Anim. Sci. 66: 2701-2710.

Osborne, J.M. and Dehority, B.A., 1989. Synergism in degradation and utilization of intact forage cellulose, hemicellulose, and pectin by three pure cultures of ruminal bacteria. Appl. Environ. Microbiol. 55: 2247-2250.

Pell, A.N. and Schofield, P., 1993a. Microbial adhesion and degradation of plant cell walls. In: H.G. Jung, D.R. Buxton, R.D. Hatfield and J. Ralph (Editors), Forage Cell Wall Structure and Digestibility. American Society of Agronomy Inc., Crop Science Society of America Inc., Soil Science Society of America Inc., Madison, pp. 397-423.

Pell, A.N. and Schofield, P., 1993b. Computerized monitoring of gas production to measure forage digestion *in vitro*. J. Dairy Sci. 76: 1063-1073.

Pond, W.G., 1987. Thoughts on fiber utilization in swine. J. Anim. Sci. 65: 497-499.

Pritchard, P.J., Dryburgh, E.A. and Wilson, B.J., 1973. Carbohydrates of spring and winter field beans (*Vicia faba* L.). J. Sci. Food Agric. 24: 663-668.

Rai, S.N. and Mudgal, V.D., 1996. Effect of alkali and (or) steam treatment of wheat straw or cellulase augmented concentrate mixture on rumen fermentation in goats. Small Rum. Res. 19: 219-225.

Raibaud, P., 1992. Bacterial interactions in the gut. In: R. Fuller (Editor), Probiotics - The Scientific Basis. Chapman Hall, Londen, pp. 9-28.

Ralph, J. and Helm, R.F., 1993. Lignin/hydroxycinnamic acid/polysaccharide complexes: Synthetic models for regiochemical characterisation. In: H.G. Jung, D.R. Buxton, R.D. Hatfield and J. Ralph (Editors), Forage Cell Wall Structure and Digestibility. American Society of Agronomy Inc., Crop Science Society of America Inc., Soil Science Society of America Inc., Madison, pp. 201-246.

Redgewell, R.J. and Selvendran, R.R., 1986. Structural features of cell wall polysaccharides of onion *Allium cepa*. Carbohydr. Res. 157: 183-199.

Rihouey, C., Morvan, C., Borissova, I., Jauneau, A., Demarty, M. and Jarvis, M., 1995. Structural features of CDTA-soluble pectins from flax hypocotyls. Carboh. Poly. 28:159-166.

Robinson, P.H., Fadel, J.G. and Tamminga, S., 1986. Evaluation of mathematical models to describe neutral detergent residue in terms of its susceptibility to degradation in the rumen. Anim. Feed Sci. Technol. 15: 249-271.

Robles, A.Y., Belyea, R.L., Martz, F.A. and Weiss, M.F., 1980. Effect of particle size upon digestible cell wall and rate of *in vitro* digestion of alfalfa and orchardgrass forages. J. of Anim. Sci. 51: 783-790.

Sakata, T., Adachi, M., Hashida, M., Sato, N. and Kojima, T., 1995. Effect of n-butyric acid on epithelial cell proliferation of pig colonic mucose in short-term culture. Dtsch. Tierärztl. Wschr. 102: 163-164.

SAS/STAT$^{(R)}$ Users Guide, 1989. Version 6, fourth edition, Vol. 2. SAS Institute Inc., Cary, N.C., 846 pp.

Schofield, P., Pitt, R.E. and Pell, A.N., 1994. Kinetics of fiber digestion from *in vitro* gas production. J. Anim. Sci. 72: 2980-2991.

Schofield, P. and Pell, A.N., 1995. Measurement and kinetic analysis of the neutral detergent-soluble carbohydrate fraction of legumes and grasses. J. Anim. Sci. 73: 3455-3463.

Schols, H.A., 1995. Structural characterization of pectic hairy regions isolated from apple pectin. PhD thesis, Wageningen Agricultural University, 157 pp.

Schols, H.A., Lucas-Lokhorst, G. and Voragen, A.G.J., 1993. Isolation and characterization of cell wall polysaccharides from soy-beans. Carbohydr. Netherl. 9: 7-10.

Schooneveld-Bergmans, M.E.F., 1997. Wheat glucuronoarabinoxylans, biochemical and

physical aspects. PhD thesis, Wageningen Agricultural University, 125 pp.

Selvendran, R.R., 1983. The chemistry of plant cell walls. In: G.C. Birch and K.J. Parker (Editors), Dietary Fibre. Applied Science Publishers, Barking, pp. 95-147.

Shulze, H., Van Leeuwen, P., Verstegen, M.W.A., Huisman, J., Souffrant, W.B. and Ahrens, F., 1994. Effect of level of dietary neutral detergent fiber on ileal apparent digestibility and ileal nitrogen losses in pigs. J. Anim. Sci. 72: 2362-2368.

Smith, J.G. and German, J.B., 1995. Molecular and genetic effects of dietary derived butyric acid. Food Technol. 49: 87-90.

Spagnuolo, M., Crecchio, C., Pizzigallo, M.D.R. and Ruggiero, P., 1997. Synergistic effects of cellulolytic and pectinolytic enzymes in degrading sugar beet pulp. Biores. Technol. 60: 215-222.

Steward, C.S. and Bryant, M.P., 1988. The rumen bacteria. In: P.N. Hobson (Editor), The Rumen Microbial Ecosystem. Elsevier Applied Science, London and New York, pp. 21-75.

Sunvold, G.D., Hussein, H.S., Fahey Jr, G.C., Merchen, N.R. and Reinhart, G.A., 1995. *In vitro* fermentation of cellulose, beet pulp, citrus pulp, and citrus pectin using fecal inoculum from cats, dogs, horses, humans, and pigs and fuminal fluid from cattle. J. Anim. Sci. 73: 3639-3648.

Sutton, A.L. and Patterson, J.A., 1996. Effects of dietary carbohydrates and organic acid additions on pathogenic *E. coli* and other microorganisms in the weanling pig. Proceedings of the 5th international symposium on animal nutrition, Kaposvár, Hungary, pp. 31- 61.

Tamminga, S., 1993. Influence of feeding mangement on ruminant fiber digestibility. In: H.G. Jung, D.R. Buxton, R.D. Hatfield and J. Ralph (Editors), Forage Cell Wall Structure and Digestibility. American Society of Agronomy Inc., Crop Science Society of America Inc., Soil Science Society of America Inc., Madison, pp. 571-602.

Theander, O. and Aman, P., 1977. The chemistry of fibre components of forage and their determination. In: Quality of Forage. Proceedings of the NJF, Roughage Symposium, Uppsala, pp. 1-26.

Theander, O. and Westerlund, E., 1993. Quantitative analysis of cell wall components. In: H.G. Jung, D.R. Buxton, R.D. Hatfield and J. Ralph (Editors), Forage Cell Wall Structure and Digestibility. American Society of Agronomy Inc., Crop Science Society of America Inc., Soil Science Society of America Inc., Madison, pp. 83-104.

Theodorou, M.K., Williams, B.A., Dhanoa, M.S., McAllan, A.B. and France, J., 1994. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. Anim. Feed Sci. Technol. 48: 185-197.

Tilley, J.M.A. and Terry, R.A., 1963. A two stage technique for the *in vitro* digestion of forage crops. J. Agric. Sci. Camb. 118: 1-8.

Titgemeyer, E.C., Cameron, M.G., Bourquin, L.D. and Fahey Jr, G.C., 1991. Digestion of cell wall components by dairy heifers fed diets based on alfalfa and chemically treated oat hulls. J. Dairy Sci. 74: 1026-1037.

Tidwell, E.K., Johnson, K.D., Bracker, C.E., Patterson, J.A. and Cherney, J.A., 1989. Plant tissue degradation measurement using image analysis. Agron. J. 81: 837-840.

Travis, A.J., Murison, S.D. and Chesson, A., 1993. Estimation of plant cell wall thickness and cell size by image skeletonization. J. Agric. Sci. Camb. 120: 279-287.

Travis, A.J., Murison, S.D., Perry, P. and Chesson, A., 1997. Measurement of cell wall volume using confocal microscopy and its application to studies of forage degradation. Ann. Bot. 80: 1-11.

Umadevi Sajjan, S. and Wankhede, D.B., 1981. Carbohydrate composition of winged bean (*Psophocarpus tetragonolobus*). J. Food Sci. 46: 601-602.

Van de Vis, J.W., 1994. Characterization and mode of action of enzymes degrading galactan structures of arabinogalactans. PhD thesis, Wageningen Agricultural University, 161 pp.

Van Dijk, L., 1996. Karakterisering van arabinoxylanen uit mais. MSc thesis, Food Chemistry Department, Wageningen Agricultural University.

Van Houtert, M.F.J., 1993. The production and metabolism of volatile fatty acids by ruminants fed roughages: A review. Anim. Feed Sci. Technol. 43: 189-225.

Van Laar, H., Tamminga, S., Williams, B.A., Verstegen, M.W.A. and Engels, F.M., 1999. Fermentation characteristics of cell-wall sugars from soya bean meal, and from separated endosperm and hulls of soya beans. Anim. Feed Sci. Technol. 79: 179-193. (Chapter 3)

Van Laar, H., Tamminga, S., Williams, B.A. and Verstegen, M.W.A., 2000a. *In vitro* fermentation characteristics of soya bean meal and maize, and their cell wall fractions, using pig faeces and sheep rumen fluid. Submitted to Animal Feed Science and Technology. (Chapter 2)

Van Laar, H., Fransen, C.T.M., Tamminga, S., Williams, B.A., Boer, H. and Verstegen, M.W.A., 2000b. An integrated approach to analysing fermentation of the cell walls from hulls and endosperm of soya beans. (*in prep.*, Chapter 4)

Van Laar, H., Tamminga, S., Williams, B.A., Verstegen, M.W.A. and Schols, H.A., 2000c. Fermentation characteristics of polysaccharide fractions extracted from the cell walls of soya bean cotyledons. Journal of the Science of Food and Agriculture (accepted). (Chapter 5)

Van Laar, H., Tamminga, S., Williams, B.A., Verstegen, M.W.A. and Schols, H.A., 2000d. *In vitro* fermentation characteristics of polysaccharide fractions extracted from the cell walls of maize endosperm. Submitted to Journal of the Science of Food and Agriculture. (Chapter 6)

Van Laar, H., Tamminga, S., Williams, B.A. and Verstegen, M.W.A., 2000e. Fermentation of the endosperm cell walls of monocotyledon and dicotyledon plant species: The relationship between cell wall characteristics and fermentability. Submitted to Animal Feed Science and Technology. (Chapter 7)

Van Soest, P.J., 1973. Collaborative study of acid-detergent fibre and lignin. J. Assoc. Offic. Anal. Chem. 56: 781-784.

Van Soest, P.J., 1982. Nutritional exology of the ruminant. Cornell University Press, Ithaca, 476 pp.

Van Soest, P.J. and Robertson, J.B., 1977. What is fibre and fibre in food? Nutri. Rev. 35: 12-22.

Van Soest, P.J., Robertson, J.B. and Lewis, B.A., 1991. Methods for dietary fibre, neutral detergent fibre, and non-starch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74: 3583-3597.

Van Straalen, W.M. and Tamminga, S., 1990. Protein degradation of ruminant diets. In: J. Wiseman and D.J.A. Cole (Editors), Feedstuff Evaluation. Butterworths, London, pp. 57-72.

Varga, G.A. and Kolver, E.S., 1997. Microbial and animal limitations to fiber digestion and utilization. J. Nutri. 127: 819S-823S.

Verbruggen, M.A., 1996. Glucuronoarabinoxylans from sorghum grain. PhD thesis, Wageningen

Agricultural University, 131 pp.

Verbruggen, M.A., Beldman, G., Voragen, A.G.J. and Hollemans, M., 1993. Water-unextractable cell wall material from sorghum: Isolation and characterization. J. Cereal Sci. 17: 71-82.

Verbruggen, M.A., Beldman, G. and Voragen, A.G.J., 1995. The selective extraction of glucuronoarabino-xylans from sorghum endoesperm cell walls using barium and potassium hydroxide solutions. J. Cereal Sci. 21: 271-282.

Weightman, R.M., Renard, C.M.G.C., Gallant, D.J. and Thibault, J.-F., 1995. Structure and properties of the polysaccharides from pea hulls. II. Modification of the composition and physico-chemical properties of pea hulls by chemical extraction of the constituent polysaccharides. Carb. Polym. 26: 121-128.

Weimer, P.J., 1996. Why don't ruminal bacteria digest cellulose faster? J. Dairy Sci. 79: 1496-1502.

Whistler, R.L. and Saarnio, J., 1957. Galactomannan from soy bean hulls. J. Am. Chem. Soc. 79: 6055-6056.

Williams, B.A., Chuzaemi, S., Soebarinoto, Van Bruchem, J., Boer, H. and Tamminga, S., 1996. A comparison of ten rice-straw varieties grown at two different altitudes during a wet and a dry season, using the *in vitro* cumulative gas production technique. Anim. Feed Sci. Technol. 57: 183-194.

Wilman, D., Foulkes, G.R. and Givens, D.I., 1996. A comparison of four methods of estimating the rate and extent of cell wall degradation in grass silages. Anim. Feed Sci. Technol. 63: 99-109.

Wilson, J.R., 1993. Organisation of forage plant tissues. In: H.G. Jung, D.R. Buxton, R.D. Hatfield and J. Ralph (Editors), Forage Cell Wall Structure and Digestibility. American Society of Agronomy Inc., Crop Science Society of America Inc., Soil Science Society of America Inc., Madison, pp. 1-32.

Wilson, J.R. and Mertens, D.R., 1995. Cell wall accessibility and cell structure limitations to microbial digestion of forage. Crop Sci. 35: 251-259.

Zebrowska, T. and Low, A.G., 1987. The influence of diets based on whole wheat, wheat flour and wheat bran on exocrine pancreatic secretion in pigs. J. Nutri. 117: 1212-1216.

Publications of the author

Zhou, W., Kornegay, E.T., Van Laar, H., Swinkels, J.W.G.M., Wong, E.A. and Lindemann M.D., 1994. The role of feed consumption and feed efficiency in copper-stimulated growth. J. of Animal Science 72(9): 2385-2394.

Bikker, P., Karabinas, V., Van Laar, H., Verstegen, M.W.A. and Campbell, R.G., 1995. The effect of body-weight on the marginal efficiency of nitrogen utilization in pigs. Manipulating pig production V. Proceedings of the biennial conference of the Australasian Pig Science Association (A.P.S.A.), Australasian Pig Science Association Werribee, p. 187.

Van Laar, H., Voigt, J., Lammers-Wienhoven, S.C.W. and Van Bruchem, J., 1995. Lichaamseiwitsynthese bij schapen, het effect van rantsoen en methode van ^{15}N labelling, Proceedings 20^{e} Studiedag Nederlandstalige Voedingsonderzoekers, pp. 58-59.

Van Laar, H. and Tamminga, S., 1996. Pens-afbraakkarakteristieken van suikers in sojaschroot en sojacelwanden, Proceedings 21^{e} Studiedag Nederlandstalige Voedingsonderzoekers, pp. 34-35.

Van Laar, H., Tamminga, S. and Abrahams, H., 1997. Pens-afbraakkarakteristieken van suikers in getoaste - en ongetoaste sojaschroot en sojacelwanden. Proceedings 22^{e} Studiedag Nederlandstalige Voedingsonderzoekers, pp. 26-27.

Van Laar, H., Tamminga, S. and Williams, B.A., 1997. *In vitro* digestibility characteristics of soya and maize endosperm cell walls. Proc. of the British Society of Animal Science, p. 200.

Van Laar, H. and Tamminga, S., 1997. Rate of cell wall sugar degradation of soya bean meal and corn cell walls. In: Van Arendonk, J.A.M., Ducrocq, V., Van der Honing, Y., Madec, F., Bonneau, M., Pollot, G.E., Peters, K., Fernandez, J., Bruns, E.W., Potucek, E. and T.W. Beeksma-Rotmensen (Eds), Book of Abstracts, 48th annual EAAP meeting, p. 117.

Van Laar, H. and Tamminga, S., 1998. Why plant anatomy is important in analyzing fibre digestibility. Annual Report of the Wageningen Institute of Animal Sciences (WIAS) 1997, pp. 41-43.

Van Laar, H., Tamminga, S. and Williams, B.A., 1998. *In vitro* afbraak karakteristieken van suikers uit hul en endosperm celwanden van de sojaboon. Proceedings 23e Studiedag Nederlandstalige Voedingsonderzoekers, pp. 23-24.

Van Laar, H., Tamminga, S., Williams, B.A., Diekema, B., Burgers, W., Groeneveld, D. and Hoogendoorn, M., 1998. *In vitro* fermentation characteristics of soya bean cell walls separated from hulls and endosperm. Proc. of the British Society of Animal Science, p. 71.

Van Laar, H., Tamminga, S. and Williams, B.A., 1999. *In vitro* afbraak karakteristieken van pectine uit de soja celwand. Proc. 24^{e} Studiedag Nederlandstalige Voedingsonderzoekers, pp. 42-43.

Van Laar, H., Tamminga, S., Williams, B.A., Verstegen, M.W.A. and Engels, F.M., 1999. Fermentation characteristics of cell-wall sugars from soya bean meal, and from separated endosperm and hulls of soya beans. Anim. Feed Sci. Technol. 79: 179-193.

Fransen, C.T.M., Van Laar, H., Tammiga, S., Kamerling, J.P. and Vliegenthart, J.F.G., 2000. CP-MAS NMR analysis of carbohydrate fractions of soy bean hulls and endosperm. (*in prep.*)

Van Laar, H., Tamminga, S., Williams, B.A. and Verstegen, M.W.A., 2000a. *In vitro* fermentation characteristics of soya bean meal and maize, and their cell wall fractions, using pig faeces and sheep rumen fluid. Submitted to Animal Feed Science and Technology.

Van Laar, H., Fransen, C.T.M., Tamminga, S., Williams, B.A., Boer, H. and Verstegen, M.W.A., 2000b. An integrated approach to analysing fermentation of the cell walls from hulls and endosperm of soya beans. (*in prep.*)

Van Laar, H., Tamminga, S., Williams, B.A., Verstegen, M.W.A. and Schols, H.A., 2000c. Fermentation characteristics of polysaccharide fractions extracted from the cell walls of soya bean cotyledons. Journal of the Science of Food and Agriculture (accepted).

Van Laar, H., Tamminga, S., Williams, B.A., Verstegen, M.W.A. and Schols, H.A., 2000d. *In vitro* fermentation characteristics of polysaccharide fractions extracted from the cell walls of maize endosperm. Submitted to Journal of the Science of Food and Agriculture.

Van Laar, H., Tamminga, S., Williams, B.A. and Verstegen, M.W.A., 2000e. Fermentation of the endosperm cell walls of monocotyledon and dicotyledon plant species: The relationship between cell wall characteristics and fermentability. Submitted to Animal Feed Science and Technology.

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Curriculum vitae

Harmen van Laar was born on the 22nd of August 1971, in Ede. He spent his childhood on the farm of his parents in Wekerom. After elementary school (Johannes Bogerman school) in Wekerom, he went to the secondary school (Christelijke Streeklyceum) in Ede. Besides school, he spent his time with small-scale rabbit farming and air rifle shooting at the shooting club 'Soranus'. In 1989, he started with the study of Animal Science at the Wageningen Agricultural University. During this study, he stayed four months at the Virginia Polytechnic Institute and State University in the United States, and six weeks at the 'Oskar Kellner' Institute in Rostock, Germany. Besides his classes, he started at the Wageningen Student Budo Club 'De Grondleggers' a study of budo, especially jiu-jitsu. In January 1995, he graduated *cum laude* with thesis subjects on Animal Nutrition, Business Management, and Human and Animal Physiology. After graduation, he started as a PhD-student on a project financed by the Dutch Foundation for Technological Research (STW) at the former Department for Animal Nutrition (now Animal Nutrition Group) of the Wageningen Agricultural University (now Wageningen University and Research Centre). Here, he conducted research on the relationship between composition and fermentation of soya bean and maize cell walls, which resulted in this thesis. He was also active as a member of the educational committee of the Graduate School Wageningen Institute of Animal Sciences (WIAS) and the PhD-student council. In 1998, he was awarded a Fulbright Fellowship to conduct research at the University of Minnesota in the United States for six months. During the PhD-period, also his study of jiu-jitsu progressed, by attaining the rank of 1st dan in March 1996 and that of 2nd dan in November 1997. He stayed at the Animal Nutrition Group until September 1st 1999. Since September 1999, he has been working at the Institute for Animal Nutrition 'De Schothorst' in Lelystad, where he is working on the further development of the gas production technique and its application in feed evaluation.

Levensloop

Harmen van Laar werd op 22 augustus 1971 te Ede geboren. Hij groeide op in Wekerom, op het boerenbedrijf van zijn ouders. Na de lagere school (Johannes Bogerman School) in Wekerom, ging hij naar het toenmalige Christelijke Streeklyceum te Ede. Naast school, hield hij zich in deze tijd bezig met onder meer de konijnenhouderij op kleine schaal en met sportschieten bij de schietvereniging 'Soranus'. In 1989 begon hij met de studie Zoötechniek aan de Landbouwuniversiteit in Wageningen. Onderdeel van zijn studie waren een verblijf van vier maanden aan de Virginia Polytechnic Institute and State University in de Verenigde Staten van Amerika, en van zes weken in Rostock aan het 'Oskar Kellner' Instituut in Duitsland. Naast zijn studie begon hij bij de Wageningse Studenten Budo Vereniging 'De Grondleggers' aan een studie van het budo, in het bijzonder het jiu jitsu. In januari 1995 studeerde hij met lof af, met de afstudeervakken Veevoeding, Bedrijfskunde, en Fysiologie van Mens en Dier. Gelijk na het afstuderen begon hij als onderzoeker in opleiding (OIO) op een door de Stichting Technische Wetenschappen (STW) gefinancierd project bij de toenmalige Vakgroep Veevoeding (nu Leerstoelgroep Veevoeding) van de Landbouwuniversiteit Wageningen (nu Wageningen Universiteit en Researchcentrum). Het onderzoek richtte zich op de relatie tussen de samenstelling en de fermentatie van soja- en maïscelwanden, hetwelk in dit proefschrift beschreven staat. Naast zijn onderzoek was hij lid van de onderwijscommissie van de onderzoekschool WIAS en de AIO-raad. In 1998 kreeg hij een Fulbrightbeurs om voor zes maanden onderzoek te doen aan de University of Minnesota in de Verenigde Staten van Amerika. Tijdens zijn OIO-schap vorderde ook zijn studie van het jiu jitsu, hij behaalde zijn 1^{e} dan in maart 1996 en zijn 2^{e} dan in november 1997. Hij was tot 1 september 1999 werkzaam bij de Leerstoelgroep Veevoeding. Sinds 1 september 1999 is hij werkzaam bij de Stichting Instituut voor de Veevoeding 'De Schothorst' in Lelystad, waar hij zich bezig houdt met het verder ontwikkelen van de gasproductiemethode en toepassing daarvan in de voederwaardering.