Fiber fermentation in pigs and poultry

Sonja de Vries

Sense and nonsense of its manipulation

FIBER FERMENTATION IN PIGS AND POULTRY
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FIBER FERMENTATION IN PIGS AND POULTRY
Sense and nonsense of its manipulation

Sonja de Vries

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PROPOSITIONS

1. Recalcitrant fiber structures that resist fermentation in the pig are too recalcitrant to be targeted by common feed processing technologies.
   (this thesis)

2. The assumption of additivity in feed formulation systems is limiting the optimal use of fiber-rich feedstuffs.
   (this thesis)

3. Considering the similarities in composition and glycemic index between spelt (*Triticum spelta*) and common wheat (*Triticum aestivum*) (Ranhotra et al. 1996; Marques et al. 2007), the recent popularity of spelt indicates that in society, marketing often overshadows scientific evidence.

4. Although barefoot running might reduce collision forces on the lower limbs (Lieberman et al 2010), abandoning running shoes will increase sole damage and overuse injuries, both in human and horses.

5. From a chemist's perspective, low carb-high fiber products are a fallacy.

6. People should be fed like a pig instead of planning their meals based on calorie intake, when weight control is the objective.

7. Those that are stopped by a glass ceiling lack the motivation or capacity to pull it down.

Propositions belonging to the doctoral thesis:
"Fiber fermentation in pigs and poultry - Sense and nonsense of its manipulation "

Sonja de Vries
Wageningen, 20 June 2014

Efficiënter veevoer

Veel goedkope grondstoffen voor diervoeders bevatten vezels die voor varkens en kippen slecht verteerbaar zijn. Ook hun darmflora kan de vezels niet aan. Daardoor gaat een deel van de energie verloren. Onderzoekers van Wageningen UR testen in samenwerking met bedrijven innovatieve technologieën om bijproducten van de raapzaad- en maïsbewerking te benutten. Door deze voor te bewerken kunnen de darmbacteriën van de dieren de vezels gemakkelijker afbreken. Zo halen ze meer energie uit de voeding en is minder akkergrond voor de veevoerproductie nodig. Hiermee werkt Wageningen UR aan de kwaliteit van leven.

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VOORWOORD - PREFACE

Men zegt dat een proefschrift in ons vakgebied door slechts een handjevol mensen van A tot Z gelezen wordt. Dat betekent dat de overige 390 exemplaren van deze oplage na een blik op de kaft, wat geblader en het lezen van stukjes als: het *Voorwoord*, de *Acknowledgements*, het *Curriculum Vitae*, en - met een beetje geluk - de *General Discussion* in de kast belandt. De aandacht die het daarna ontvangt is waarschijnlijk meer gerelateerd aan die kietelende titel, het leuke plaatje op de voorkant (Inge dank daarvoor!) en de naam (-sbekendheid) van de auteur, dan aan de kwaliteit van de inhoud. Als je het zo bekijkt is het wellicht niet de beste strategie om ruim 4 jaar te spenderen aan de inhoud van het proefschrift waarna er nog slechts luttele uurtjes over zijn om de meest gelezen en bekeken fragmenten te componeren. Maar ja, als onderzoeker houd je nu eenmaal van die inhoud. Bovendien hoop ik stiekem toch dat de afzonderlijke publicaties die voortkomen uit dit proefschrift (Hoofdstuk 3 tot en met 9) enige interesse ontvangen uit de wetenschappelijke wereld.

Maar wat houdt die inhoud waar ik al die jaren aan gewerkt heb dan in? Voor veel mensen in mijn omgeving is het toch enigszins in het ongewisse gebleven waar ik me nu precies mee bezig hield. Iets met onderzoek, varkens, kuikens, technologieën, voer en mest verzamelen. Het resultaat is dit boekje. Dat maakt het wellicht niet duidelijker maar wel tastbaar. Bij het bedenken van de titel hebben vele opties de revue gepasseerd: *A not-so-comprehensive-but–far-from-complete researchers guide through the fiber Galaxy, Fiber for dummies* of *What's eating de Vries et al.* Uiteindelijk is het gebleven bij: *Fiber fermentation in pigs and poultry – Sense and nonsense of its manipulation*. Misschien niet de meest pakkende titel maar wel redelijk zelfverklarend. Het gaat dus over vezels en hoe deze worden afgebroken in het dier. En ook over technologieën die de afbreekbaarheid van deze vezels beïnvloeden…. of niet.

Bij het afstuderen van mijn MSc ontving ik twee boekjes: "*Krabbelschrift: voor iedereen die zich even verveelt op kantoor*" en "*Promoveren, een wegwijzer voor de beginnende wetenschapper*". Nu, 4 jaar-en-een-beetje later, kan het gebruik van deze boekjes kenmerkend worden gevonden voor mijn AIO-periode. Boekje 1 heb ik nog regelmatig onder ogen bij het openen van mijn bureaula, maar van de 48 bladzijden zijn er nog 47 onbekladderd. De momenten dat ik mij heb verveeld zijn dus op één hand te tellen. Bij het openen van boekje 2 moest ik als beginnend AIO toch even slikken. Hoofdstukken als '*Begeleiders: Geen tijd, niets gelezen, te weinig afspraken. Elkaar voor de gek houden. Mag ik ook nog wat zeggen' en 'Dissertitis: Eenzaamheid, weer niks gedaan vandaag, er komt niets uit mijn pen, verveling, wie zit er op mijn proefschrift te wachten? Stoppen of doorgaan?*' beloofden een hobbelige weg met menig obstakel. Ik heb ze gelukkig over kunnen slaan en heb mijn AIO-tijd ervaren als boeiend, leerzaam, afwisselend, uitdagend, maar vooral ook, LEUK! En nee, je leest hier geen advertentie van het PNN (Promovendi Netwerk Nederland).

Nu had ik mezelf voorgenomen om niet te verzanden in de zoetsappige, individuele, maar dan toch eigenlijk niet-zo-persoonlijke - want redelijk afgezaagde - lijst van dankbetuigingen. Echter, er zijn toch echt een aantal personen welke zo'n substantiële bijdrage hebben geleverd aan dit proefschrift, direct of indirect, dat ik er niet omheen kan (en wil) die hier te noemen. Een goed proefschrift, begint met goede begeleiding. Wouter, Henk, Mirjam en Walter, bedankt voor jullie kritische blikken, waardevolle

adviezen en leuke discussies. Walter, als dagelijks begeleider heb jij natuurlijk het meest met me te stellen gehad. Wat heb ik het getroffen met zo'n begeleider! Omdat je niet moet proberen te verwoorden wat een ander al heeft gedaan refereer ik hier naar Herman Lelieveldt (Boekje 1; p.50), daar wordt je omschreven als 'De professional'. Bedankt voor het zijn van die professional en daarnaast jezelf. Mirjam, bedankt voor alle suikerpraat. Ik heb ontzettend veel van je geleerd. Na onze laatste discussie in 'het Oude Pakhuis' kon ik het niet laten om mijn laatste stelling te deponeren. Volgens mij beschik jij over voldoende van beide, dus dat zit wel goed! René, officieel geen begeleider maar toch nog coauteur op twee papers! Daarnaast ben ik door jouw toedoen bij Diervoeding beland. Het feit dat we beiden fan zijn van zowel kippen als onderwijs zorgde voor voldoende gespreksstof. Bedankt!

Naast goede begeleiding is een goede werksfeer evenzo van belang. Lieve diervoedingscollega's, bedankt voor jullie gezelschap, zowel tijdens als buiten werktijd! De s***jes noem ik hier in het bijzonder. Harma, studiegenootje, huisgenootje, bestuursgenootje, collega-AIO, officemate, secretaresse, paranimf en ook nu weer collega. Wat we ook van elkaar zijn, het liefst noem ik je vriendin. Lotte, het kostte zelfs minder dan 4 jaar om je te desensibiliseren. Nu knuffel jij mij! Volgend streven: je eerste €-weddenschapje volbrengen. Iets met een borstel? Myrthe, over koetjes, kalfjes of statistiek. Met jou kan ik praten! Hoewel soms misschien wat te hard. Trekken wij ons niets van aan. Toch?!

Annemieke, hoewel we niet precies tegelijk begonnen en eindigden, hebben we toch het grootste deel van ons PhD-traject samengewerkt. Ik vond het leuk om een kijkje in de chemische keuken te nemen. Bedankt voor alle hulp, uitleg en geduld.

Ruurd, thank you for giving me the opportunity to visit your group. It was a pleasure to perform an experiment at SRTC with all freedom and support from your side. I am very happy with the results (Chapter 9), which in my opinion can be considered the top off of my thesis. Apart from the professional support I received from the colleagues from the Department of Agricultural, Food and Nutritional Science of the University of Alberta, I am also very grateful for those that accompanied me during my spare hours in Edmonton. Georg und deiner Familie, vielen Dank für die Gastfreundschaft. Zahid, Amy, Laura, Alice, Miranda, Thomas and Candace, thank you for the nice dinners, pub- and movie evenings, hikes, and skiing-trips.

Angela en haar paranimfen, in plaats van door het raam naar buiten gaan we nu door de deur naar binnen. Ik vind het super dat jij bij mij op het podium staat, en zie er naar uit binnenkort ook naast jou op het podium te staan! Wie had dat ruim 15 jaar geleden gedacht? (zeker dhr. Nagelkerke niet!)

Tabellen invullen, 100-en buisjes zagen, kippenmest föhnen, varkenspoep rapen en magimixen. Thuis, in Wageningen en in Edmonton. Harmen: je hebt er weer een stapel certificaten bij. Tige tank foar al dyn help. Mar noch mear foar alle geselligens en humor!

Beste lezer, of je nu gaat voor alle 10 hoofdstukken of de samenvatting, ik waardeer het ten zeerste dat je tot hier geboeid bent gebleven door mijn geneuzel. Je bent al op pagina 2. De rest is een eitje. Ik sla dit boek nu dicht. Ome Hans: Het krijgt een mooi plekje naast dat van u.

Saja

CONTENTS

Voorwoord – Preface i

1. General Introduction 1
2. Technologies to improve degradation of fiber-rich feed ingredients 9
3. Technologies to improve *in vitro* degradability of barley 26
4. Technologies to improve *in vitro* degradability of DDGS 32
5. Acid-extrusion to improve degradability of DDGS in pigs 48
6. Degradation of RSM polysaccharides in pigs 64
7. Enzymes to improve degradability of rapeseed meal in broilers 82
8. Marker methods to evaluate fiber degradability in broilers 100
9. Interaction between fermentable fiber and digestive utilization of the diet 112
10. General Discussion 128

References 145

List of abbreviations 159

Summary (EN) 161

Samenvatting (NL) 164

Acknowledgements 167

About the author 169

Colophon 175

‘DDGS and rapeseed meal should be considered by-products rather than co-products’

Chapter 1

General Introduction

The increased availability of fiber-rich feedstuffs, such as by-products from the biofuel industry, and their competitive pricing compared with traditional feedstuffs, has stimulated the use of these products as ingredient for animal diets. As these protein-rich products typically contain substantial amounts of fiber, the nutritional implications of the inclusion of these fiber fractions in animal diets have attracted more interest.

THE FIBER CONCEPT

Pig and poultry diets consist almost exclusively of feedstuffs of plant origin, which contain varying levels of plant cell wall material. Plant cell walls are mainly (70-90 %[35]) constructed of non-starch polysaccharides (NSP) that are interconnected and associated with proteins and lignin via covalent and non-covalent linkages[36]. Although the chemical constitution of these cell wall fractions varies widely, they can be considered alike from a nutritional viewpoint, as they are not enzymatically digested and exert similar physiological properties. Hence, often a physiological based definition is used to describe the fraction of feed resistant to enzymatic digestion[34]. This fraction, the vast majority of which consists of plant cell wall components is generally referred to as fiber, or dietary fiber. Several methods are available to analyze fiber fractions, varying in the chemical components that are measured by the analyses[34]. Usually, cellulose, most hemicelluloses, and lignin are included, but the exact chemical components included in the fiber fraction depend on the definition and methods used (Textbox 1.1, Figure 1.1). Resistant starch – which is by definition resistant to enzymatic digestion, but mostly not originating from the cell wall[37] – is often included based on its similar behavior in the gastrointestinal tract. In animal nutrition, historically the gravimetrically based crude fiber (CF), and later neutral (NDF) and acid detergent fiber (ADF) methods, have been used. Alternatively, the fiber fraction is calculated as the organic material from feed remaining after subtraction of ash, crude protein (CP), crude fat, starch, and ethanol-soluble sugars[38] from the dry matter content (DM). Although these physiologically based fractions are convenient and might correlate well with degradability of specific fiber fractions in the animal[26] they do not allow detailed evaluation of specific NSP structures.

Table 1.1 Typical chemical composition (%, wt/wt; as-fed basis) of maize dried distillers grain with solubles (DDGS) and rapeseed meal reported in literature.

	DDGS[14, 39-43]	Rapeseed meal[31, 38, 44, 45]
Crude protein	25-31	34-40
Crude fat	9-11	2-4
Starch	4-11	2-5
Total dietary fiber	29-46	32-35
Crude fiber	7	10-13
Neutral detergent fiber	20-44	21-35
Acid detergent fiber	7-18	16-22
Lignin	2	10-13
Ash	3-4	6-10

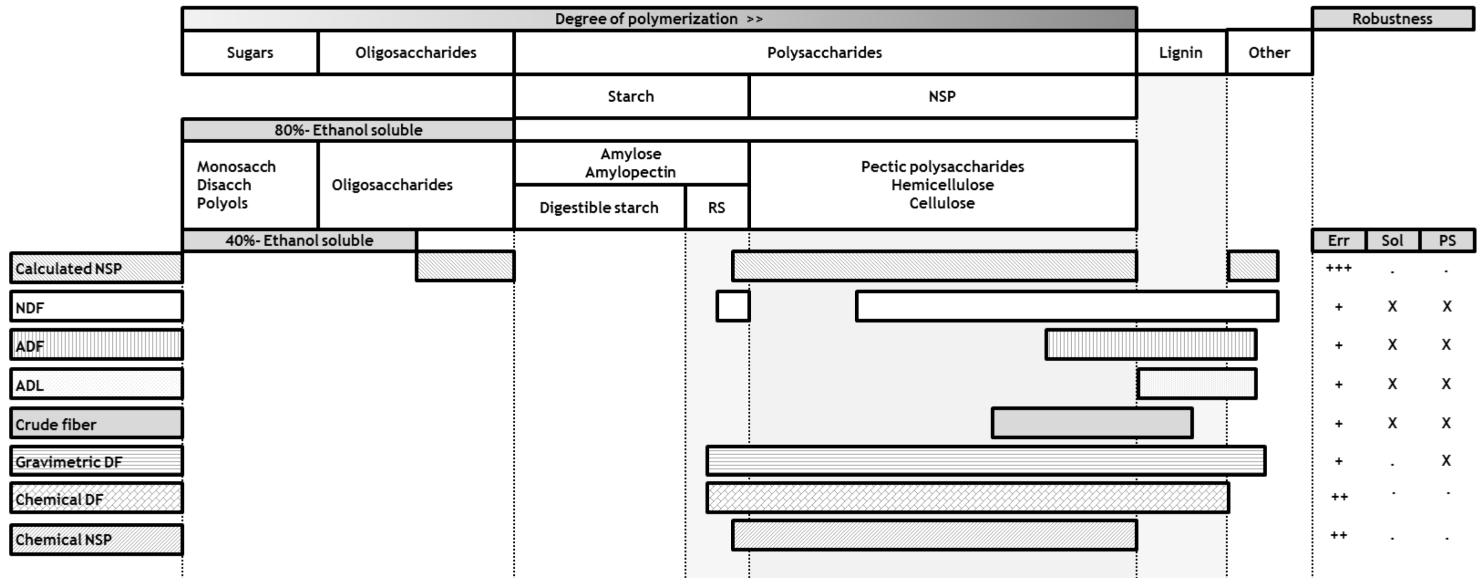

Figure 1.1 Indicative chemical compositions of fiber fractions when calculated as rest fraction (calculated NSP[38]) or analyzed by the detergent system (NDF, ADF, ADL[46]), crude fiber (CF), enzymatic-gravimetric dietary fiber (DF, e.g. Prosky method[47]), enzymatic-chemical DF (e.g. Uppsala method[48]), or enzymatic-chemical NSP methods (e.g. Englyst method[49, 50]). Robustness indicates the sensitivity of the methods to analytical errors (Err) and specific sample characteristics as solubility and particle size. Where '+' indicates smallest and '+++' greatest analytical variability, '.' minor and 'x' substantial influence of solubility (Sol) of polysaccharides or particle size (PS) of samples. Sacch= saccharides.

FIBER-RICH FEEDSTUFFS FOR PIG AND POULTRY DIETS

The quantity and type of NSP, as well as their structural arrangement in the cell wall, widely varies between plant species as well between different tissues within the plant[51, 52]. Hence, not only the level but also the type of NSP and their physicochemical properties vary considerably between feedstuffs. Particularly feedstuffs derived from biofuel production often contain high levels of NSP, due to concentration of cell wall fractions in the residual by-product not converted into fuel. This thesis focuses on two fiber-rich feedstuffs that are widely used as protein source in pig and poultry diets and differ in the type of fiber present: maize dried distillers grain with solubles (DDGS) and rapeseed meal (RSM). Both products are by-products (Textbox 1.2, Table 1.1) and have undergone extensive processing of which procedures and conditions differ considerably between production plants and batches[53, 54]. Dried distillers grain with solubles is a by-product from the bio-ethanol production, where entire grain kernels are ground, cooked (premixing at 40-60 °C and cooking at 90-165 °C) and enzymatically hydrolyzed (thermostable amylases, 60 C, > 30 min), after which yeast are added to ferment sugars to ethanol. After distillation, solids and solubles are separated by centrifugation. Solubles are concentrated by condensation and added back to the solids, to a maximum of 25 % of the total product. The resulting DDGS is dried using rotary drum driers (250-600 °C, product temperature approximately 100 °C,

Textbox 1.1

FIBER DEFINITION: WHAT'S IN A NAME?

Traditionally crude fiber, which became an official AOAC[13] method in 1890, was used to measure the indigestible organic matter of food en feed. Although the method, based on chemical extraction with alkali and acid solutions, is robust to analytical variation it recovers only part of the fiber fraction[26] (Figure 1.1). Depending on the fiber structure of the material, a variable, but considerable, part of hemicellulose (80-85 %), cellulose (0 to 60 %), and lignin (10 to 95 %) may be solubilized and not recovered in the CF fraction[26]. In an attempt to more completely describe the non-digestible plant cell wall constituents in food and feed, the term dietary fiber was introduced in 1953. Debates about the appropriate definition as well as adequate methods to analyze DF, have been ongoing ever since[34]. The definition currently used by AOAC is: *"Dietary fiber consists of the remnants of edible plant cells, polysaccharides, lignin, and associated substances resistant to (hydrolysis) digestion by the alimentary enzymes of humans"* and includes oligosaccharides, pectic polysaccharides hemicellulose, cellulose, lignin, gums, and some minor associated plant cell wall substances. Several methods to analyze DF, or its specific components as non-digestible oligosaccharides NSP, RS, or lignin are available[34] (Figure 1.1). Depending on the material, a significant fraction of the polysaccharides is not included in the detergent and crude fiber methods. Easily solubilizable NSP, such as β-glucan and pectic polysaccharides, might be excluded from NDF and CF, whereas some pectic polysaccharides that precipitate in strong acid will be included in the ADF fraction[26]. In the DF and NSP methods (including calculated NSP), starch is removed enzymatically (α-amylase and amyloglucosidase), which means some enzyme-resistant starch may be included in the fiber fraction. Generally, physically enclosed starch and native starch granules (referred to as RS type 1 and 2, respectively) are well removed by these procedures whereas DMSO or KOH is needed to remove retrograded and chemically modified starch (RS type 3 and 4). Apart from the chemical composition of the cell wall fraction, also the physical characteristics of the sample may influence the recovery of polysaccharides using gravimetric procedures. Particle size, for example, considerably contributes to the amount of fiber that is lost during the filtration step of gravimetric methods. Due the character of the methods, the detergent and crude fiber methods are robust to analytical variation, whereas the more sophisticated enzymatic chemical methods are less producible and less suited for routine analyses[26] containing enzyme mixture.

typically < 1 h) and usually pelleted[14, 55-58]. Several feedstocks can be used for ethanol production, but maize, wheat, barley, and sorghum or mixtures thereof, are most common[14, 55, 56]. In this thesis, only DDGS from maize was studied. Rapeseed meal remains after extraction of oil from preconditioned (30-40 °C, 30-45 min), flaked, and cooked (65-105 °C, 20-40 min) rapeseed by screw-pressing and subsequent solvent extraction (hexane, 50-60 °C). The remaining cake is toasted (95-115 °C, 30-40 min), cooled, hammer milled, and sometimes pelleted[59, 60]. Rapeseed can derive from several plant species of the *Brassicaceae* family, of which *Brassica napus*, *Brassica rapa ssp. campestris*, and *Brassica juncea* (also referred to as mustard seed) are most common[60]. In this thesis various batches of RSM from the *B. napus* species, all from the 'canola' classification (Textbox 1.2) were used. Apart from the chemical and physical processing technologies discussed above, cell wall degrading enzymes, as cellulases, hemicellulases, and pectinases, may be used during RSM production[61]. The extensive processing conditions undergone by both DDGS and RSM, have their adverse effect on protein quality[62, 63] and will inevitably impact the fiber fraction, and thereby its degradability as well.

FIBER IN MONOGASTRIC NUTRITION: BONUS OR BURDEN?

Non-starch polysaccharides can be partly fermented by the microbiota residing in the gastrointestinal tract of the animal. The end products of NSP fermentation, short chain fatty acids (SCFA), can be absorbed and used as energy source[64-66]. Dietary fiber can, however, also interfere with digestive processes via various pathways, thereby often reducing digestion and absorption of other nutrients from the diet. The relative importance of each of the mechanisms, via which NSP can affect the digestive utilization of a feedstuff, differs between pigs and poultry due to considerable differences in digestive physiology between these species[2]. In either case, however, high inclusion levels of NSP-rich feedstuffs may reduce the nutritional value of the diet. In growing pigs, typically over 50 % of NSP is degraded[38] and even more so in adult sows[67]. Compared to pigs, retention time of digesta in the chicken is low[2], providing less opportunity for fermentation of fiber. Hence, in chicken, broilers as well as adult, generally less than 20 % of NSP is degraded[21, 23, 25, 68-73].*Two major factors that can be altered by the presence of specific fibers in the diet are digesta retention time and digesta viscosity. Generally, poorly fermentable fiber will decrease retention time in the hindgut, whereas viscous fiber may increase gastric retention time and reduce small intestinal retention time. This affects the time available for digestion and fermentation and thereby the degradation of the diet. Furthermore, viscous fiber may impair enzyme accessibility of nutrients, potentially reducing nutrient degradation. It follows that the resulting effects of fiber inclusion on the digestive utilization of the diet are complex. Adverse, as well as beneficial effects on digestion of other nutrients have been found[74-82], depending e.g. on the species, age of the animal, fiber type, and level.† Secondary effects of fiber on utilization of other nutrients are usually more

* Degradation of NSP strongly depends on the amount and type of NSP present, resulting in ranges between 0 and 100 %. Values given are overall averages. For detailed discussion of NSP degradation in pigs and poultry, see chapter 2.

† Discussed into more detail in chapter 2.

pronounced in poultry than in pigs[2]. The small intestinal digesta of pigs is more diluted (ca. 2-fold) compared to poultry[2], providing a better buffering capacity to prevent changes in the physical properties of the feed matrix. In addition, due to the longer gastric retention of digesta, fiber may be substantially degraded already in the stomach, abolishing some of the potential physical effects they may have in the small intestine. Still, adverse effects of fiber on digestion of other nutrients can be substantial, especially in young pigs[2, 83, 84].

IMPROVING DIGESTIVE UTILIZATION OF FIBER-RICH FEEDSTUFFS

The increased use of fiber-rich feedstuffs in pig and poultry diets requires an optimal utilization of these ingredients. Hence, the animal feed industry explores opportunities to improve degradability of these feedstuffs and maximize their inclusion levels in pig and poultry diets. Processing and enzyme technologies can modify the physicochemical characteristics of NSP from feed ingredients, thereby affecting their degradability. In this way, fermentability of NSP and thus their potential energetic utilization might be enhanced. In addition, technologies can be aimed at alleviation of adverse effects on digestion and absorption of other nutrients, which might be particularly of interest for young pigs and poultry.

In current feed formulation systems interactions between feed ingredients are assumed to be absent. As discussed above, however, fiber may interfere with digestive processes, thereby affecting digestion and utilization of other dietary components. Ignoring these possible interactions between the presence of specific fibers and the digestive utilization of the diet may lead to inaccurate estimations of the nutritive value of feed ingredients and, especially, diets.

Textbox 1.2

BIOFUEL BY-PRODUCTS : DDGS AND RAPESEED MEAL

DDGS

Several by-products from the production of bio-ethanol are commonly used in animal nutrition, already for more than 50 years[14]. The two main types of ethanol production from maize are dry milling and wet milling. The wide range of by-products that are produced differ substantially in composition and nutritional values. Maize gluten feed, maize gluten meal, maize germ meal, and crude maize oil originate from the wet milling process. Wet distillers grain, condensed distillers solubles (CDS), modified wet distillers grain, dried distillers grain (DDG), and dried distillers grain with solubles (DDGS). Due to continuous improvements in ethanol production technologies, composition of those products is subject to change and new products such as high protein or defatted DDGS enter the market.

Rapeseed meal

Rapeseed meal is by-product from the oil production from rapeseed; a yellow-flowering plant belonging to the *Brassicaceae* family. In Canada, the term 'canola' is used to market *B. napus, B. rapa,* as well as *B. juncea,* yielding oil with less than 2 % erucic acid and meal containing less than 30 μg glucosinolates[31], also referred to as 'double-zero rapeseed' quality.

AIM AND OUTLINE OF THIS THESIS

The research described in this thesis is part of the collaborative project between Agrifirm Group, Duynie Holding, Nutreco Nederland B.V., and Wageningen University (Animal Nutrition Group and Food Chemistry Group), carried out within the framework of the Carbohydrate Competence Center (CCC).

The overall aim of the project was to improve digestive utilization of fiber-rich feedstuffs for pigs and poultry. In this thesis, focus was on degradability of fiber fractions from two fiber-rich feedstuffs, DDGS and RSM, in growing pigs and broilers. First, the effects of processing and enzyme technologies on fiber-rich feedstuffs, published in literature, were reviewed (Chapter 2). Based on this review, processing technologies widely differing in their mode of action were selected and combined with specific cell wall degrading enzyme treatments. The effects of processing technologies, enzymes, and their combination, on model feedstuffs that differed in quantity and type of NSP (i.e. β-glucans, arabinoxylans, or pectins), were studied. *In vitro* studies in barley (Chapter 3), maize and maize DDGS (Chapter 4), and RSM[33], were performed to evaluate effectiveness of the selected technologies. Technologies that effectively modified degradation of fiber fractions *in vitro*, were selected to be studied *in vivo* (Chapter 5, 6, 7). To obtain better understanding of fiber degradation in the animal and to identify limiting structures in the degradation of NSP, degradation of polysaccharides was studied in detail in growing pigs (Chapter 5, 6) and broiler chickens (Chapter 7, 8). In the final stage of the project the interaction between inclusion of specific fermentable fiber sources and the degradation of fiber-rich feedstuffs was investigated (Chapter 9). Finally, the results of this thesis were summarized and discussed in Chapter 10.

Improving digestive utilization of fiber-rich feedstuffs in pigs and poultry by processing and enzyme technologies: A review

Chapter 2

Technologies to improve fiber degradation

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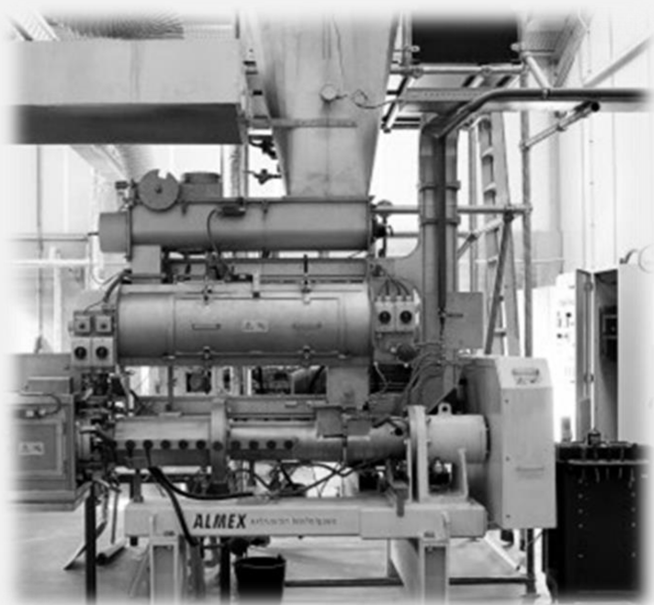

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ABSTRACT

The effects of processing technologies, whether or not combined with cell wall degrading enzymes, on the physicochemical properties of non-starch polysaccharides (NSP) and the resulting effects on NSP degradation in both pigs and poultry were reviewed. Evaluation of the effects of processing technologies on digestion of NSP is hampered by the potential shift of polysaccharides recovered in the fiber fractions of common, gravimetric, fiber analysis methods. Results from in vivo studies describing effects of processing technologies or enzyme treatments on crude fiber, neutral detergent fiber, or acid detergent fiber digestibility, instead of NSP digestibility, should therefore, be interpreted with care. Detailed information on the composition of the NSP-fraction and degradability of its components will help to identify and understand modifications that occur during processing.

Processes based on mechanical modification of feedstuffs that are commonly used in the feed industry, such as hammer and roller milling increase solubility of the NSP-fraction resulting in a 6 to 7 % unit increase in coefficient of apparent total tract digestibility (CATTD) of the fiber fraction in both pigs and poultry. Dry thermal processes have a minor impact on physicochemical properties of feedstuffs and consequently, the effects on the coefficient of apparent ileal digestibility (CAID) and CATTD of the fiber fractions in pigs and poultry are limited. Hydrothermal processes that include high shear forces such as expander processing and extrusion cooking are more effective and increase solubility but also viscosity. The CATTD of fiber fractions in pigs can be increased on average 3 % units by hydrothermal processing of feeds and feed ingredients, although some studies have reported unchanged or even decreased digestibility values. In poultry, CATTD of fiber fractions can be increased 4 to 16 % units by hydrothermal processing. Increased digesta viscosity resulting from technological processing of feed and feed ingredients can be counteracted by the addition of specific enzymes. Enzyme addition to heat processed diets and diets containing heat processed ingredients results in a 3- to 4-fold reduction in viscosity compared with enzyme addition to unprocessed diets, or diets containing unprocessed ingredients. In addition, modifications in cell wall architecture obtained by processing technologies will improve the accessibility of NSP to enzymes. As a result, the effects of enzyme addition on digestibility of the fiber fraction are 1.5 to 6 times larger, when applied to heat processed diets compared with unprocessed diets.

INTRODUCTION

The animal feed industry has been confronted with increased costs of raw materials over the last decade, mainly because of increased demands for cereals and oil seeds by emerging markets and increased use of these products for energy production[85]. Utilization of by-products from the bio fuel and food industries as feed ingredients is important to reduce feed costs and enhance the sustainable use of feed resources for animal production. Many of these products contain high levels of plant cell wall material, mainly composed of non-starch polysaccharides (NSP), which cannot be digested by mammalian enzymes, but can be partly degraded by the microbial community residing in the intestinal tract of animals. The structural arrangement of NSP in plant cell walls often limits their accessibility to microbial enzymes, and thereby their degradation[35]. Furthermore, NSP can interfere with digestive processes and associate with other nutrients, thereby reducing digestibility and absorption of these nutrients[86].

As indicated by Zijlstra et al.[32], feed processing and enzyme technologies can be valuable tools to enhance digestive utilization of nutrients from NSP-rich feedstuffs. Modifications in chemical structure and physicochemical properties of NSP caused by processing are however, poorly understood and strongly depend on process conditions and the type of NSP[87]. In addition, the relationship between NSP structure and their degradation in the digestive tract of the animal is unclear, and hinders development of successful technologies to improve the utilization of NSP and potential nutrients present in NSP-rich by-products. Knowledge of the chemical structure or physicochemical properties of NSP, in relation to gastrointestinal degradation, will enable development of more specific and effective processing technologies.

This review aims to identify and quantify effects of processing technologies, whether or not combined with cell-wall degrading enzymes, on digestive utilization of NSP-rich feed ingredients by pigs and poultry. First, a brief introduction on the degradation of NSP in relation to the cell wall architecture, and its effects on digestive processes in the animal is provided. In the second part of this review, the effects of processing technologies, whether or not in combination with cell wall degrading enzymes, on the physicochemical properties of NSP and the resulting effects on NSP degradation in both pigs and poultry are discussed.

METHODOLOGY

Peer reviewed scientific articles concerning feed processing for pigs and poultry, whether or not combined with enzyme addition, were selected for paragraph 4 “Improving digestive utilization of NSP-rich feedstuffs by processing and enzyme technologies”. Selection was based on the description of the processes or enzyme technologies used, reported changes in physicochemical properties of interest (NSP-solubility, viscosity, hydration properties) or reported digestibility of the fiber fraction [i.e. NSP, dietary fiber (DF), crude fiber (CF), neutral detergent fiber (NDF), or acid detergent fiber (ADF)].

The contribution of NSP from the feed ingredient of interest to the total dietary NSP-fraction, was calculated using data from original publications, or if not reported, data from Centraal Veevoeder Bureau[38]. Water holding capacity (WHC) is defined as

the amount of water retained by fiber without addition of mechanical forces. Water binding capacity (WBC) is defined as the amount of water retained by fiber after centrifugation, according to the definition of hydration properties as described by Nelson[88].

DIGESTION OF NSP-RICH FEEDSTUFFS

Degradation of NSP in pigs and poultry as influenced by cell wall architecture

In pigs, NSP degradation by microbial enzymes mainly occurs in the large intestine, although some degradation has been reported to occur in the stomach and may reach considerable levels at the terminal ileum[89-91]. Pettersson and Åman[23] suggested that in poultry, NSP degradation mainly occurs in the crop or gizzard, based on the observation that only small differences between NSP digestibility in digesta collected from the middle and last section of the small intestine and excreta existed. Digestibility values of NSP found in the various sections of the gastrointestinal tract could, however, be biased by reverse peristalsis of the soluble fraction of the digesta, that occurs in birds[92, 93].

The degree of NSP degradation in monogastric animals is related to their solubility, and depends on the type of polymers and their structural associations with other cell wall components[35, 51, 94]. Solubilization of NSP from the cell wall is facilitated by a decreasing chain length, irregular polysaccharide structure – preventing crystalline structures[95] -, low degree of substitution[96], or weak linkages with other polysaccharides and cell wall components[51, 94].

The type of NSP, their intracellular connections and thereby their solubility, vary between plant species and within plant tissues. Grains from monocotyledonous plants, including cereals, contain mainly arabinoxylan, β-glucan and cellulose, whereas grains from dicotyledonous plants as legumes and oil seeds contain mainly pectin, cellulose and xyloglucan[51]. Secondary cell wall tissues of the grain, (i.e. the hull and the pericarp and testa) are mainly composed of insoluble, lignified and strongly linked NSP, such as cellulose and (glucuronoarabino) xylan. Parenchymatous tissues are, however, mainly composed of soluble arabinoxylan and β-glucan in cereal grains and mainly pectin in dicotyledonous grains, with much weaker intracellular bonds[51].

Differences in NSP solubility are reflected in digestibility coefficients in pigs. Easily solubilizable arabinoxylan from parenchymatous tissues is readily degraded with coefficients of apparent total tract digestibility (CATTD) of 0.82 for oats[97], 0.73 to 0.83 for rye[96] and 0.68 to 0.94 for wheat[97, 98]. Whereas insoluble, branched chain arabinoxylan from lignified, secondary cell wall tissues in wheat and rye remains virtually undegraded[96, 98]). β-Glucan from both parenchymatous as well as secondary cereal cell wall tissue is almost completely degraded[83, 89, 97, 98], but differences in coefficient of apparent ileal digestibility (CAID) show that β-glucan from the endosperm is more easily degraded than that in the aleurone layer or the pericarp and testa[83, 89, 97, 98] In pigs, the CATTD of cellulose from non–lignified parenchymatous tissues is 0.78 in oats[98], 0.84 in rye[96] and 0.43 to 0.60 in wheat[89, 98]. Degradation of cellulose from lignified materials which is highly crystalline[99] such as the aleurone layer and the pericarp and testa of rye and wheat, is much lower in pigs[35, 96, 98].

Arabinosyl and galacturonic acid residues, originating from pectin from both the pea cotyledon and the hull are readily digested, whereas xylosyl and glucosyl residues originating from glucuronoxylan and cellulose from the pea hull, are more difficult to digest[100]. *In vitro* fermentation studies indicate that pectic arabinan and arabinogalactan, present as side chains[101], are more easily fermented by some bacterial species present in the pig's intestinal tract, than the (rhamno) galacturonan backbone[102].

Poultry, have a relatively short colon and a rapid digesta transit, and as a consequence the fermentative capacity of this species is limited almost exclusively to the soluble NSP fraction. The CATTD of NSP from diets containing cereals that are relatively high in soluble NSP, such as barley, wheat and oats have been reported to range between 0.28 and 0.40[23, 69]. In contrast, the NSP-fraction from diets containing cereal products that consist mainly of insoluble NSP (e.g. wheat bran and maize) are less well digested, with CATTD of 0.08 for NSP from maize[21] and 0.16 to 0.19 for NSP from wheat bran and barley[69]. The CATTD of the NSP-fraction from diets containing dicotyledonous materials ranges between 0 and 0.12[21, 25, 69, 71, 72]. Soybean meal NSP, containing pectin that consist for 60 % of neutral sugar side chains[101] that seem to be well fermented[102] and lacking the so called 'smooth regions' formed by homogalacturonan[103] are relatively well degraded, whereas those of peas are poorly degraded.

Antinutritive properties of NSP

Non-starch polysaccharides can directly and indirectly interfere with the digestion and absorption of other nutrients. First, the structural arrangement of NSP in the cell wall can affect digestibility of the NSP-fraction itself as well as that of other nutrients encapsulated in the cell, limiting the accessibility of these nutrients by digestive enzymes[69, 84, 97]. Surface activity of NSP can cause them to bind to the surface of feed particles after ingestion, reducing the accessibility and absorption of nutrients from the diet[86, 104]. Second, as reviewed by several author[86, 105-107], NSP contribute to the physical properties of the digesta, such as viscosity and hydration properties, thereby influencing digesta transit time, bulking properties[108], microbial activity[69, 97, 109] gut physiology and function[69, 110-113] and endogenous losses[114], again potentially reducing nutrient digestion and absorption. The impact of these antinutritive properties on nutrient digestion can be considerable. In poultry, starch and protein digestion were reduced up to 14 % units and fat digestion up to 24 % units, after addition of isolated soluble NSP from wheat and rye to the diet[74-76], as reviewed by Smits and Annison[86].

IMPROVING DIGESTIVE UTILIZATION OF NSP-RICH FEEDSTUFFS BY PROCESSING AND ENZYME TECHNOLOGIES

Processing technologies

Effects of processing technologies on physicochemical properties of NSP

Processing technologies are based on mechanical, thermal and/or chemical modification of the feedstuff. Mechanical forces, as pressure and shear, may cause attrition, abrasion and breakage of particles, thereby breaking the seed coat, reducing particle size and fiber length and opening the cell wall structure. Thermal processes can break weak bonds between polysaccharides and glycosidic linkages within polysaccharides. In starch-rich products, heating, particularly under moist conditions, causes starch gelatinization. This causes the cell to swell and break, thereby increasing the surface area of the cell and disruption of the cellular integrity[99]. Heating can, however, also cause cross-linking between nutrients, for example via the Maillard reaction, especially during dry heat processes[99]. Thermal treatment in either acidic or alkaline conditions leads to partial hydrolysis of NSP as demonstrated by[115], where bivalent organic acids were used to completely degrade complex polymers of wheat straw into monomers. Degradation of polysaccharides and disruption of the physical binding forces, which occur during processing, affect physicochemical properties of the NSP-fraction (Table 2.1).

Particle size. Particle size of feedstuffs determines the surface area available for contact with digestive enzymes in the gastrointestinal tract, thereby influencing their degradation, but also affects physicochemical characteristics of feedstuffs, such as their hydration properties. Many processing technologies used in animal feed production have an effect on particle size (Table 2.1), either a primary effect in the case of milling, or a secondary effect as is the case for most thermal treatments. Milling, mainly hammer milling, is common practice in feed production. The magnitude of particle size reduction, the type of deformation and the uniformity of the particles depend on the milling conditions and equipment used and differ between products because of differences in grain size, crystallinity and brittleness[116]. The effect of (hydro) thermal processes on particle size varies considerably between processes and products (Table 2.1). Processes like steam cooking-flaking and infrared radiation-flaking break the seed coat and endosperm, resulting in an increase in surface area while leaving the grain intact, whereas expander processing and extrusion, that involve high shear forces, completely disrupt the grain structure[87]. In most studies, heat treatments are applied to ground products in which the whole grain structure has already been disrupted. Consequently, the results are less dependent on the grain structure and are affected by altered milling properties resulting from changes in the texture or brittleness of the product obtained during processing. Mild hydrothermal processes like steam cooking-flaking, result in increased particle size in finely ground material[116], whereas no effect or a reduced particle size is observed in coarsely ground material[116-120]. As shown in Table 2.1, processes including high shear forces such as expander processing and extrusion cooking, result in reduced particle size[116, 119, 121, 122], the effects of shear being larger for coarsely ground materials compared with finely ground materials[116]. Pelleting may cause agglomeration of small particles, as indicated by the increased particle size

of hammer milled barley after pelleting, whilst pelleting decreases particle size of roller milled barley[121].

Solubility. Solubilization of NSP from the cell wall is hampered by covalent and non-covalent cross-links between the various polysaccharides and other cell wall components. Solubilization of NSP during processing technologies, mainly depends on the type of crosslinks that have to be broken[94]. As shown in Table 2.1, β-glucan, especially that of parenchymatous tissue, is easily solubilized, and crops containing a relatively large proportion of these polysaccharides such as barley, show a moderate to large increase in solubility of the NSP-fraction after milling and mild thermal treatments[116]. Arabinoxylan from parenchymatous tissue is easily solubilized, whilst that from secondary cell wall tissue, as abundant in oat and barley hulls and wheat bran, require more severe processing technologies such as extrusion[116, 123]. Disengagement of cellulose from the cell wall, particularly in lignified tissues, requires severe physical and chemical treatments to break down its crystalline structure and to modify or remove lignin[124, 125]. On the other hand, also milling has been shown to break down the crystalline structure of cellulose[126]. It might be expected that cellulose from feedstuffs being less lignified than the lignocellulosic materials discussed by Sun and Cheng[124] and Mosier et al.[125], can be modified to some extent, during processes commonly used in feed industry. Part of the pectin present in common feedstuffs, mainly that from the primary cell wall, is relatively soluble[45, 51]. Solubilization of pectin however, might be limited by its structural arrangement in the cell wall through intermolecular ionic bridges, ester linkages and hydrogen bonds[51]. For example, the NSP-fraction from peas, consisting predominantly of pectin, can be solubilized during both milling and thermal treatments[116, 127, 128] (Table 2.1), whereas NSP from the secondary cell wall tissues require more severe processing conditions than those from parenchymatous tissues[128, 129]. The so called 'hairy' regions of pectin, characterized by a rhamnogalacturonan backbone substituted with side chains of neutral sugars (mainly arabinan and arabinogalactan) seem to be more easily solubilized than the 'smooth' regions[130, 131]. Some pectin from secondary cell walls might be tightly bound to cellulose[132] making solubilization difficult. During thermal treatments, pectin can be solubilized and degraded by acid hydrolysis, β-elimination and de-esterification. Neutral and alkaline conditions promote β-elimination of mainly highly esterified pectin, whereas heating under acidic conditions favors hydrolysis of polymeric pectin with a low degree of esterification[99, 131].

Viscosity. Solubilization, degradation and loss of side chains during processing can affect viscous properties of NSP, and thereby rheological properties of the digesta. Because viscosity is related to molecular weight[133], a decrease in viscosity after degradation of NSP, as generally observed after milling, can be expected[134]. During thermal processing however, cell wall structures can be disrupted, resulting in increased solubilization without actual degradation of the polymers, often resulting in increased viscosity. As shown in Table 2.1, the viscosity of – in particular – soluble β-glucan and arabinoxylan is high and increases during heating[135], whereas cellulose contributes little to the viscosity of the NSP-fraction[136, 137]. Consequently, thermal processing of β-glucan- and arabinoxylan-rich feedstuffs increases viscosity of these products[122, 138-141].

Table 2.1. Influence of processing technologies on particle size, solubility, viscosity and hydration properties of various non-starch polysaccharide (NSP) sources in diets for pigs and poultry.

Process	Feedstuff	NSP[1]	Particle size[2]	NSP solubility[2]	Viscosity[2]	Hydration property[2]		Reference
						WHC[3]	WBC[4]	
Mechanical processing								
Hammer milling (fine vs. coarse)								
	Barley	67 – 100	---/-	+++				[116, 119, 142]
	Maize	100	--	+++				[116]
	Oats	100	---	+				[116]
	Peas	100	---	++				[116]
	Pea hulls	100		+++		+/++	--/+	[127, 143]
	Wheat	100	---/-	++				[116, 142, 144]
	Wheat bran	100		++		-	--/0	[143, 145]
Milling (Roller vs. hammer)								
	Wheat	100		+				[138]
Thermal processing, moderate or no mechanical forces								
Drum Drying								
	Wheat flour	100		0/+				[146]
Boiling								
	Wheat bran	100		+		+	+	[145]
Roasting								
	Wheat bran	100		0		+	0	[145]
Infrared irradiation								
	Wheat	100		-/+	0/+			[138]
	Wheat bran	100		+				[145]
Toasting								
	Peas	100		+				[128]
	Pea hulls	100		0				[147]
Autoclaving								
	Rye	100		0	--			[148]
	Wheat	100		+				[146]
	Wheat bran	100		+		0	0	[145]
Steam cooking								
	Rice	100	+					[118]
	Wheat bran	100		0		+	0	[145]
Thermal processing, severe mechanical forces								
Steam flaking								
	Wheat	100		++				[146]
Infrared irradiation-flaking								
	Barley	67 – 84	-		++/+++			[119, 140]
	Wheat	100		0/+				[138]
Steam cooking-flaking								
	Barley	82 – 100	-/+	+				[116, 120]
	Maize	72 – 100	-/+	+		++		[116, 117, 120]
	Oats	100	-/+	++				[116]
	Peas	100	-/++	+++				[116]
	Rice	100	0/-			0		[84]
	Wheat	100	-/+	0				[116]
Pelleting								
	Barley	100	--/+					[121]
Expander processing								
	Barley	100	--/0	0/+				[120, 121, 149]
	Wheat products[5]	100		0/+				[149]
Extrusion cooking								
	Barley	100	---/-	-/+	+++		+	[116, 141, 150]
	Maize	100	--	0				[116]

Process	Feedstuff	NSP[1]	Particle size[2]	NSP solubility[2]	Viscosity[2]	Hydration property[2] WHC[3]	WBC[4]	Reference
	Oats	100	--/0	++				[116]
	Peas	100	---/-	+/+++				[116, 151]
	Pea hulls	100		+/+++		-/+	---/0	[127]
	Soybean meal[6]	88		---/+				[20]
	Wheat	100	---/0	+/++	+++	+++	+++	[116, 122, 152]
	Wheat flour	100		+/++				[146, 152]
	Wheat bran	100	-/0	+/+++	+++	-/+++	0/ +++	[122, 123, 145]
Popping								
	Wheat	100		+/ +++				[146]
Chemical processing								
Acid								
	Rye	100		+	-			[148]
Acid autoclaving								
	Rye	100		++	+			[148]

[1] Contribution of NSP from processed feedstuff to total NSP content of the sample (%).
[2] Change in physicochemical properties of the processed feed ingredient compared with the untreated control. Where --- indicates a decrease of >50 %, -- a decrease of 33-50 %, - a decrease of 10-33 %, 0 a change of -10-10 %, + an increase of 10-50 %, ++ an increase of 50-100 % and +++ an increase of >100 %.
[3] Water holding capacity (WHC), defined as the amount of water retained by the product without addition of mechanical forces.
[4] Water binding capacity (WBC), defined as the amount of water retained by the product after centrifugation.
[5] Wheat bran/wheat middlings (2:1).
[6] Untoasted extruded soybean meal compared with toasted soybean meal.

The more severe processes with severe mechanical forces, such as extrusion cooking, have the largest effects on viscosity (Table 2.1). These findings correspond with results of several *in vivo* studies, where thermal treatments increased small-intestinal digesta viscosity, especially in young chickens and piglets[7, 29, 30, 138, 153-155]. Likewise, heat treatment of pectin-containing products results in increased digesta viscosity[20, 116] (Table 2.1). Pectin can exert high viscosity and has the ability to gel under specific circumstances. High methyl-esterified pectin is able to form gels under moist acidic conditions and in the presence of sugar especially after heat treatment, whereas low methyl-esterified pectin only forms gels in the presence of divalent cations[131].

Hydration properties. Both WHC and WBC are widely used to describe the functionality of fibers[35, 95]. Water holding capacity of a diet seems to be a better predictor of the physical properties of digesta *in vivo*, especially when gel forming polysaccharides such as pectin are present[156]. During processing, physicochemical changes of components other than the fiber fraction such as starch gelatinization during thermal treatments, may dominate hydration properties of single feedstuffs and complete diets. Parenchymatous cell wall tissues, especially pectin, show in general higher hydration properties compared with secondary cell wall tissues, because of the more hydrophilic nature of these tissues and in the case of pectin its electric charge[143, 157]. Milling increases the surface area of the product, potentially increasing water absorption rate[95] and WHC as occurs for example in pea hulls[127, 143] (Table 2.1). On the other hand, depending on the material[158], the fiber matrix

may collapse during milling, resulting in reduced WHC and WBC in wheat bran[143]. In contrast to the increase in WHC, WBC of pea hulls was reduced after milling[127] (Table 2.1;), indicating disruption of the fiber matrix. Possibly, the water entrapping cell wall structure was damaged and the product was not able to physically bind water whereas solubilization of the polymers resulting possibly from increased porosity and surface area, increased its capability to absorb water and swell. Dry thermal processes have less influence on hydration properties compared with hydrothermal processes (Table 2.1). The latter increase WHC and WBC in most products[117, 122, 123, 141, 145]. Products containing high amounts of cellulose, such as pea hulls, appear to be less affected by hydrothermal processes[127].

Effects of processing technologies on digestion of NSP

Published studies often include only information on the fiber content as such and not on the chemical structure of NSP, which makes it difficult to correlate alterations in nutritional value to changes in the physicochemical properties of NSP. CF, NDF and ADF represent a variable part of the NSP-fraction, as already recognized by other authors[51, 86]. In order to consider changes in digestibility of the fiber fraction due to processing technologies, it is important to realize that processing will solubilize part of the NSP-fraction and can reduce particle size, such that polysaccharides that are recovered in CF, NDF and ADF fractions will differ before and after processing. Evaluation of the effects of processing technologies on digestibility of fibrous fractions should preferably focus on changes in the total NSP-fraction instead of the less specific CF, NDF and ADF fractions. Studies in literature reporting the effects of various processing technologies on the CAID and CATTD of the fiber fraction in pigs and poultry are summarized in Table 2.2 to 2.4.

Mechanical processing. As shown in Table 2.3, *in vivo* studies comparing milled feedstuffs to whole grains or coarsely milled feedstuffs show increased CATTD of the fiber fraction in growing pigs up to 6 % units[142, 144, 159], although in some studies the contrast in particle size may have been too small to observe differences[142, 159, 160]. In broilers, hammer milling decreased particle size (> 3- fold) and increased CAID of the fiber fraction by 7 % units, compared with crushing of peas[28] (Table 2.4). Hammer milling of flaxseed did not improve CAID of the fiber fraction in broilers[19], but the contribution of NSP from flaxseed to the total NSP content of the diet in this study was only 14 %.

Thermal processing. Dry heat processing techniques like infrared radiation, often erroneously referred to as micronization, have a minor impact on particle size, NSP-solubility, viscosity and hydration properties of the product, which is reflected in a lack of effect of these treatments on digestibility of the fiber fraction in both pigs (Table 2.3) and poultry (Table 2.4). Surprisingly, baking increased CAID and CATTD of NSP by 10 to 12 % units[161], values much higher than expected from a dry heat process where no mechanical forces are involved. As indicated by the authors, the improvement in NSP-digestibility can be partly caused by the increased resistant starch content of the analyzed NSP-fraction in the baked diet. Although even after correction for glucosyl originating from resistant starch, NSP digestibility increased by 7 to 9 % units.

Table 2.2. Influence of processing technology on change in coefficient of apparent ileal digestibility (CAID) of total and soluble non-starch polysaccharides (NSP), crude fiber (CF), neutral detergent fiber (NDF) and acid detergent fiber (ADF) in pigs.

			Body weight	CAID change[2]					Reference
				NSP		CF	NDF	ADF	
Process	Feedstuff	NSP[1]	(kg)	Total	Soluble				
Toasting									
	Peas (dried)								
		100	20-50	-16 – 24					[128]
Baking									
	Barley								
		88	>50	9.8 – 12.3					[161]
Pelleting									
	Barley								
		88	>50	-1.8					[12]
Extrusion cooking									
	Barley								
		100	>50	-17.5					[151]
		76	>50	-3.3	19.7				[150]
	Maize								
		58	<20			1.4			[162]
		26 – 54	20-50			-5.2	-3.9	-21.0	[163]
	Peas								
		100	20-50	1.5					[151]
	Wheat bran /potato starch (4:1)								
		100	20-50	5.0					[151]

[1] Contribution of NSP from processed feedstuff to the total dietary NSP content (%).
[2] Change in CAID of the processed diet compared with the untreated control diet in % units.

Thermal treatments that include either moisture addition, (e.g. steam cooking), or a combination of moisture addition with pressure, shear, or both, (e.g. steam-pelleting, expander-processing, extrusion cooking, steam cooking-flaking), can considerably improve digestibility of the fiber fraction in pigs (-18 – 24 % units; Table 2.2 and 2.3) and poultry (4 to 16 % units; Table 2.4). Nevertheless, some conflicting results for hydrothermal treatments have been found[128, 151]. In the study of Canibe and Bach Knudsen[128] (Table 2.2), decreased digestibility of the NSP-fraction in their second experiment (-16 % units) might be explained by the increased cellulose content of this fraction. The reason for the reduced digestibility of the NSP fraction of barley (-18 % units) in the study of Sun et al.[151] remains however, unclear. The largest effects are found for feedstuffs that contain high levels of easily solubilizable NSP, such as β-glucan in barley and pectin in peas (Table 2.3 and 2.4). Mild treatments such as steam cooking might only affect feedstuffs as barley (Table 2.3), whereas more severe processing conditions (i.e. high temperature and shear) like expander processing and extrusion cooking, are required for feedstuffs that contain complex arabinoxylans, such as. maize[116]. As indicated previously, improvements in digestibility of fiber fractions observed after thermal treatments can partly be explained by the reduced particle size obtained during processing.

Table 2.3. Influence of processing technology on change in coefficient of apparent total tract digestibility (CATTD) of total and soluble non-starch polysaccharides (NSP), crude fiber (CF), neutral detergent fiber (NDF) and acid detergent fiber (ADF) in pigs.

Process	Feedstuff	NSP[1]	Body weight (kg)	CATTD change[2]					Reference
				NSP		CF	NDF	ADF	
				Total	Soluble				
Hammer milling (fine vs. coarse)									
	Barley	83 – 93	20-50			-2.9 – 6.4[3]	-1.5		[142, 159]
	Wheat	80	20-50				2.2		[142]
	Wheat based[4]	100	20-50				1.0		[144]
Toasting									
	Barley	38 – 44	<20				-5.6 – -0.6		[11]
	Peas	100	20-50	-4.5 – 1.0					[128]
Baking									
	Barley	88	>50	9.7 – 11.0					[161]
Infrared radiation									
	Maize	54 – 61	<20			-4.1 – 2.5			[164]
Steam cooking									
	Barley	56	<20			2.5			[7]
	Maize	25	<20			0.4			[164]
Pelleting									
	Barley	83	20-50			1.9 – 4.0	2.6 – 3.4	0.8 – 5.2	[121]
	Barley[5]	100	>50	-2.4					[12]
	Peas[6]	100	20-50				5.5	7.4	[165]
	Maize/wheat based[7]	100	20-50	1.0 – 9.0[8]		-2.6 – 16.4	1.5 – 10.2	-9.4 – 13.3	[166]
Infrared radiation-flaking									
	Barley	72	<20				4.6		[154]
	Maize	48	<20				-1.4		[154]
Expander processing									
	Barley	83	20-50			0.2 – 6.5	-1.2 – 2.9	2.3 – 10.1	[121]
	Barley based[9]	100	20-50				0.6		[167]

Process	Feedstuff	NSP[1]	Body weight (kg)	CATTD change[2]					Reference
				NSP		CF	NDF	ADF	
				Total	Soluble				
	Barley/Wheat products[10]	77 – 82	20-50			-0.2 – 1.5	-1.1 – 4.2	0.9 – 2.0	[149]
	Peas	44	20-50				20.1		[167]
	Pea based[11]	100	20-50				5.5		[167]
	Wheat based[4]	100	20-50				0 – 2.0		[144]
Expander processing – pelleting									
	Barley	83				1.6 – 10.2	-0.2 – 5	-1.0 – 8.1	[121]
Extrusion cooking									
	Barley	72	<20				2.5		[154]
		100	20-50	-8.0					[151]
		76	>50	3.8	6.5				[150]
	Maize	48 – 58	<20			-2.4 – 3.0	-1.7		[154, 164]
		26 – 54	20-50			3.7	5.8	-8.8	[163]
	Peas	44 – 100	20-50	5.0			-8.1 – 13.5	-7.6 – 5.3	[151, 165, 167]
	Wheat bran / potato starch (4:1)	100	20-50	18.5					[151]

[1] Contribution of NSP from processed feedstuff to the total dietary NSP content (%).
[2] Change in CATTD of the processed diet compared with the untreated control diet in % units.
[3] Fine milling compared to whole grain.
[4] Grinding and expander process applied to complete diet containing wheat (400 g/kg), barley (129 g/kg), soybean meal (231 g/kg), rapeseed meal (100 g/kg), and wheat feed (100 g/kg).
[5] Pelleting process applied to complete diet containing barley (873 g/kg) and soybean meal (100 g/kg).
[6] Pelleting process applied to complete diet containing peas (850 g/kg).
[7] Pelleting process applied to complete diets containing wheat (123 - 354 g/kg), maize (123 - 354 g/kg), soybean meal (49 - 140 g/kg), barley (35 - 100 g/kg), wheat bran (0 - 180 g/kg), maize bran (0-180 g/kg), soybean hulls (0-180 g/kg), and sugar beet pulp (0 – 90 g/kg).
[8] Change in digestibility (% units) of total dietary fiber as analyzed by the method of Prosky et al.[47].
[9] Expander process applied to complete diet containing barley (305 g/kg), wheat (300 g/kg), and soybean meal (258 g/kg).
[10] Barley and wheat bran-wheat middlings (3:2:1 and 9:2:1).
[11] Expander process applied to complete diet containing peas (400 g/kg), barley (384 g/kg), and soybean meal (66 g/kg).

Table 2.4. Influence of processing technology on coefficients of change in apparent ileal digestibility (CAID) and apparent total tract digestibility (CATTD) of non-starch polysaccharides (NSP), crude fiber (CF), neutral detergent fiber (NDF) and acid detergent fiber (ADF) in poultry.

Process	Feed ingredient	NSP[1]	Age[2]	Change in digestibility[3]					Reference
				CAID	CATTD				
				NSP	NSP	CF	NDF	ADF	
Hammer milling									
	Flaxseed	14	18	-1.3					[19]
	Peas (dehulled)	90	17	7					[28]
Infrared irradiation									
	Barley	55	25		1.2				[29]
Steam cooking - flaking									
	Barley	56	4 – 21				16.0		[153]
	Maize	40	4 – 21				6.5		[30]
Expander processing									
	Barley	55	28		3.8				[29]
Extrusion cooking									
	Soybean meal	88	25	9.9 – 13.0					[20]

[1] Contribution of NSP from processed feedstuff to the total dietary NSP content (%).
[2] Age of the animal in days.
[3] Change in CAID and CATTD of the processed diet compared with the untreated control diet in % units.

Combinations of processing technologies and cell wall degrading enzymes

Effects of combinations of processing technologies and cell wall degrading enzymes on digestion of NSP

Cell wall degrading enzymes can be used to specifically cleave polymers or remove side chains, thereby preventing network or junction zone formation, and to cleave tightly bound nutrients from the cell wall[1, 3, 4]. A large body of literature describing the effects of cell wall degrading enzymes on NSP digestion exists as reviewed by many authors[1, 3-5, 32, 106]. Results indicate that cell-wall degrading enzyme extracts, such as cellulases, glucanases, pectinases and xylanases, can successfully improve NSP digestibility, provided that enzyme activities match the substrate and enzymes are applied in the correct dose[32]. Feed processing can result in increased digesta viscosity, which can negatively affect digestion of other nutrients. Increased digesta viscosity is mainly observed after heat treatments such as extrusion cooking, infrared radiation and steam cooking, especially in poultry[30, 116, 140, 145, 146]. Cell wall degrading enzymes may be used to control viscous properties of processed feedstuffs by degrading the viscous polymers that were solubilized during processing. In addition, modifications in cell wall architecture obtained by processing technologies such as milling, can improve the accessibility of NSP for enzymes. Combinations of processing technologies followed by enzyme treatments could therefore, be of special interest.The reducing effect of enzyme addition on viscosity of the feed ingredient and digesta are 3 to 4 times larger in diets containing heat-processed ingredients than in the corresponding control diets[29, 30, 139, 141, 148, 153, 168] (Table 2.5). In pigs, enzyme addition to a pelleted diet results in a 6-fold improvement in CAID of NSP and a 3-fold improvement in CATTD of NSP compared with enzyme addition to the corresponding unprocessed diet[12]. Similarly, enzyme addition to a diet containing extruded barley, results in a 1.5 times higher CATTD of β-glucan in broilers compared with enzyme treatment of the unprocessed diet[141]. The additional effects of enzyme addition to milled diets compared with enzyme addition to unmilled diets are less clear. Fine milling of flaxseed increases the effect of enzyme supplementation on viscosity and NSP digestibility compared with coarse milled flaxseed. In contrast, the effect of enzyme was positive in crushed peas, but negative when peas were more finely milled[28]. Possibly the enzymes were effective in solubilizing and degrading NSP in the crushed peas, whereas there was no additive effect of enzyme addition in ground peas where NSP-containing cell wall structures were already broken down to a degree.

Table 2.5. Additional effect of enzyme addition to (partly) processed diets compared with enzyme addition to unprocessed diets on viscosity and change in coefficients of apparent ileal digestibility (CAID) and apparent total tract digestibility (CATTD) of the fiber fraction in pigs and poultry.

Processing treatment			Diet characteristics		Enzyme activities	Species	Additional effect of enzyme addition			
								Change in NSP		
Processed ingredient	NSP[1]	Processing technology	Diet ingredients[2]	Diet form			Viscosity[3]	CAID	CATTD	Reference
Barley										
	48	unprocessed	barley, maize, soybean meal	pellet	cellulase, endo-β-1:3,1:4-glucanase, xylanase	broilers	-12[5]		5.4[6]	[141]
		extrusion					-53[5]		8.6[6]	
	44	unprocessed	barley, maize, soybean meal	mash	xylanase, β-glucanase	broilers	-64			[29]
		infrared irradiation flaking					-86			
		expander processing					-73			
	56	unprocessed	barley, soybean meal	mash	xylanase, protease, α-amylase	broilers	-89			[153]
		steam cooking flaking					-95			
Flaxseed										
	14	coarse milling	maize, soybean meal,flaxseed	mash	pectinase, cellulase, xylanase, glucanase, mannanase, galactanase	broilers	-21	1.6		[19]
		fine milling					-37	7.0		
Maize										
	40	unprocessed	maize, soybean meal, wheat middlings	mash	xylanase, protease, α-amylase	broilers	-7			
		steam cooking flaking					-30			[30]
Peas (dehulled)										
	90	crushing	peas	mash	pectinase	broilers		7		[28]
		milling						-8		
	90	crushing	peas	mash	α-galactosidase	broilers		3		[28]
		milling						-5		
Rye										
	54	unprocessed	rye, soybean meal	mash	pentosanase	broilers	-19 – -33			[148]
		autoclaving					-56 – -74			

Processing treatment			Diet characteristics		Enzyme activities	Species	Additional effect of enzyme addition			
								Change in NSP		
Processed ingredient	NSP[1]	Processing technology	Diet ingredients[2]	Diet form			Viscosity[3]	CAID	CATTD	Reference
Wheat										
	92	coarse milling fine milling	wheat, soybean meal	mash	xylanase	finishing pigs	2 13			[168]
	97	unprocessed pelleting	wheat, maize gluten meal	mash	xylanase	broilers	-71 -63			[155]
	52	unprocessed autoclaving	wheat, soybean meal	mash	pentosanase	broilers	-17 -40			[148]
Complete diet										
	100	pelleting extrusion	wheat, maize, soybean meal	pellet	cellulase, endo-β-1:3,1:4-glucanase	broilers	-3[6] -3[6]			[169]
	100	pelleting extrusion	barley, wheat, maize, soybean meal	pellet	cellulase, endo-β-1:3,1:4-glucanase	broilers	-2[6] -10[6]		10[8] 2[8]	[169]
	100	unprocessed pelleting	barley, soybean meal	mash pellet	β-glucanase	finishing pigs		1.0 6.3	1.0 3.3	[12]
	100	unprocessed cold pelleting steam pelleting	barley, wheat, soybean meal, rye	mash pellet	β-glucanase, arabinoxylase	broilers	-4[7] -18[7] -19[7]			[139]

[1] Contribution of NSP from processed feed ingredient to the total dietary NSP content (%).
[2] NSP-containing ingredients included in the diet. Listed from highest to lowest inclusion level.
[3] Change in ileal digesta viscosity (%) of animals fed the enzyme treated diet compared with animals fed the untreated control diet, unless stated otherwise.
[4] Change in CAID or CATTD of NSP (% units) of the enzyme treated diet compared with the untreated control diet, unless stated otherwise.
[5] Change in extract viscosity (%) of the enzyme treated diet compared with the untreated control diet.
[6] Change in CATTD of β-glucan in % units.
[7] Change in extract viscosity (%) of the enzyme treated diet compared with the untreated control diet.
[8] Change in CATTD of dietary fiber (% units) of the enzyme treated diet compared with the untreated control diet.

CONCLUSIONS

Evaluation of the effects of processing technologies on digestion of NSP is hampered by the potential shift of polysaccharides recovered in the fiber fractions of common, gravimetric, fiber analysis methods such as CF, NDF, or ADF. Processes based on mechanical modification of feedstuffs such as hammer and roller milling increase solubility of the NSP-fraction resulting in increased CATTD of the fiber fraction up to 6 or 7 % units in both pigs and poultry. Dry thermal processes have a minor impact on physicochemical properties of feedstuffs and consequently the effects on CAID and CATTD of the fiber fractions in pigs and poultry are limited. Hydrothermal processes that include high shear forces such as expander processing and extrusion cooking are more effective and increase solubility but also viscosity. Results on the CATTD of fiber fractions are hampered by differences between analytical methods, but range between 0 to 19 % units in pigs and 4 to 16 % units in poultry, although some studies have reported decreased digestibility values.

Enzyme addition to heat processed diets and diets containing heat processed ingredients results in a 3- to 4-fold reduction in viscosity, compared with enzyme addition to unprocessed diets, or diets containing unprocessed ingredients. In addition, modifications in cell wall architecture obtained by processing technologies can improve the accessibility of NSP to enzymes. As a result, the effects of enzyme addition on digestibility of the fiber fraction are 1.5 to 6 times larger, when applied to heat processed diets compared with unprocessed diets.

Effects of processing technologies combined with cell wall degrading enzymes on in vitro degradability of barley

Chapter 3

Technologies to improve *in vitro* degradability of barley

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ABSTRACT

Effects of processing technologies and cell wall degrading enzymes on in vitro degradation of barley were tested in a 5 × 2 factorial arrangement: five technologies (unprocessed, wet-milling, extrusion, autoclaving, and acid-autoclaving), with or without enzymes. Digestion in the upper gastrointestinal tract (Boisen incubation) and subsequent, large intestinal fermentation (gas production technique) were simulated in duplicate. All technologies increased digestion of DM (13-43 % units) and starch (22-51 % units) during Boisen incubation, compared with the unprocessed control ($P < 0.01$). Wet-milling, extrusion, and acid-autoclaving increased CP digestion by 29-33 % units ($P < 0.01$), whereas autoclaving did not. Xylanase and β-glucanase addition increased digestion of DM (~20 % units), starch (~20 % units), and CP (~10 % units) in unprocessed and autoclaved barley ($P < 0.01$). Wet-milling, extrusion, and acid-autoclaving, reduced the extent (50 %) and maximum rate (60-75 %) of fermentation ($P < 0.01$), which appeared to reflect the reduced amount of starch present in the Boisen residues. In conclusion, wet-milling, extrusion, and acid-autoclaving successfully improved in vitro starch and CP digestion in barley, half of which is related to the cell wall matrix disruption. Addition of xylanases and β-glucanases improved in vitro starch and CP digestion only in unprocessed barley or barley poorly affected by processing.

INTRODUCTION

Cereal products contain plant cell wall material, mainly composed of non-starch polysaccharides (NSP) that cannot be digested by mammalian enzymes, but can be partly degraded by the microbial community residing in the intestinal tract of animals. The structural arrangement of NSP in plant cell walls often limits degradation of NSP and other nutrients[35]. Feed processing and enzyme technologies can enhance digestive utilization of nutrients from cereals such as barley. Here, the effects of particle size reduction, hydrothermal treatment with or without shear, and acid hydrolysis, in combination with addition of xylanases and β-glucanases on the degradability of barley during *in vitro* digestion and fermentation were investigated. It is hypothesized that the effectiveness of enzyme addition to improve degradation depends on technological pre-treatment.

MATERIALS AND METHODS

Effects of processing technologies and addition of xylanases and β-glucanases (enzyme addition) on *in vitro* degradation of barley were tested in a 5 × 2 factorial arrangement. Digestion in the upper gastrointestinal tract (Boisen incubation) and subsequent, large intestinal fermentation (gas production technique) were simulated in duplicate *in vitro*. Barley was hammer milled using a 3.2 mm sieve. *Wet-milling* was performed using a refiner (Sprout-Waldron, Muncy, Pennsylvania) with a feed rate of 101 $kg \cdot h^{-1}$ and water rate of 489 $litre \cdot h^{-1}$, and maximum temperature of 35 °C. *Extrusion* (77 % DM) was performed using a co-rotating double screw extruder (M.P.F.50, Baker Perkins, Peterborough, United Kingdom) with a feed rate of 28 $kg \cdot h^{-1}$, and a screw speed of 250 rpm. Product temperature at the die was 114 °C. Afterwards, samples were milled (ZM 100; Retsch, Haan, Germany; at 12000 rpm; 3 mm sieve). *Autoclaving* (77 % DM) was performed using a Varioklav 25T tabletop (Thermo Scientific, Waltham, Massachusetts) for 30 min at 120 °C. *Acid-autoclaving* (100 g/L DM) was performed after soaking (4 h, pH 3.4) in maleic acid (10 g/kg DM), and autoclaved as described above. After wet-milling, autoclaving, and acid-autoclaving, samples were freeze-dried. *Xylanases and β-glucanases* (Shearzyme 500L and Ultraflo L; Novozymes, Bagsvaerd, Denmark) were added in the first step of Boisen incubation (25 μl/g barley, each). Digestion in stomach and small intestine was simulated using a modified method of Boisen and Fernández[17, 33]. Samples were not ground prior to incubation, pH during the first incubation step was adjusted to 3.5, and amyloglucosidase was added during the second incubation step. After incubation, samples were centrifuged (10 min; 3030 × *g*), decanted, soaked with demi-water (5 min), centrifuged, and decanted. Residues were freeze-dried. Fermentation in the large intestine was simulated using a cumulative gas production method[170]. After incubation, samples were freeze-dried. Fecal inoculum was prepared from pig feces, collected from five sows. Geometric mean diameter (GMD) of unprocessed barley was analyzed in duplicate using the wet sieve method and of wet-milled and extruded barley using Coulter Counter (Beckman Coulter, Brea, California). Samples were milled (MM 2000; Retsch, Haan, Germany; amplitude 80; 1 min) and analyzed in duplicate for content of DM (ISO 6496[171]), N (AOAC 698.06[13]), total starch (AOAC 996.11), NDF (AOAC 2002.04) and insoluble NSP (Englyst method[49]). Disappearance

of nutrients from unprocessed and processed barley during Boisen incubation (*in vitro* digestion) were calculated using unprocessed barley as the reference. Cumulative gas production corrected for DM (DMCV; ml/g DM) was modeled according to a monophasic model and maximum rate of gas production (R_{max}) and time of R_{max} (T_{max}) were calculated[172]. Results were analyzed by ANOVA, with the GLM procedure of SAS (SASInstitute, 2008) using processing technology, enzyme addition, and its interaction as fixed effects. Model residuals were tested for homogeneity and arcsine root transformed if required.

RESULTS AND DISCUSSION

Analyzed chemical composition of barley before and after processing is presented in Table 3.1. All processing technologies increased *in vitro* DM and starch digestion of barley compared with the unprocessed control (Figure 3.1, $P < 0.01$). Wet-milling, extrusion, and acid-autoclaving increased CP digestion compared with the unprocessed control ($P < 0.01$), whereas autoclaving did not. Enzyme addition increased DM, starch, and CP digestion in unprocessed and autoclaved barley only ($P < 0.01$).

Table 3.1. Dry matter (DM) content (g/kg barley), chemical composition (g/kg DM) and geometric mean diameter (GMD, µm) of unprocessed and processed barley.

Item	Unprocessed	Wet-milled	Extruded	Autoclaved	Acid-autoclaved
DM	878	968	879	958	996
Starch	585	512	572	574	484
CP	94	93	94	97	96
NDF	151	n.d.[1]	n.d.	n.d.	n.d.
I-NSP[2]	191	241	215	208	215
GMD	1210	61	470	n.d.	n.d.

[1] not determined.

[2] Insoluble non-starch polysaccharides.

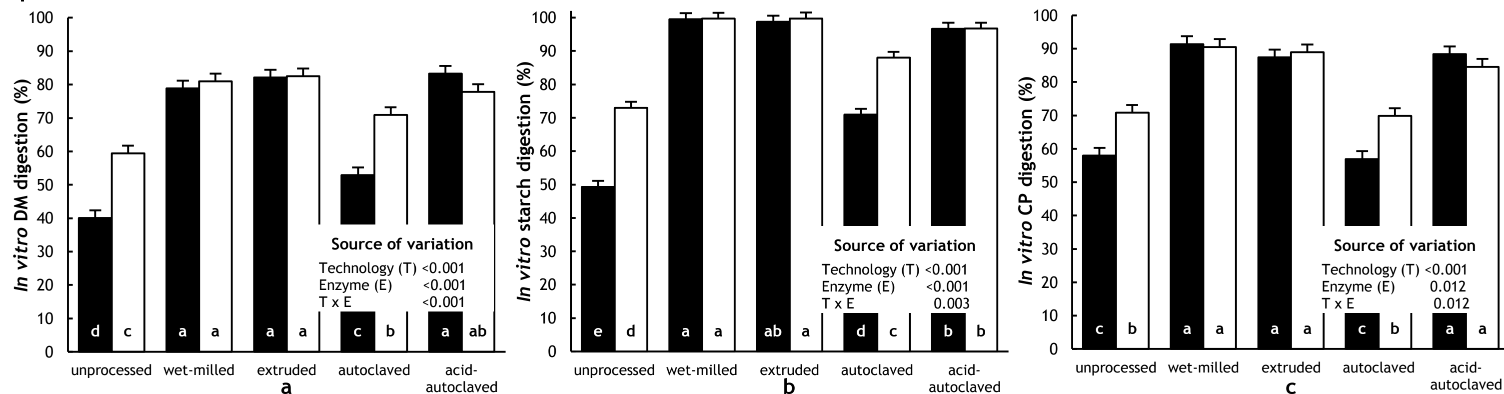

Figure 3.1. In vitro digestion of DM (panel a), starch (panel b), and CP (panel c) of unprocessed and processed barley, with (white bars) or without (black bars) addition of β-glu canases and xylanases (25 µl/g). Means without a common label differ significantly ($P < 0.05$). Error bars indicate SEM.

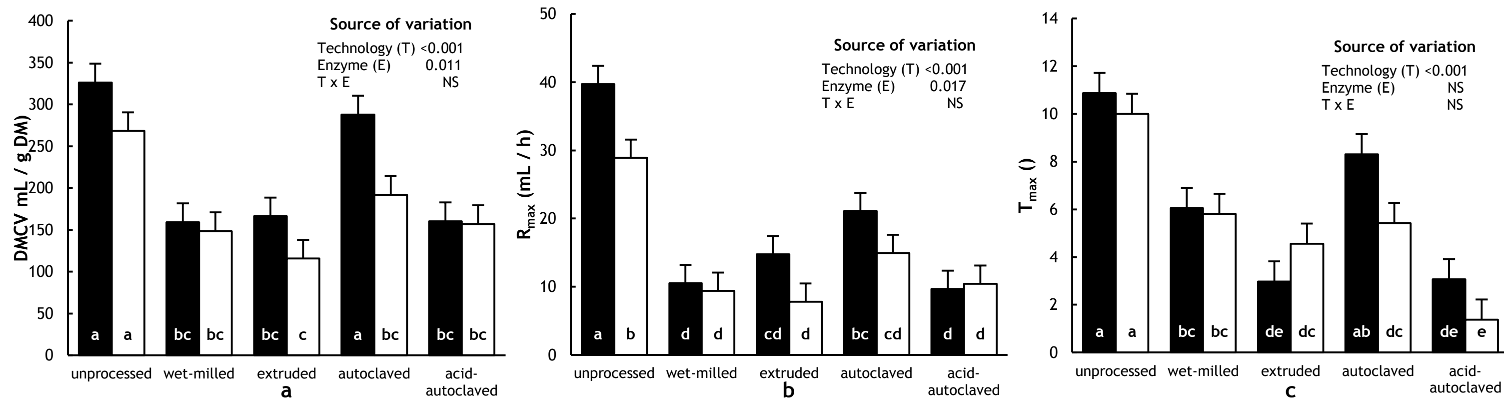

Figure 3.2. Dry matter corrected volume (DMCV, panel a), maximum rate of gas production (R_{max}, panel b), and time at which R_{max} occurs (T_{max}, panel c), of unprocessed and processed barley, with (white bars) or without (black bars) addition of β-glucanases and xylanases (25 µl/g). Means without a common label differ significantly ($P < 0.05$). Error bars indicate SEM.

These results illustrate that wet-milling (particle size reduction), as well as extrusion, effectively improved starch digestion. Autoclaving alone improved starch digestion only to a limited extent, potentially caused by retrogradation. Acid-autoclaving, however, increased starch digestion by 47 % units, almost to the same extent as wet-milling and extrusion. Possibly, less retrograded starch is formed during acid-autoclaving compared to autoclaving, due to the moist and acidic conditions used during this process[173]. The improved CP digestion after wet milling (particle size reduction), extrusion (particle size reduction, shear, and temperature) and acid-autoclaving (temperature, high moisture, and acid hydrolysis) indicates that improvement in CP digestion can be obtained by multiple mechanisms. The absence of improved CP digestion following autoclaving indicates that either thermal treatment without shear forces is less effective, or that there are specific interactions between nutrients, such as in the Maillard reaction. These reactions are less likely to occur under acidic and excess moist conditions[174, 175], which might explain the higher CP digestion in acid-autoclaved barley compared with autoclaved barley. Enzyme addition improves starch and CP digestion, but only in barley that is unprocessed or poorly affected by processing. In wet-milled, extruded, and acid-autoclaved barley there was no additional effect of enzyme addition on starch and CP digestion, which indicates that the cell wall matrix is no longer limiting digestibility in processed barely. This implies that the improvement in starch and CP digestion by wet-milling, extrusion, and acid-autoclaving is partly related to disruption of the cell wall matrix.

It appeared that the gas production results reflected the amount of starch present in the Boisen residues, hiding potential effects of technologies on the fermentation characteristics of barley NSP (Figure 3.2). This is illustrated by strong, negative correlations between DMCV, R_{max}, and T_{max} and starch digestion during Boisen incubation (r=-0.93, -0.93, and -0.81, respectively; $P < 0.01$).

In conclusion, there were clear interactions between processing technologies and enzyme addition on *in vitro* digestion of starch and CP of barley. Wet milling, extrusion, and autoclaving successfully improved *in vitro* digestion of starch (~50 % units) and CP (~30 % units) in barley, whereas addition of xylanases and β-glucanases improved *in vitro* digestion of starch (~20 % units) and CP (~10 % units) only when processing technology was relatively ineffective. This indicates that at least 50 % of the improvement in starch and CP digestion as obtained by processing technology is related to cell wall matrix disruption.

Processing technologies and cell wall degrading enzymes to improve nutritional value of dried distillers grain with solubles for animal feed
An *in vitro* digestion study

Chapter 4

Technologies to improve *in vitro* degradability of DDGS

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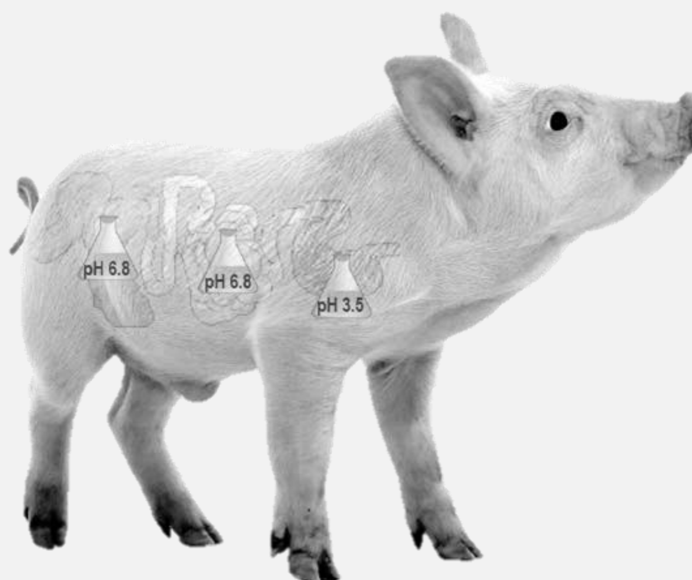

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ABSTRACT

Currently, the use of maize dried distillers grain with solubles (DDGS) as protein source in animal feed is limited by the inferior protein quality and high levels of non-starch polysaccharides (NSP). Processing technologies and enzymes that increase NSP degradability might improve digestive utilization of DDGS, enhancing its potential as a source of nutrients for animals. The effects of various combinations of processing technologies and commercial enzyme mixtures on in vitro digestion and subsequent fermentation of DDGS were tested. Wet-milling, extrusion, and mild hydrothermal acid treatment increased in vitro protein digestion but had no effect on NSP. Severe hydrothermal acid treatments, however, effectively solubilized NSP (48 to 78 %). Addition of enzymes did not affect NSP solubilization in unprocessed or processed DDGS. Although, the cell wall structure of DDGS seems to be resistant to most milder processing technologies, in vitro digestion of DDGS can be effectively increased by severe hydrothermal acid treatments.

INTRODUCTION

Maize dried distillers grain with solubles (DDGS), a by-product from bioethanol production, is increasingly being used as protein source in animal feed[14] and its availability is expected to increase even further in the future[56]. Compared with protein sources such as soybean meal, the nutritional value of DDGS is lower due to its inferior protein quality, partly caused by the excessive pre-treatment and drying conditions during the ethanol production process, and high level of non-starch polysaccharides (~ 30 %)[14, 56]. Non-starch polysaccharides (NSP), although partly fermented by the microbial community residing in the intestinal tract of the pig, are not completely degraded. Less than 50 % of the NSP from DDGS are degraded by the pig, leaving more than 30 % of DDGS' energy unused[40, 176]. In addition, especially in young pigs, NSP may affect digestion of other nutrients, both directly due to physical hindrance and indirectly due to physiological changes in the gut[83, 84].

Hence, the animal feed industry explores opportunities to improve degradability of the NSP-fraction from feedstuffs, thereby enhancing its potential as a source of nutrients for animals. Commonly used feed processing technologies, such as hammer milling and pelleting, effectively improve the degradability of easily solubilizable NSP, but might not be sufficient to affect more recalcitrant NSP structures, such as arabinoxylans in maize (Chapter 2). Therefore, more effective technologies are required to modify cell wall structure and allow exogenous or endogenous enzymes to degrade the complex NSP structures[177]. Mechanical forces open the cell wall structure and reduce particle size, thereby increasing the surface area accessible for microbial and endogenous enzymes. Thermal processes can break weak bonds between polysaccharides and glycosidic linkages within polysaccharides, but excessive heating may increase protein and amino acid damage[99]. Hydrothermal pre-treatments using acid catalysts are established methods to improve extractability of lignocellulosic material[124]. Potential protein damage and high residual acid or mineral concentrations limit the use of extremely high processing temperatures and high acid concentrations for processing animal feedstuffs. Instead, relatively mild acid treatments – i.e. having a low combined severity factor (CSF) – using dicarboxylic organic acids, such as maleic acid, could be of special interest[178]. In addition, cell wall degrading enzymes, such as xylanases, can be used to specifically cleave polymers or remove side-chains[32]. It is hypothesized that the effectiveness of enzymes to improve NSP degradability will depend on the extent to which the cell wall structure is modified during processing.

This manuscript describes two successive experiments in which the effects of various combinations of processing technologies and enzymes on *in vitro* digestion and subsequent fermentation of maize and DDGS are tested. In experiment 1, the effects of particle size reduction, hydrothermal treatment with or without shear, and acid hydrolysis in the presence or absence of cell wall degrading enzymes on physicochemical properties and *in vitro* degradation of maize and DDGS were investigated. Based on the results, experiment 2 was designed to investigate the effects of more severe hydrothermal acid treatments on *in vitro* digestion of DDGS.

MATERIALS AND METHODS

Experimental design

In experiment 1, effects of processing technologies and cell wall degrading enzymes on physicochemical properties and *in vitro* degradation of maize and DDGS were tested in a 5 × 2 factorial arrangement: five processing technologies (unprocessed, wet-milling, extrusion, autoclaving, and hydrothermal acid treatment), each with or without the addition of cell wall degrading enzyme mixtures. Enzymatic digestion in the upper gastrointestinal tract and subsequent large intestinal fermentation were simulated in duplicate.

In experiment 2, effects of hydrothermal acid treatments – varying in type of acid and acidity level – and cell wall degrading enzymes on *in vitro* degradation of DDGS were tested in a 4 × 2 factorial arrangement: four hydrothermal acid treatments (unprocessed, maleic 2.9, maleic 2.3, sulfuric 2.9), with or without enzyme addition. The concentration of acid used for maleic 2.3 was twice the concentration used for maleic 2.9. The concentration of sulfuric acid was chosen to result in a similar pH and thus a similar CSF as maleic 2.9. Solubilization of non-starch polysaccharides during the enzymatic digestion procedure was tested in triplicate.

Materials

Whole maize grain (*Zea mays*) and unpelleted DDGS were obtained from a commercial bioethanol plant (Abengoa Bioenergy, France). Maize was milled using a hammer mill, at 1475 rpm using a 3.2 mm sieve, whereas DDGS was used as mash.

Processing and enzyme technologies

Wet-milling. Maize and DDGS were milled using a laboratory scale refiner (Sprout-Waldron), at a feed rate of 102 $kg \cdot h^{-1}$ using 480 liter water per h. Diameter of the discs was 30 cm with the distance between discs set at 0.07 mm and a rotation speed of 3000 rpm. Product temperature when leaving the refiner was 35 °C. Product was collected in bins after the process reached a steady-state and subsamples were taken under continuous mixing. Samples were cooled to room temperature, frozen (-20 °C), and freeze-dried.

Extrusion. Maize and DDGS were mixed with water using a paddle-mixer (Type F60; Halvor Forberg, Bygland, Norway) to reach a dry matter (DM) content of 80 %. Within 30 minutes after mixing, samples were extruded using a co-rotating double screw extruder (M.P.F.50; Baker Perkins, Peterborough, United Kingdom), without additional steam conditioning. The extruder had a screw length/diameter ratio of 25. The screw configuration was as follows: 4 1.5D feed screw elements, 1 1D single lead element, 3 1D feed screw elements, 1 1D single lead element, 2 1D feed screw elements, 2 4D 90 degree forwarding block paddles, 1 1.5D feed screw elements, 1 4D 90 degree forwarding block paddles, 1 1.5D feed screw elements, 2 4D 90 degree forwarding block paddles, and 2 1.5D single lead elements. A die with two orifices (6 mm) was used; no die face cutter was used. Feeding rate was set at 30 $kg \cdot h^{-1}$, screw speed was 250 rpm. Barrel temperatures in the ten segments of the extruder were set at 30, 50, 72, 82, 90, 105, 115, 120, 120, and 120 °C. Product temperature at the die was 115 °C. Samples were collected after the process reached a steady-state and

cooled to room temperature. Samples were frozen (-20 °C) without further freeze-drying.

Extruded maize samples were ground in an ultracentrifuge mill (ZM 100; Retsch, Haan, Germany) at 12000 rpm using a 3 mm sieve. Extruded DDGS samples were ground using a mortar and pestle.

Autoclaving. Maize and DDGS were mixed with water by hand to reach a DM content of 80 %. Samples were then autoclaved (Varioklav 25 T tabletop; Thermo Scientific, Waltham, MA, USA) during 30 minutes at 120 °C, starting when the preset temperature was reached. Samples were cooled to room temperature, frozen (-20 °C), and freeze-dried.

Hydrothermal acid treatment. For experiment 1, 1.4 g maleic acid (> 98.0 % pure, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 1L water and added to 100 g maize or DDGS DM. For experiment 2, 6.5 g maleic (maleic 2.9), 13 g maleic (maleic 2.3), or 28 g sulfuric (sulfuric 2.9) acid was dissolved in 1L water and added to 100 g DDGS DM. Samples were mixed by hand, soaked (4 h), and autoclaved as described above. In experiment 1, the pH of the solution after autoclaving was 3.6 for maize and 4.2 for DDGS, corresponding to a CSF of -1.53 and -2.13, respectively. In experiment 2, the pH of the solution after autoclaving was 2.9 for maleic 2.9, 2.3 for maleic 2.3, and 2.9 for sulfuric 2.9, corresponding to a CSF of -0.83, -0.23, and -0.83, respectively.

Cell wall degrading enzyme treatment. The enzyme treatment comprised a combination of commercial food-grade enzyme mixtures commonly used in bakery and brewing processes: Shearzyme®500L and Ultraflo™L (Novozymes, Bagsvaerd, Denmark), with mainly endo-1,4-β-xylanase and endo-1,4-β-glucanase activities. The declared enzyme activity by the manufacturer was 500 FXU-S/g for Shearzyme ®500L and 45 FBG/g for Ultraflo™L. Each enzyme was added to the substrates dissolved in buffer solution during the first incubation step of the Boisen and Fernández[13] procedure at a concentration of 25 μL/g of substrate.

In vitro digestion and fermentation

Enzymatic digestion in the stomach and small intestine was simulated using a modified method of Boisen and Fernández[17], as described by Pustjens et al.[33] Further milling prior to the procedure was omitted, pH during the first incubation step was adjusted to 3.5, and amyloglucosidase[116] was added during the second incubation step. Briefly, 10 g sample were mixed with phosphate buffer (250 ml, 0.1M, pH 6.0) and HCL solution (30 ml, 0.2M) and pH was adjusted to 3.5, using 1 M HCL or 1 M NaOH. Pepsin solution (10 ml, 25g/L, porcine pepsine: 2000 FIP U/g, Merck, Darmstadt, Germany) was added and samples were incubated for 75 minutes at 40 °C. Afterwards, phosphate buffer (100ml, 0.2M, pH 6.8) and NaOH solution (40 ml, 0.6M) were added and pH was adjusted to 6.8. Pancreatin solution (10 ml, 100g/L, porcine pancreatin: grade IV, Sigma-Aldrich, St. Louis, MO, USA) and amyloglucosidase (55mg, amyloglucosidase from Aspergillus Niger: 120U/g, Sigma-Aldrich, St. Louis, MO, USA) were added and samples were incubated for 3.5 h at 40 °C. Supernatant was boiled for 30 min. and frozen (-20 °C). Residues were washed with demineralized water after

centrifugation (3030 *g* for 10 min.) to remove free glucose, decanted, frozen (-20 °C), and freeze-dried.

Fermentation in the large intestine was simulated using a cumulative gas production method as described by Williams et al.[170]. Briefly, 0.5 g Boisen residue were mixed with 89 ml buffer solution containing macro- and micro-minerals and fecal inoculum. Samples were incubated for 72 h at 39 °C in shaking water baths (40 rpm). Cumulative gas production during incubation was measured using a fully automated time related gas production system[179]. After incubation samples were frozen (-20 °C) and freeze-dried. Fecal inoculum was prepared from pig feces, collected from five sows that were fed commercial diets containing barley (303 g/kg), wheat middlings (200 g/kg), maize (100 g/kg), rapeseed meal (75 g/kg), soya hulls (50 g/kg), wheat (50 g/kg), and linseed (15 g/kg) as the main feed ingredients. Fermentation was performed in two separate runs, executed in two subsequent weeks.

Analytical methods

The geometric mean diameter (GMD) of unprocessed products was analyzed using the wet sieve method and calculated according to the ASABE method[180]. Geometric mean diameter of wet-milled and extruded samples was analyzed using A Coulter Counter (Beckman Coulter, Brea, California). Water binding capacity (WBC) was analyzed by soaking 250 mg of raw material or freeze-dried residue in 10 mL of water for 24 h at room temperature. Samples were centrifuged (3274 *g* for 20 min) at room temperature and subsequently drained inverted for 15 min. Water binding capacity was calculated as the weighed quantity of water retained per g of dry material.

Prior to chemical analyses, samples were ground in a mixer mill (Retsch MM 2000) at an amplitude of 80, for 1 min. Unprocessed and processed products and residues of enzymatic digestion were analyzed for the content of DM (103 °C overnight), nitrogen (AOAC 968.06[13]; using a Thermo Quest NA 2100 Nitrogen and Protein Analyser; Interscience, New York), total starch (AOAC 996.11; using a commercial test kit, Megazyme international Ltd., Ireland), and total (DDGS) or insoluble (maize) NSP analyzed as neutral sugars and uronic acids, according to the procedure described below. Neutral sugar composition was analyzed by gas chromatography according to the method of Englyst and Cummings[49]. After pre-treatment with 72 % (wt/wt) H_2SO_4 for 1 h at 30 °C, samples were hydrolyzed with 1 M H_2SO_4 at 100 °C for 3 h. Constituent sugars were derivatized into alditol acetates and analyzed using a GC (Focus-GC, Thermo Scientific, Waltham, Massachusetts). Inositol was used as an internal standard. Uronic acid content was analyzed according to the automated colorimetric m-hydroxydiphenyl assay[21] using an auto-analyzer (Skalar Analytical B.V., Breda, The Netherlands). Galacturonic acid was used for calibration. NSP content was calculated as the sum of neutral sugars and uronic acids minus glucosyl from starch. Protein content was calculated from the N-content using a protein conversion factor of 5.7[181]. Unprocessed products were additionally analyzed for the content of ether extract using a Soxhlet device with petroleum ether (AOAC 920.39) and ash (AOAC 942.05).

Calculations and statistical analysis

The CSF of the hydrothermal acid treatments was calculated as:

Log{t x exp[(T-100)/14.75]}–pH, where *t* is the treatment time (min), *T* is temperature (°C) and *pH* the acidity level.

Solubilization of nutrients in unprocessed and processed maize and DDGS during the enzymatic digestion procedure (*in vitro* digestion) was calculated using the unprocessed products as the reference. Total gas production volume corrected for DM (mL/g DM) was calculated from the cumulative gas production at 72 h. Gas production data for each bottle were modeled according to a monophasic model and the maximum rate of gas production was calculated as described by Groot et al.[172]

The effects of experimental treatments on *in vitro* digestion of DM and nutrients and on parameter estimates of the gas production curves during *in vitro* fermentation were analyzed by analysis of variance, using the GLM procedure of SAS (version 9.2, SAS Institute Inc., Cary, NC). Processing technology, enzyme addition, and its interaction were included as fixed effects in the model. For treatment effects on the parameter estimates of gas production curves, the effect of run (first or second) was included in the model if found to be significant. Interactions between run and treatment effects were tested but found to be not significant in all cases and excluded from the model. Model residuals were tested for homogeneity and normality, to verify model assumptions. Least square means were compared using a least significant differences procedure. Data are presented as least square means and standard error of the mean (SEM) unless stated otherwise. Differences among means with $P < 0.05$ were accepted as representing statistically significant differences.

RESULTS AND DISCUSSION

Chemical and physical characteristics. In contrast to maize, DDGS contained only small amounts of starch (3 %) whilst protein (29 %) and NSP (27 %) content were concentrated 3- to 4-fold (Table 4.1 and Supplemental information Table 4.3), as expected[42]. Non-starch polysaccharides in maize DDGS are mainly composed of cellulose and (arabino) xylans[43], as confirmed by the sugar composition found here (Table 4.1). Unpublished results from our research group indicate that also yeast β-glucans (7 %) and possibly mannans (<2 %) are present. Although detailed structural characterization of the NSP in DDGS is lacking, it can be speculated that glucuronoarabinoxylans – abundant in the water-unextractable solids (WUS) of maize[182] – are dominating. These heteroxylans are highly substituted with monomeric arabinosyl and glucuronic acid residues, as well as oligomer-side chains containing arabinosyl, xylosyl, and galactosyl residues, and are highly cross-linked by diferulic acid bridges[182, 183]. The ratio of arabinosyl and uronyl to xylosyl residues is indicative for the degree of substitution and thus related to the structure of the (glucurono) arabinoxylans present in DDGS. Higher arabinosyl:xylosyl (Ara:Xyl) and uronyl:xylosyl (UA:Xyl) ratios, indicate that relatively more xylopyranosyl units from the xylan-backbone are substituted with arabinofuranosyl or glucuronic acid residues, generally suggesting a higher degree of branching. The Ara:Xyl and UA:Xyl ratios (Table 4.1) found for the unprocessed DDGS (0.71 and 0.20, respectively), are within the ranges described for maize[182] and maize fiber[177].

Table 4.1. Analyzed nutrient composition (g/100 g DM) of the unprocessed maize and unprocessed, wet-milled, extruded, autoclaved, or hydrothermal acid treated (maleic acid) maize dried distillers grain with solubles (DDGS)

	Maize	DDGS				
		Unprocessed	Wet-milled	Extruded	Autoclaved	Maleic acid
Dry matter, g/100g fresh	88	89	96	83	96	95
Protein	9	29	29	30	30	31
Starch	68	3	3	3	3	3
Non-starch polysaccharides	8	27	29	31	27	28
Molar composition of NSP[1]						
Rhamnosyl	1	0	0	0	0	0
Arabinosyl	21	22	21	21	21	19
Xylosyl	30	30	30	31	31	31
Mannosyl	2	5	4	4	4	4
Galactosyl	5	5	5	5	6	5
Glucosyl	30	31	33	31	31	34
Uronyl	12	7	7	7	7	7
Ara:Xyl[2]	0.68	0.74	0.70	0.67	0.66	0.60
UA:Xyl[3]	0.38	0.24	0.22	0.21	0.23	0.22
Particle size (µm)	534	526	97	377	n.a.[4]	n.a.[4]
Water binding capacity (g/g DM)	1.5	2.3	3.0	3.0	2.1	2.9

[1] Mol%; presented as anhydrosugar moieties
[2] Molar ratio of arabinosyl:xylosyl
[3] Molar ratio of uronyl:xylosyl
[4] Not analyzed

Particle size of DDGS was within the expected range[184]. Wet-milling effectively reduced geometric mean diameter (GMD) by 82 %, but also extrusion reduced GMD (28 %). Water binding capacity (WBC) was higher in DDGS compared with maize (Table 4.1). Compared with unprocessed DDGS, WBC was higher in wet-milled, extruded, and hydrothermal acid treated DDGS, whereas it remained unchanged after autoclaving.

Table 4.2. Analyzed non-starch polysaccharide (NSP) content (g/100g DM) and molar composition of NSP of unprocessed and hydrothermal acid treated maize dried distillers grain with solubles (DDGS, experiment 2). Hydrothermal acid treatment was performed using maleic acid at two levels of acidity: maleic 2.9 (pH 2.9) and maleic 2.3 (pH 2.3), or using sulfuric acid (sulfuric 2.9; pH 2.9).

	Unprocessed	Maleic 2.9	Maleic 2.3	Sulfuric 2.9
CSF[1]		-0.83	-0.23	-0.83
Non-starch polysaccharides	28	24	23	26
Molar composition of NSP[2]				
Rhamnosyl	0	0	0	0
Arabinosyl	20	16	18	18
Xylosyl	28	23	27	27
Mannosyl	4	18	11	11
Galactosyl	5	4	5	5
Glucosyl	37	33	34	33
Uronyl	6	6	6	6
Ara:Xyl[3]	0.70	0.70	0.68	0.69
UA:Xyl[4]	0.21	0.28	0.24	0.22

[1] Combined severity factor (Chum et al., 1990).
[2] Mol%; presented as anhydrosugar moieties.
[3] Molar ratio of arabinosyl:xylosyl.
[4] Molar ratio of uronyl:xylosyl.

In vitro digestion and fermentation. This study aimed to investigate the effects of particle size reduction, hydrothermal treatment with or without shear, and acid hydrolysis, in combination with addition of commercial cell wall degrading enzyme mixtures on the degradability of maize and DDGS during *in vitro* digestion and fermentation. Enzymatic digestion in the stomach and small intestine was simulated using a two-step *in vitro* digestion procedure, which is commonly used to asses *in vitro* degradability of feedstuffs. Technically, disappearance of DM and nutrients during this procedure reflects the amount solubilized – i.e. only partly resulting from actual digestion by the added digestive enzymes – rather than the amount degraded. Because NSP cannot be degraded by mammalian enzymes, disappearance of NSP solely encompasses solubilization. Nonetheless, solubilization of NSP is related to their degradation *in vivo*, as modifications in cell wall matrix affect accessibility for microbial enzymes (Chapter 2). Together with fermentability of *in vitro* digested residues, results of this study provide an indication how processing technologies and commercial enzyme mixtures will affect degradability of NSP in the animal, which can be of use for future *in vivo* trials.

In experiment 1, wet-milling, extrusion, and hydrothermal acid treatment increased *in vitro* DM digestion of maize by 14 to 30 % units ($P < 0.01$), mainly due to increased starch (Supplemental information, Figure 4.5) and protein digestion (Figure

4.1). In DDGS, DM digestion increased only by 8 to 11 % units ($P < 0.01$), as a result of increased protein digestion (Figure 4.1). In experiment 2, hydrothermal acid treatment increased *in vitro* DM digestion of DDGS by 18 to 34 % units ($P < 0.01$), mainly due to increased protein (data not shown) and NSP solubilization (Figure 4.3).

Protein degradation. Although protein digestion in unprocessed maize and DDGS was similar (60 %), wet-milling, extrusion, and hydrothermal acid treatment improved protein digestion by 12 to 27 % units ($P < 0.01$) in maize (Figure 4.1B), whereas in DDGS this increase was only 6 to 12 % units ($P < 0.01$; Figure 4.1E). Apparently, the indigestible fraction of DDGS protein is less susceptible for modification compared to that of maize protein. This could be a result of changes in maize protein structure that occur during the bioethanol production process, as illustrated by the higher fraction of protein that is associated with cell wall material in DDGS compared with maize[185]. Alternatively, the susceptibility of yeast protein – which constitutes approximately 20 % of DDGS protein[186] – to protein and amino acid damage during extrusion and hydrothermal acid treatment, may be increased. Han and Liu[186] suggested that the major part of yeast protein will be present in the form of free amino acids, which might be more reactive[174]. Autoclaving reduced protein digestion in DDGS (7 % units, $P < 0.01$), suggesting that proteins were damaged as a result of specific interactions between molecules, such as in the Maillard reaction[187]. These reactions are less likely to occur under acidic and excess moist conditions[174, 175], which might explain the higher protein digestion in acid-autoclaved products (hydrothermal acid treatment) compared with autoclaved products.

Non-starch polysaccharide degradation. Non-starch polysaccharide solubilization of maize was increased by hydrothermal acid treatment combined with commercial enzyme mixtures only (18 % units, $P < 0.01$; Figure 1C), indicating that modification of the cell wall matrix was required to allow enzymes to work[188]. The increased WBC after wet-milling, extrusion, and hydrothermal acid treatment (Table 4.1) indicates that the cell wall matrix was, at least to a certain extent, affected by these technologies. Nonetheless, solubilization of NSP from DDGS was not affected by the processing technologies and commercial enzyme mixtures used in experiment 1 (Figure 4.1F).Except for wet-milling, processing technologies and enzyme treatment did not affect the extent and rate of fermentation of the undigested DDGS residues (Figure 4. 2). Autoclaving followed by enzyme treatment reduced, however, total gas produced at 24 h (data not shown), which is often seen as a relevant time point in the fermentation for monogastrics[189]. Possibly, protein-cell wall interactions that occur during autoclaving reduce NSP degradability. The effect of wet-milling on the maximum rate of fermentation in undigested residues of both maize (online supplement, Figure 4.3) and DDGS (Figure 4.2), demonstrates the accelerating effect of decreased particle size on fermentation[190], which could indicate higher fermentability *in vivo*.

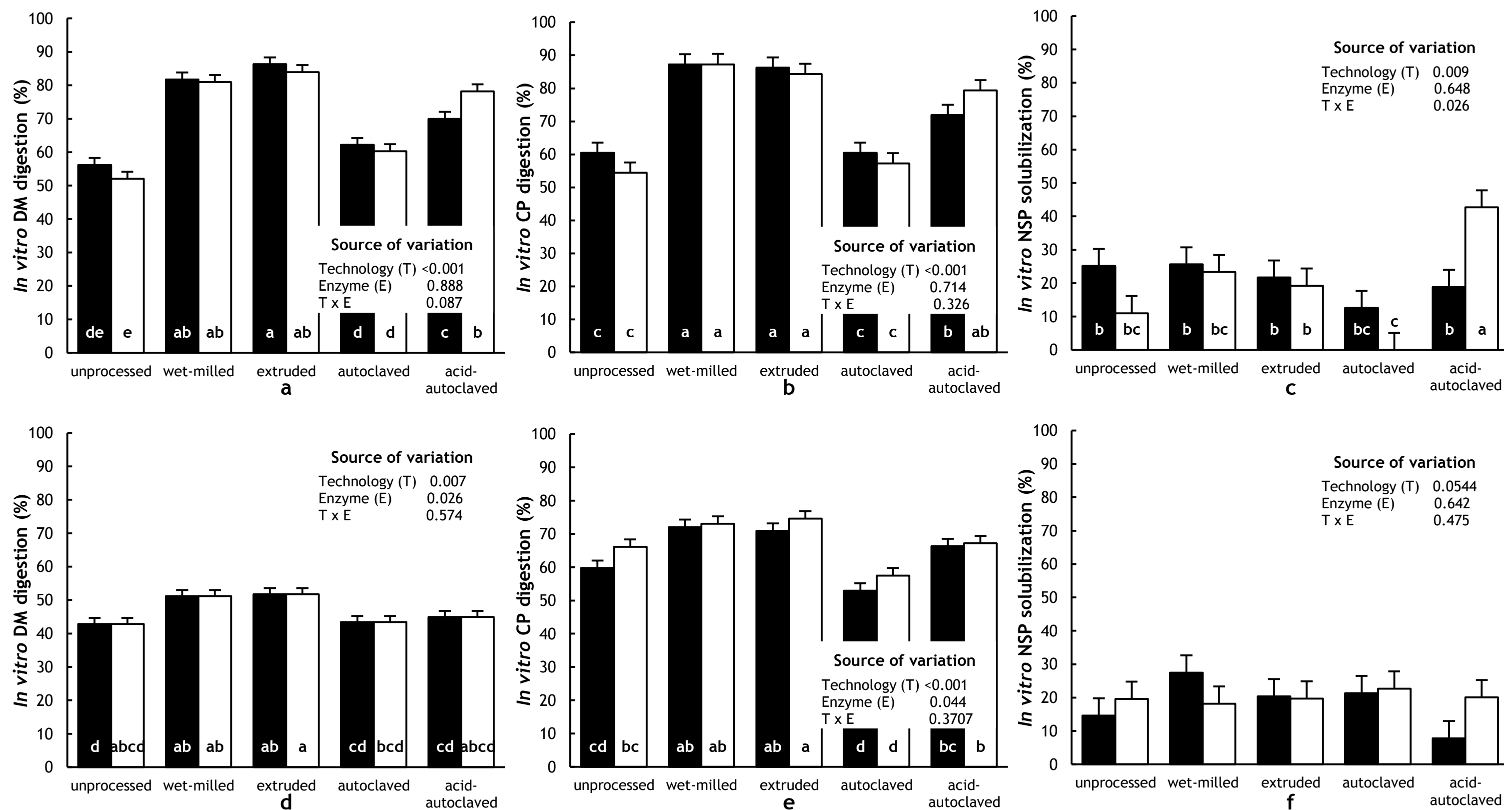

Figure 4.1. In vitro digestion of dry matter (panel a, d), protein (panel b, e), and solubilization of non-starch polysaccharides (NSP, panel c, f) from unprocessed, wet-milled, extruded, autoclaved, or hydrothermal acid treated (acid) maize and maize dried distillers grain with solubles (DDGS), with (white bars) or without (black bars) the addition of commercial enzyme mixtures. Error bars indicate SEM.

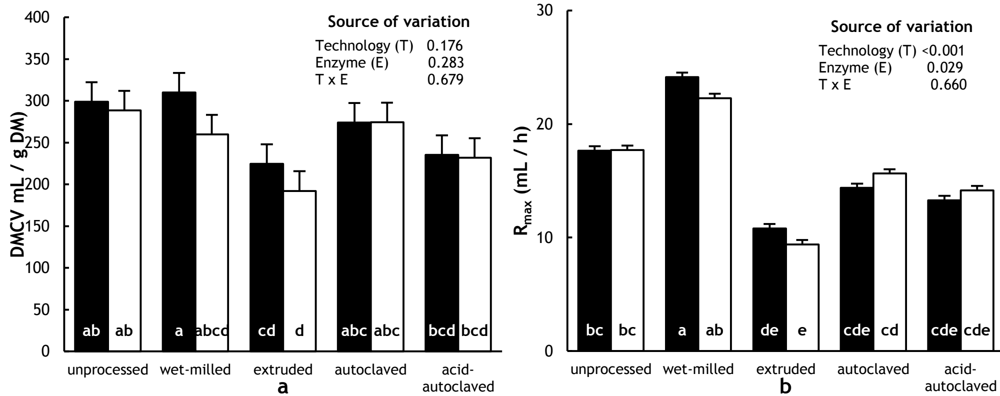

Figure 4.2. Total gas production (panel a) and maximal gas production rate (panel b) of unprocessed, wet-milled, extruded, autoclaved, or hydrothermal acid treated (maleic acid) maize dried distillers grain with solubles (DDGS), with (white bars) or without (black bars) the addition of commercial enzyme mixtures. Error bars indicate SEM.

In summary, the results of experiment 1 indicate that the cell wall structure of DDGS is hardly affected by the processing technologies and commercial enzyme mixtures employed in the present study. We concluded that more severe processing technologies are required to untangle the cell wall structure of DDGS. Therefore, in experiment 2, the effects of hydrothermal acid treatments using higher acid concentrations were tested. Hydrothermal acid treatment effectively increased solubilization of NSP from DDGS during *in vitro* digestion (~30 to 60 % units, $P < 0.01$), both using maleic acid at two levels of acidity and sulfuric acid (Figure 4.3B). At similar pH, NSP solubilization was higher when maleic acid was used compared with sulfuric acid (~ 7 % units, $P < 0.01$). Higher efficiency of maleic acid compared with sulfuric acid was also reported by Lee and Jeffries[178] for maize cobs treated at higher CSF (1.8 to 2.1). A twofold increase in the concentration of maleic acid resulted in an additional increase in solubilization of NSP by ~20 % units ($P < 0.01$). From the remaining constituent sugars in the *in vitro* digested residues it can be seen that when maleic acid at a low levels of acidity and sulfuric acid are used, mainly arabinosyl and to a lesser extent xylosyl and uronyl containing polymers are solubilized (Figure 4.3C); Ara:Xyl and UA:Xyl ratios decreased after *in vitro* digestion (Figure 4.4). This indicates that mainly highly substituted arabinoxylans are affected by hydrothermal acid treatment. Arabinosyl, which is more acid-labile compared with xylosyl, is removed from the xylan backbone, leaving lower substituted xylans in the residue. At a higher level of acidity (maleic 2.3), almost all xylan structures seem to solubilize. The Ara:Xyl ratio of xylans in the *in vitro* digested residues is decreased even more than at the lower level of acidity, whilst the UA:Xyl ratio remained at the level of unprocessed residues (Figure 4.4), suggesting that arabinosyl substituents are more easily removed from the xylan backbone than uronic-acid residues. Cellulose remains virtually insoluble in all three treatments as expected for the selected CSF, because of its rigid structure and strong anchorage in the cell wall matrix[51, 177].

Addition of commercial enzyme mixtures did not affect NSP solubilization in unprocessed or processed DDGS, neither in experiment 1 nor in experiment 2. In maize, however, enzymes increased NSP solubilization after sufficient technological processing (hydrothermal acid treatment). Apparently, xylans from maize that can be potentially degraded by the commercial enzyme mixtures used were either removed or modified during the ethanol production process. Possibly, the high amount of substituents of maize pericarp xylans that are concentrated in DDGS hinders activity of the xylanases in the enzyme mixtures used[191]. The increased protein digestibility resulting from enzyme treatment (1 to 6 % units, $P < 0.05$; Figure 4.1E) indicates, however, that enzyme treatment affects the cell wall matrix to a certain extent, such that protein associated to cell wall material is released more easily.

In conclusion, *in vitro* digestion of protein from maize, and to a lesser extent DDGS is increased by wet-milling, extrusion, and hydrothermal acid treatment. The cell wall structure of DDGS was resistant to most processing technologies, but the increased NSP solubilization after severe hydrothermal acid treatments, illustrates that *in vitro* digestion of DDGS may be effectively increased. Maleic acid was more effective than sulfuric acid. A twofold increase in maleic acid concentration resulted in additional solubilization of NSP by ~20 % units. Commercial enzyme mixtures did not affect NSP solubilization in unprocessed or processed DDGS, but increased *in vitro* protein digestibility.

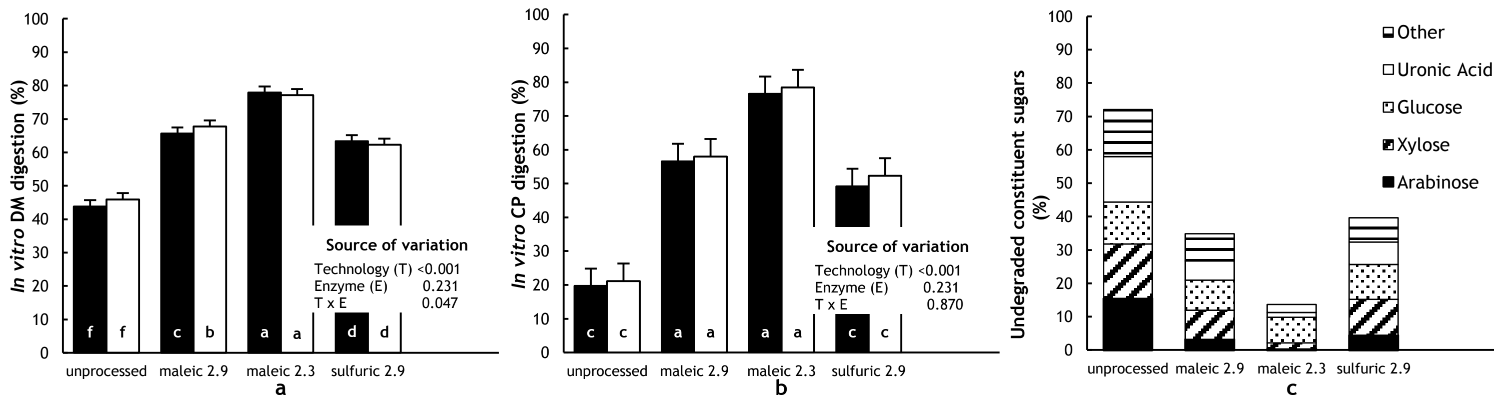

Figure 4.3. *In vitro* digestion of dry matter (panel a) and solubilization of non-starch polysaccharides (NSP, panel b) of unprocessed and hydrothermal acid treated maize dried distillers grain with solubles (DDGS), with (white bars) or without (black bars) the addition of commercial enzyme mixtures. Hydrothermal acid treatment was performed using maleic acid at two levels of acidity: maleic 2.9 (pH 2.9) and maleic 2.3 (pH 2.3), or using sulfuric acid (sulfuric 2.9; pH 2.9). Error bars indicate SEM. In panel c, the remaining constituent sugars (% of constituent sugars before incubation) in *in vitro* digested residues of unprocessed and or hydrothermal acid treated DDGS are presented.

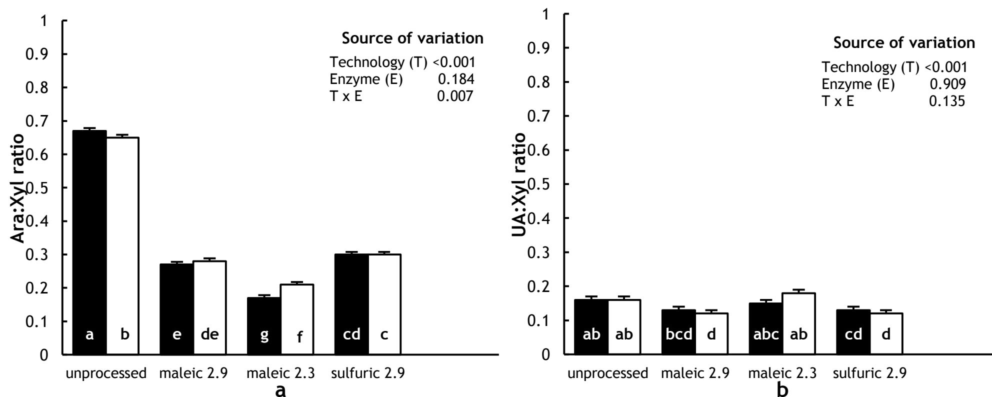

Figure 4.4. Mean molar arabinosyl:xylosyl (Ara:Xyl) and uronyl:xylosyl ratio (UA:Xyl) in in vitro digested residues from unprocessed and hydrothermal acid treated maize dried distillers grain with solubles (DDGS), with (white bars) or without (black bars) the addition of commercial enzyme mixtures. Hydrothermal acid treatment was performed using maleic acid at two levels of acidity: maleic 2.9 (pH 2.9) and maleic 2.3 (pH 2.3), or using sulfuric acid (sulfuric 2.9; pH 2.9). Error bars represent SEM.

SUPPLEMETAL INFORMATION

Table 4.3. Analyzed nutrient composition (g/100 g DM) of unprocessed, wet-milled, extruded, autoclaved, or hydrothermal acid treated (maleic acid) maize.

	Maize				
	Unprocessed	Wet-milled	Extruded	Autoclaved	Maleic acid
Dry matter (g/100g fresh)	88	98	86	98	97
Protein	9	9	9	9	9
Starch	68	65	71	71	67
Non-starch polysaccharides	8	6	6	8	7
Molar composition of NSP[1]					
Rhamnosyl	1	0	0	0	0
Arabinosyl	21	23	22	21	14
Xylosyl	30	35	31	28	32
Mannosyl	2	1	5	3	1
Galactosyl	5	4	4	2	4
Glucosyl	30	23	22	31	36
Uronyl	12	13	15	16	12
Ara:Xyl[2]	0.68	0.64	0.72	0.75	0.44
UA:Xyl[3]	0.38	0.38	0.50	0.56	0.39
Particle size (µm)	534	67	588	n.d.[4]	n.d.[4]
Water binding capacity (g/g DM)	1.5	2.1	4.0	2.1	5.9

[1] Mol%; presented as anhydrosugar moieties.
[2] Molar ratio of arabinosyl:xylosyl.
[3] Molar ratio of uronyl:xylosyl.
[4] Not determined.

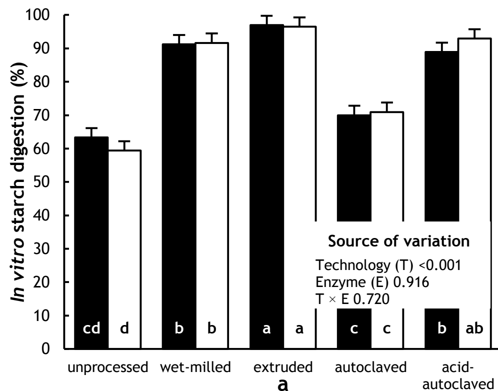

Figure 4.5. *In vitro* digestion of starch from unprocessed, wet-milled, extruded, autoclaved, or hydrothermal acid treated (acid) maize, with (white bars) or without (black bars) the addition of commercial enzyme mixtures. Error bars indicate SEM.

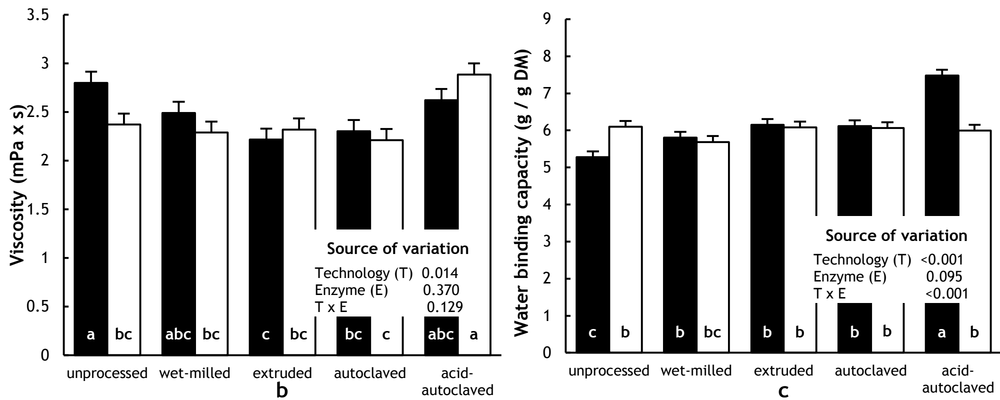

Figure 4.6. Viscosity of supernatant (panel a) and water binding capacity of residue (panel b) after enzymatic digestion simulation of unprocessed, wet-milled, extruded, autoclaved, or hydrothermal acid treated (acid) maize dried distillers grain with solubles (DDGS), with (white bars) or without (black bars) the addition of cell wall degrading enzymes. Error bars indicate SEM.

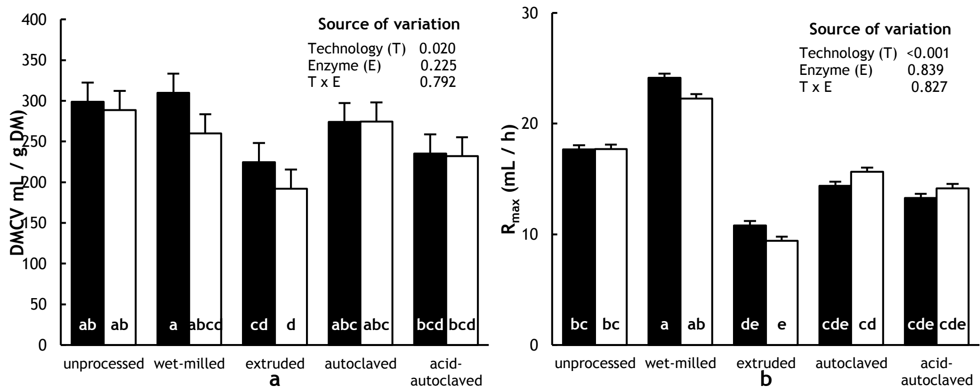

Figure 4.7. Total gas production (panel a) and maximal gas production rate (panel b) of unprocessed, wet-milled, extruded, autoclaved, or hydrothermal acid treated (maleic acid) maize, with (white bars) or without (black bars) the addition of commercial enzyme mixtures. Error bars indicate SEM.

Effects of acid-extrusion on the degradability of maize dried distillers grain with solubles in pigs

Chapter 5

Acid-extrusion to improve degradability of DDGS in pigs

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ABSTRACT

Commonly used feed processing technologies are not sufficient to affect recalcitrant non-starch polysaccharides (NSP) such as arabinoxylans present in maize dried distillers grain with solubles (DDGS). Instead, hydrothermal treatments combined with acid catalysts might be more effective to modify these NSP. The objective of this experiment was to investigate the effects of hydrothermal maleic acid treatment (acid-extrusion) on the degradability of maize DDGS in growing pigs. It was hypothesized that acid-extrusion modifies DDGS cell wall architecture and, thereby, increases fermentability of NSP. Two diets, containing either 40 % (wt/wt) unprocessed or acid-extruded DDGS, were restrictedly fed to groups of gilts (n = 11, with 4 pigs per group; initial mean BW: 20.8 ± 0.2 kg) for 18 d and performance and digestibility were analyzed. Acid-extrusion tended to decrease apparent ileal digestibility (AID) of CP (~3 % units, $P = 0.06$) and starch (~1 % unit, $P = 0.10$). Apparent digestibility of CP and starch measured at the mid colon (2 % units, $P = 0.03$ for CP and 0.3 % units, $P < 0.01$ for starch) and total tract (ATTD; 3 % units, $P < 0.01$ for CP and 0.2 % units, $P = 0.02$ for starch) were lower for the acid-extruded diet compared with the control diet. Hindgut disappearance was, however, not different between diets indicating that reduced CP and starch digestibility were mainly due to decreased AID. Acid-extrusion tended to increase AID of NSP (6 % units, $P = 0.09$) and increased digestibility of NSP measured at the mid colon (6 % units, $P < 0.01$), whereas, hindgut disappearance and ATTD of NSP did not differ between diets. Greater NSP digestibility was mainly due to greater digestibility of arabinosyl, xylosyl, and glucosyl residues, indicating that both arabinoxylan and cellulose degradability were affected by acid-extrusion. In conclusion, these results show that acid-extrusion did not improve degradation of DDGS for growing pigs. Although acid-extrusion seemed to facilitate more rapid degradation of NSP and shifted fermentation to more proximal gastrointestinal segments, total extent of NSP degradation was not affected. More than 35 % of the NSP from DDGS remained undegraded, independent of technological processing. Enzyme technologies that specifically target ester-linked acetyl, feroloyl, or coumaroyl groups were identified to be of interest for future research.

INTRODUCTION

Currently, the use of maize dried distillers grain with solubles (DDGS) as a protein source in animal feed is limited because of the inferior protein quality and high level (~30 %) of non-starch polysaccharides (NSP)[14], of complex, highly substituted, glucuronoarabinoxylans (GAX)[182, 192, 193]. Although NSP can be fermented by microbiota in the gastrointestinal tract of the pig, degradability of NSP from DDGS is typically only ~50 %[42]. In addition, especially in young pigs, NSP may affect digestion of other nutrients, both directly due to physical hindrance and indirectly due to physiological changes in the gut[97, 166].

Processing technologies can be used to modify plant cell wall architecture and improve NSP degradability, but commonly used technologies in feed production – e.g. hammer milling and pelleting – are insufficient to affect recalcitrant NSP structures[194]. Hydrothermal pretreatments using acid catalysts are established methods to degrade lignocellulosic material[124]. For animal feed applications, however, potential protein damage and high residual acid or mineral concentrations limit the use of extreme processing temperatures and high acid concentrations[195]. Instead, milder acid treatments using dicarboxylic organic acids, such as maleic acid, at lower temperatures could be of interest. In a previous *in vitro* study[192], hydrothermal treatment (autoclaving) with maleic acid effectively increased solubilization of NSP from maize DDGS. In the current experiment, the effects of hydrothermal acid treatment (acid-extrusion) on the degradability of maize DDGS, in particular its fiber fraction, in pigs were investigated. It was hypothesized that acid-extrusion modifies DDGS cell wall structure and, thereby, increases fermentability of NSP.

MATERIALS AND METHODS

The experiment was conducted at research farm ‘De Haar’ of Wageningen University, Wageningen, The Netherlands. All experimental procedures were approved by the Animal Care and Use Committee (DEC) of Wageningen University.

Materials and Experimental Diets

Pelleted maize DDGS was obtained from a commercial bioethanol plant (Abengoa Bioenergy, Rotterdam, The Netherlands) and crushed using a roller mill (Major flaking mill; E R &F Turner, Ipswich, United Kingdom).

Two diets, containing 40 % (wt/wt, as-fed basis) unprocessed (control diet) or acid-extruded DDGS (described below), were formulated to meet or exceed nutrient requirements of growing pigs[38] (Table 5.1 and 5.2). Additional sodium and potassium bicarbonate were added to the acid-extruded diet to neutralize the diet (pH > 4). Resulting differences in nutrient density between the control and acid-extruded diet were compensated for by adjusting the feeding level for the acid-extruded diet. Chromium oxide was included in the diets as an indigestible marker.

Table 5.1. Analyzed chemical composition of unprocessed and acid-extruded DDGS (%, DM basis[1])

	Unprocessed DDGS	Acid-extruded DDGS
DM, % fresh	92	72
CP[2]	31	28
Indispensable AA		
Arg	1.10	1.11
His	1.11	1.01
Ile	1.16	1.11
Leu	3.53	3.42
Lys	0.86	0.90
Reactive Lys[3]	0.84	0.88
Phe	1.50	1.46
Thr	1.20	1.15
Val	1.56	1.49
Dispensable AA		
Ala	2.28	2.21
Asp	2.00	1.98
Glu	4.07	4.13
Gly	1.19	1.16
Pro	2.81	2.76
Ser	1.48	1.43
Tyr	1.34	1.30
Total AA[4]	2.72	2.66
Total Lys:CP	0.029	0.027
Reactive Lys:CP	0.028	0.027
Total carbohydrates	32	32
Molar composition of carbohydrates[5]		
Rhamnosyl	0	0
Arabinosyl	18	18
Xylosyl	27	27
Mannosyl	5	5
Galactosyl	5	5
Glucosyl	35	36
Uronyl	9	9
Ara:Xyl[6]	0.68	0.68
UA:Xyl[7]	0.35	0.32

[1] Unless indicated otherwise.
[2] Crude protein content was calculated from the N content using a protein conversion factor of 5.9[43].
[3] Measured as O-methylisourea-reactive lysine[196].
[4] Calculated as sum of Arg, His, Ile, Leu, Lys, Phe, Thr, Val, Ala, Asp, Glu, Gly, Pro, Ser, and Tyr.
[5] Mol%; presented as anhydrous sugar moieties.
[6] Molar ratio of arabinosyl:xylosyl.
[7] Molar ratio of uronyl:xylosyl.

Table 5.2. Composition of experimental diets (%, as-fed basis)

Ingredient	Control diet	Acid-extruded diet[1]
Unprocessed DDGS (89 % DM)	40.00	
Acid-extruded DDGS (72 % DM)		49.40
Maleic Acid	-	1.80
Water	9.40	-
Maize starch	25.65	25.65
Casein	2.00	2.00
Soy protein isolate	2.00	2.00
Fishmeal	6.00	6.00
Soy oil	3.50	3.50
Sucrose	7.00	7.00
Limestone	1.10	1.10
Mineral and vitamin premix[2]	1.00	1.00
Monocalcium phosphate	0.70	0.70
Potassium bicarbonate	0.70	1.50
Salt	0.30	0.30
Sodium bicarbonate	0.15	0.50
L-Lysine HCL	0.40	0.40
L-Threonine	0.05	0.05
L-Tryptophan	0.02	0.02
Cr2O3	0.025	0.025
Calculated nutrient composition[3]		
GE, kcal/kg	4228	4105
Digestible phosphorus	0.40	0.39
Sodium	0.31	0.30
Digestible lysine	1.04	1.01
Digestible methionine + cysteine	0.57	0.55
Digestible threonine	0.63	0.61
Digestible tryptophan	0.17	0.16

[1]The acid-extruded diet was fed at 103 % of the control diet to compensate for differences in nutrient density between the diets.
[2]Provided per kg of diet: Vitamin A (retinyl acetate), 6,000 IU; vitamin D (cholecalciferol), 1,200 IU; vitamin E (DL-α-tocopherol), 40 mg; vitamin B1 (thiamin), 1.0 mg; vitamin B2 (riboflavin), 3 mg; vitamin B6 (pyridoxine-HCl), 1 mg; vitamin K3 (menadione), 1.5 mg; vitamin B12 (cyanocobalamin), 15 µg; choline chloride, 150 mg; niacin, 20 mg; pantothenic acid (d-calcium pantothenate), 10 mg; folic acid, 0.2 mg; Co, 0.2 mg, as $CoSO_4{\cdot}7H_2O$; Cu, 15 mg, as $CuSO_4{\cdot}H_2O$; Fe, 80 mg, as $FeSO_4{\cdot}H_2O$; I, 0.7 mg, as KI; Mn, 30 mg, as MnO; Se, 0.2 mg, as Na_2SeO_3; Zn, 50 mg, as $ZnSO_4{\cdot}H_2O$;.
[3]Calculated based on data from CVB[38], Stein and Shurson[41], and Cervantes-Pahm and Stein[197]. Digestible phosphorus and AA contents indicate apparent ileal digestible contents.

Hydrothermal Acid Treatment

Dried distillers grain with solubles was mixed with maleic acid (5 %, wt/wt DM basis) and water to reach a DM content of 40 % (wt/wt), using a paddle-mixer (Type F60; Halvor Forberg, Bygland, Norway). After soaking overnight, the DDGS mixture was extruded using a co-rotating double screw extruder (M.P.F.50; Baker Perkins, Peterborough, United Kingdom), without additional steam conditioning. The extruder had a length/diameter ratio of 25. The screw configuration was as follows: four 1.5D feed screw elements, one 1D single lead element, three 1D feed screw elements, one 1D single lead element, two 1D feed screw elements, two 4D 90 degree forwarding block paddles, one 1.5D feed screw elements, one 4D 90 degree forwarding block paddles, one 1.5D feed screw elements, two 4D 90 degree forwarding block paddles, and two 1.5D single lead elements. A die with two orifices (6 mm) was used; no die face cutter was used. Feeding rate was 54 $kg{\cdot}h^{-1}$ and screw speed was 250 rpm. Barrel

temperatures in the nine subsequent segments of the extruder were set at 30, 50, 72, 82, 90, 105, 115, 120, and 120 °C. Product was collected after the process reached a steady state and cooled to room temperature. Pressure and product temperature at the die were 125 ± 10 psi and 104 ± 1.4 ºC, respectively and DM content of the extruded DDGS was 72 % (wt/wt).

Animals and Experimental Procedures

A total of 48 gilts (initial BW: 20.8 ± 0.2 kg; Topigs 20 × Talent; Van Haaren, Horssen,) were allocated to one of the two treatments based on BW and housed in groups of four pigs per pen. The experiment was performed in two rooms of six pens each. Dietary treatments were balanced across both rooms. Diets were fed as mash and mixed into a slurry with water (1 : 3) in the feed through, which was long enough to allow simultaneous eating of all pigs. The daily feed allowance was 2.4 times energy requirements for maintenance (100 kcal ME/kg of $BW^{0.75}$) in two equal meals at 7.00 and 16.00 h. Feed refusals were collected 30 min after feeding. Pigs had free access to water. After a 5 d gradual transition from a commercial starter diet to the experimental diets, pigs were allowed to adapt to the experimental diets for 14 d, followed by 4 d collection of feces. At d 24 (room 1) or d 25 (room 2) animals were anesthetized the abdominal cavity was opened and the gastrointestinal tract from stomach to anus was removed from the abdominal cavity, after which animals were euthanized. Before removal, the different segments of the gastrointestinal tract were isolated using tie-wraps, to prevent mixing of digesta from different segments. The gastrointestinal tract was segmented and digesta samples were collected by gentle finger-stripping. Pigs were fed approximately 4 h before dissection to ensure presence of fresh digesta in the terminal ileum. Feed intake per pen was recorded throughout the experiment. Pigs were weighed at the start of the experiment (d 0), adaption period (d 5), feces-collection period (d 19) and at the end of the experiment (d 24 or 25) after consuming their morning meal. Feces were collected directly from the rectum, two times daily after feeding. Digesta from the last 100 cm of the small intestine (terminal ileum) and middle 50 cm of the colon (mid colon) were collected by gentle finger-stripping immediately after dissection. Digesta and feces were pooled per pen by weight, immediately frozen (-20 °C) and freeze-dried.

Chemical Analyses

Before chemical analyses, individual freeze-dried samples were ground in a mixer mill (MM 2000, Retsch GmbH, Haan Germany) at an amplitude of 80, during 1 min. All chemical analyses were performed in duplicate using standard laboratory methods[13, 171]. Feed refusals were analyzed for content of air-dry matter (AOAC 930.15). Diets, digesta, and feces were analyzed for content of DM (AOAC 930.15), ash (AOAC 942.05), chromium[198], nitrogen (diets by the Kjeldahl method: ISO 5983, digesta and feces by the Dumas method: AOAC 990.03; using a Thermo Quest NA 2100 Nitrogen and Protein Analyser, Interscience B.V., Breda, The Netherlands), total starch (AOAC 996.11; using a commercial test kit, Megazyme international Ltd., Ireland), and total NSP measured as neutral sugars and uronic acids. Before neutral sugar and uronic acid analyses of diets, NSP were extracted, as described by Jonathan et al.[199]. Briefly, starch was gelatinized and enzymatically degraded, after which NSP were precipitated using acidified ethanol. Neutral sugar composition was analyzed according to the

method of Englyst and Cummings[49]. After pre-treatment with 72 % (wt/wt) H_2SO_4 for 1 h at 30 °C, samples were hydrolyzed with 1 M H_2SO_4 at 100 °C for 3 h. Constituent monosaccharides were derivatized into their corresponding alditol acetates and analyzed using a GC (Focus-GC, Thermo Scientific, Waltham, MA). Inositol was used as an internal standard. Uronic acid content was analyzed according to the automated colorimetric m-hydroxydiphenyl assay[200] using an auto-analyzer (Skalar Analytical B.V., Breda, The Netherlands). Galacturonic acid was used for calibration. Non-starch polysaccharide content was calculated as the sum of neutral sugars and uronic acids minus glucosyl originating from remaining starch in the NSP extract from diets, or in digesta and feces (measured enzymatically as described above). Nitrogen content of diets was corrected for differences between analytical methods (Kjeldahl vs. Dumas method) using: $N_{Dumas} = N_{Kjedahl} \times 0.9885 + 0.0103$[201]. Crude protein content was calculated as N × 6.25 (ISO 5983).

Diets, DDGS, and ileal digesta were analyzed for content of amino acids[202] and DDGS samples were analyzed for reactive lysine[196]. Before amino acid and reactive lysine analysis diets were defatted using a Soxhlet apparatus and petroleum ether and subsequently ground in a mixer mill (MM 2000, Retsch GmbH, Haan Germany) at an amplitude of 80 for 2 min. Amino acids were analyzed using a 5 mg sample that was hydrolyzed using 1 ml of 6 M HCl during 23 h at 110 °C in glass tubes, sealed under vacuum. The tubes were opened, norleucine was added to each tube as an internal standard, and the tubes were then dried under vacuum (Savant SpeedVac Plus, SC210A, Thermo Scientific, Waltham, MA). Amino acids were dissolved in 2 ml of loading buffer (sodium acetate; pH 2.2). Amino acids were separated by ion exchange chromatography using a Biochrom 20 AA analyzer (Biochrom, Cambridge, UK) and analyzed by post column derivatization with ninhydrin, using photometric detection at 570 nm or 440 nm (proline). Reactive lysine was analyzed using 5 mg samples that were incubated during 7 d with 1 ml O-methylisourea (OMIU) to convert all lysine molecules with a free ε-amino group into homoarginine. Homoarginine was measured in the dried sample according to the amino acid analysis procedure described previously. The amount of OMIU-reactive lysine was calculated from the amount of homoarginine using the molecular weights of homoarginine and lysine.

Diets and feces were analyzed for content of ether extract (crude fat) using a Soxhlet apparatus and petroleum ether after hydrochloric acid hydrolysis (AOAC 920.39). Diets were additionally analyzed for content of NDF (AOAC 2002.04), and total and phytate bound phosphorus (enzymatic-colorimetric; using a commercial test kit, K-PHYT 07/11, Megazyme international Ltd., Bray, Ireland).

The control diet and pooled samples of digesta and feces from pigs fed the control diet (n=1, 6 pens with 4 pigs per pen) were analyzed for content of esterified coumaric, ferulic, and diferulic acid as described by Appeldoorn et al.[193].

Calculations and Statistical Analysis

Apparent ileal and total tract digestibility were calculated according to the marker method with Cr_2O_3 as a marker[203]. The unprocessed control diet was used as the reference. to calculate digestibility of the control and acid-extruded DDGS diet. Body weight, ADG, and ADFI were analyzed using a general linear model (PROC GLM, SAS version 9.2, SAS Institute Inc., Cary, NC). All other data were analyzed using a generalized linear model with beta-distributed error for the response variable and a

logit link function (PROC GLIMMIX, SAS version 9.2, SAS Institute Inc., Cary, NC). Diet and the blocking factor room were included as fixed effects. The interaction between the blocking factor room and dietary treatment was tested, but found to be not significant and excluded from the model. Pen was the experimental unit. Distributions of the means and residuals were examined to verify model assumptions. Significance of differences was tested using type III likelihood ratio statistics. Data are presented as back-transformed means and pooled SEM unless otherwise stated. Differences among means with $P < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Diets and Animal Performance

In one pen (acid-extruded diet), two pigs showed signs of diarrhea during the experiment. Laboratory analyses of feces by the Dutch animal health service (GD, Deventer, The Netherlands) confirmed that these pigs suffered from *E. Coli* infection, and this pen was excluded from analyses. The pigs in all other pens had firm feces.

The acid-extruded diet had a slightly lower nutrient content and greater ash content compared to the control diet (Table 5.3), due to the inclusion of acid and additional bicarbonate. By adjusting feeding level for the acid-extruded diet (103 % of the control diet) we endeavored to equalize nutrient intake of both treatment groups. As pigs receiving the acid-extruded diet did not always consume their total daily allowance (Table 5.4), this was, however, not fully accomplished. Crude protein and lysine content of the DDGS (Table 5.1) were within ranges reported previously for maize DDGS[14, 40, 204]. The total and reactive lysine:CP ratios indicate that the DDGS was of moderate quality, with substantial protein damage[14]. Hydrothermal treatments may reduce protein quality due to specific interactions between protein and amino acids with other components, such as the Maillard reaction[187]. The total and reactive lysine:CP ratios in unprocessed and acid-extruded DDGS were, however, similar (Table 5.1), indicating that little additional protein damage occurred during hydrothermal acid treatment[187]. Extrusion conditions used in the current study were moderate, whilst the content of potentially reducing sugars in DDGS is relatively low (<10 %)[43], reducing the risk of protein damage[205, 206]. In addition, the Maillard reaction occurs at a slower rate under acidic conditions[175]. As discussed above, a considerable part of the DDGS protein was heat-damaged prior to acid extrusion, presumably as a result of exposure to the severe pretreatment conditions[55, 57, 58] during the dry-grind ethanol production process. This may have reduced the chance for protein damage during hydrothermal acid treatment.

The sugar composition of the DDGS indicated the presence of cellulose and GAX, as expected[192]. The slightly lower starch (~1 % unit), NSP (~3 % units), non-glucosyl polysaccharide (NGP; NSP – glucosyl; ~2 % units), and NDF (~6 % units) contents of the acid-extruded diet (corrected for differences in nutrient density between diets) indicated some degradation of polysaccharides occurred during processing. The resulting saccharide fragments are not recovered in the NSP extract, but are still present in the diet fed to the pigs; therefore ATTD was calculated using the unprocessed control diet as the reference.

Table 5.3. Analyzed chemical composition of experimental diets (%, DM basis[1]).

	Control diet	Acid-extruded diet[2]
DM, % as-fed	87.55	86.89
CP[3]	22.83	22.54
Starch	27.79	25.87
Crude fat	10.77	10.73
Ash	6.28	7.09
NDF	19.63	13.46
Phosphorus	0.74	0.76
Phytate bound phosphorous	0.08	0.13
Non-starch polysaccharides (NSP)	18.78	15.73
Molar composition of NSP[4]		
Rhamnosyl	0	0
Arabinosyl	20	20
Xylosyl	36	34
Mannosyl	4	5
Galactosyl	5	4
Glucosyl	26	27
Uronyl	9	10
Ara:Xyl[5]	0.55	0.60
UA:Xyl[6]	0.26	0.29
Indispensable AA		
Arg	0.87	0.91
His Ile	0.75	0.73
Ile	0.89	0.91
Leu	2.05	2.11
Lys	1.40	1.45
Phe	1.02	1.04
Thr Trp	0.85	0.90
Val	1.11	1.13
Dispensable AA		
Ala	1.15	1.21
Asp	1.69	1.82
Glu	3.15	3.25
Gly	0.87	0.88
Pro	1.52	1.65
Ser	0.90	0.92
Tyr	0.81	0.82
Total AA[7]	19.04	19.75

[1] Unless indicated otherwise.
[2] The acid-extruded diet was fed at 103 % of the control diet to compensate for differences in nutrient density between the diets.
[3] Crude protein content was calculated from the N content using a protein conversion factor of 6.25 (ISO 5983).
[4] Mol%; presented as anhydrous sugar moieties.
[5] Molar ratio of arabinosyl:xylosyl.
[6] Molar ratio of uronyl:xylosyl.
[7] Calculated as sum of Arg, His, Ile, Leu, Lys, Phe, Thr, Val, Ala, Asp, Glu, Gly, Pro, Ser, and Tyr.

Table 5.4. Growth performance of growing pigs fed diets containing 40 % (wt/wt) unprocessed (control) or acid-extruded DDGS as the only NSP source[1]

	Control	Acid-extruded	Pooled SEM	*P* - value
No. of observations[2]	6	5	-	-
Initial mean BW, kg	20.8	20.7	0.10	0.247
Final mean BW, kg	32.8	31.0	0.42	0.003
ADG, g/pig	530	475	7.24	<0.001
ADFI, g/pig	860	815	7.24	<0.001
G:F, g/g	0.62	0.58	0.006	0.001

[1]Recorded between start of adaptation period and end of the experiment (19 or 20 d), with the exception of initial mean BW, which was recorded at start of the experiment before feeding the experimental diets.
[2] Number of replicate pens of 4 pigs each.

Feed intake and weight gain were lower for pigs fed the acid-extruded diet compared with pigs fed the unprocessed control diet ($P < 0.05$, Table 4). As feed allowance was restricted to 2.4 times the energy requirements for maintenance, the reduced feed intake of the pigs fed the acid extruded diet was related to orts in this group. This might have resulted from altered physico-chemical properties of this diet such as increased water binding capacity[192, 207] and reduced pH[208] or from reduced palatability of the diet due its acidity[208]. Gain-to-feed ratio was reduced for pigs fed the acid-extruded diet ($P < 0.05$). Overall, G:F was high, probably related to an increased relative weight of the gastrointestinal tract and its contents in response to the high fiber diets fed in this experiment[209, 210].

DRY MATTER AND NUTRIENT DIGESTIBILITY

Ileal and total tract digestibility of DM and nutrients are presented in Table 5.5 and 5.6. Apparent ileal digestibility (AID) of CP from the DDGS was calculated to be ~60 % (calculated by difference method; AID for protein sources other than DDGS as reported in literature[38]), which is in the range reported for maize DGGS previously[40, 211].

Acid-extrusion tended to decrease AID of CP (~3 % units, $P = 0.06$), AA (~3 % units, $P = 0.07$), and starch (~1 % unit, $P = 0.10$). Digestibility of CP and starch measured at the mid colon and ATTD were lower for the acid-extruded diet compared with the control diet, but hindgut disappearance did not differ between diets. Therefore, the reduced CP and starch digestibility in pigs fed the acid-extruded diet were mainly due to decreased AID. Apparent total tract digestibility of crude fat tended to be lower (0.4 % units, $P = 0.10$) for the acid-extruded diet compared with the control diet. Little protein damage of DDGS was found after acid-extrusion and also AID of lysine seemed not to be affected by acid-extrusion. Moreover, the majority (> 90 %) of starch originated from maize starch, which was not exposed to acid-extrusion. Hence, reduced digestibility of the acid-extruded diet presumably resulted indirectly from changes in digestive or absorptive processes rather than from chemical modification of the nutrients present in DDGS. The acid-extruded diet contained ~3 % low molecular weight sugars that were formed during the acid-extrusion process (Table 5.2, as discussed above) and the remaining NSP in the acid-extruded diet were more soluble (18 % of hemicellulose was water soluble in the acid-

Table 5.5. Apparent digestibility (%) measured at the distal ileum (ileum), middle of the colon (colon) or in feces (total tract), as well as hindgut disappearance (%) of DM and nutrients in growing pigs fed diets containing 40 % (wt/wt) unprocessed (control) or acid-extruded DDGS as the only NSP source.

	Control	Acid-extruded	Pooled SEM	*P* - value
No. of observations[1]	6	5	-	-
Ileum				
DM	68.6	65.3	1.71	0.090
CP	68.1	64.8	1.59	0.063
Starch	97.5	96.4	0.56	0.096
Colon				
DM	79.7	79.4	0.38	0.375
CP	76.4	74.3	0.58	0.030
Starch	99.6	99.3	0.06	0.006
Total tract				
DM	80.6	79.8	0.36	0.065
CP	78.0	75.4	0.49	0.006
Starch	99.7	99.5	0.05	0.024
Crude fat	97.9	97.5	0.10	0.104
NDF	59.9	63.7	1.43	0.030
Hindgut disappearance[2]				
DM	12.0	14.5	1.62	0.152
CP	10.0	10.3	1.59	0.820
Starch	2.2	3.0	0.52	0.144

[1] Number of replicate pens of 4 pigs each.
[2] Calculated by difference between ileal and total tract digestibility.

extruded DDGS vs. 15 % in unprocessed DDGS, no further data presented). Literature indicates that the presence of high levels of non-absorbed water-soluble low molecular weight sugars can increase osmolality and water content of the chyme[212]. This may increase passage rate and reduce absorption of nutrients, possibly explaining the decreased AID and G:F observed. Indeed, observed DM contents of digesta and feces were lower in pigs fed the acid-extruded diet compared with those fed the control diet (8 vs. 11 %, wt/wt; $P < 0.01$) in ileum, 19 vs. 24 % ($P < 0.01$) in colon and 25 vs. 28 % ($P < 0.01$) in feces). Alternatively, increased microbial activity in the distal ileum as indicated by the numerically greater ileal NSP degradability and by high concentrations of low molecular weight arabinosyl and xylosyl sugars[213, 214], may have influenced apparent digestibility values by increasing the production of microbial biomass and endogenous losses and reducing hydrolysis and absorption of nutrients[80, 215]. Furthermore, although the amount of acid added to the acid-extruded diet was within the range of concentrations of organic acids applied more often in pig diets, this bivalent acid is stronger (pKa1=1.93, pKa2=6.14)[216] and effects of maleic acid on digestive and digestive and metabolic processes cannot be excluded[208, 217, 218].

Table 5.6. Apparent digestibility (%) of amino acids in growing pigs fed diets containing 40 % (wt/wt) unprocessed (control) or acid-extruded DDGS as the only NSP source, measured at the distal ileum.

	Control	Acid-extruded	Pooled SEM	*P* - value
No. of observations[1]	6	5	-	-
Indispensable AA				
Arg	78.2	77.1	0.68	0.267
His	70.9	71.5	0.74	0.587
Ile	68.9	66.4	1.26	0.085
Leu	76.0	74.3	0.62	0.087
Lys	79.5	79.5	0.64	0.959
Phe	76.7	75.7	0.57	0.290
Thr	60.6	57.5	2.04	0.173
Val	66.9	64.5	1.17	0.072
Dispensable AA				
Ala	67.5	66.0	1.34	0.269
Asp	60 .3	56.2	2.14	0.093
Glu	74.3	70.4	1.44	0.026
Gly	52.0	48.5	2.70	0.231
Pro	68.8	63.1	1.90	0.018
Ser	65.6	64.2	1.99	0.499
Tyr	76.6	75.1	0.57	0.104
Total AA[2]	70.3	67.8	1.19	0.067

[1] Number of replicate pens of 4 pigs each.
[2] Calculated as sum of Arg, His, Ile, Leu, Lys, Phe, Thr, Val, Ala, Asp, Glu, Gly, Pro, Ser, and Tyr.

NON-STARCH POLYSACCHARIDE DEGRADATION

Apparent total tract digestibility of NSP was ~63 %, and 20 (control diet) to 27 % (acid-extruded diet) was degraded before the end of the ileum (Table 5.7). Although detailed structural characterization of the NSP in DDGS is lacking, it is expected, based on the composition of maize and maize fiber fractions, that complex, highly substituted GAX were dominating[182, 192, 193]. These heteroxylans originate from a complex cell wall matrix where hemicellulose, cellulose, and lignin, are intertwined. They consist of a xylan-backbone substituted with monomeric arabinofuranosyl and glucuronic acid residues, acetyl and feruloyl esters, as well as oligomer side chains containing arabinosyl, xylosyl, galactosyl, and feruloyl residues, and are highly cross-linked by diferulic acid bridges[182, 183, 193]. The ratio of arabinosyl and uronyl to xylosyl residues is indicative for the degree of substitution and thus related to the structure of the GAX present in the feed or of the undegraded GAX remaining in digesta and feces. The greater Ara:Xyl in ileal digesta compared with the feed together with a higher relative degradation of xylosyl compared to arabinosyl (Table 5.7; Figure 5.1), indicates that mainly linear, lowly substituted xylan-fragments were degraded in the upper gastrointestinal tract. This was followed by a reduction in in the Ara:Xyl and UA:Xyl in the mid colon, indicating that more complex, highly substituted, xylan-fragments, were degraded mainly the caecum and proximal colon.

Table 5.7. Apparent digestibility (%) measured at the distal ileum (ileum), middle of the colon (colon) or in feces (total tract), as well as hindgut disappearance (%) of non-starch polysaccharides (NSP) and its constituent sugars in growing pigs fed containing 40 % (wt/wt) unprocessed (control) or acid-extruded DDGS as the only NSP source.

	Control	Acid-extruded	Pooled SEM	*P* - value
No. of observations[1]	6	5	-	-
Ileum				
NSP[2]	20.3	26.6	3.27	0.092
NGP[3]	19.2	25.6	4.66	0.208
Arabinosyl	12.1	23.0	4.55	0.047
Xylosyl	33.2	37.2	4.62	0.424
Glucosyl	23.4	27.7	5.45	0.447
Uronyl	39.8	40.8	3.91	0.817
Colon				
NGP[3]	57.6	64.0	1.61	0.004
Arabinosyl	56.8	65.1	1.75	0.001
Xylosyl	50.5	59.2	2.00	0.003
Glucosyl	62.7	69.1	1.82	0.008
Uronyl	77.7	78.7	1.46	0.509
Total tract				
NSP[2]	63.8	63.0	1.28	0.551
NGP[3]	61.8	61.4	0.73	0.694
Arabinosyl	60.6	61.0	0.08	0.757
Xylosyl	55.3	56.1	1.38	0.566
Glucosyl	68.6	66.8	2.35	0.479
Uronyl	79.8	78.0	0.59	0.055
Hindgut disappearance[4]				
NSP[2]	43.0	36.8	4.48	0.203
NGP[3]	42.1	36.0	5.44	0.299
Arabinosyl	48.0	38.5	5.68	0.133
Xylosyl	20.1	20.5	7.04	0.955
Glucosyl	45.3	38.3	6.55	0.320
Uronyl	40.0	37.2	3.54	0.457

[1] Number of replicate pens of 4 pigs each.
[2] Non-starch polysaccharides; monosaccharides represent anhydrous sugar moieties.
[3] Non-glucosyl polysaccharides; monosaccharides represent anhydrous sugar moieties.
[4] Calculated by difference between ileal and total tract digestibility.

More than 35 % of the NSP from DDGS remained undegraded. Based on the sugar composition of the unfermented NSP found in feces and previous observations on degradation of maize GAX, it is expected that the unfermented residue contained dense substituted xylans with large oligomeric side chains. These are firmly anchored in the cell wall through ester-linkages[193]. To further identify limiting structures in degradation of NSP from DDGS, the content of esterified coumaric and ferulic acid in feed and pooled samples from ileum and colon contents and feces were analyzed (Figure 5.2). These phenolic acids cross-link polysaccharides, as well as lignin within the cell wall[27, 183, 193, 219] and have been shown in maize GAX to be resistant to hydrothermal and enzymatic treatment. Hence, they may contribute to the recalcitrance of maize GAX structures. Contents in feed were 3.6 g/kg (wt/wt, DM basis) for monoferulic acid, 0.2 g/kg for diferulic acid, and 0.3 g/kg for coumaric acid, and increased 2- to 4-fold in digesta and feces. It was calculated that the DDGS cell wall fraction contained approximately 2 to 3 % of phenolic acids. Especially the content of diferulic acids (~0.1 %) is lower than reported for maize bran[183].

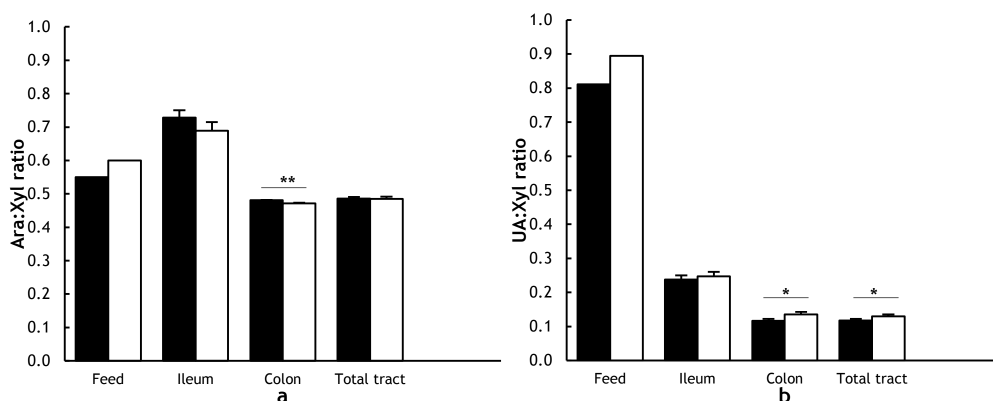

Figure 5.1. Mean molar arabinosyl:xylosyl ratio (Ara:Xyl, panel a) and uronyl:xylosyl ratio (UA:Xyl, panel b) in unprocessed (control) or acid-extruded diets (n=1) as well as in digesta from ileum and colon, and feces from growing pigs fed diets containing 40 % (wt/wt) unprocessed (control, n=6) or acid-extruded (n=5) DDGS as the only NSP source. Error bars represent SEM. Asterisks indicate significant trends (*, $P < 0.10$) or differences (**, $P < 0.05$).

Monoferulic acid was degraded more easily than diferulic acid; whereas coumaric acid was degraded to the least extent (Figure 5.3C). These preliminary data indicated that phenolic acid associated linkages within the cell wall were partly degraded by the pigs' microbiota. The fact that coumaric acid – and to a smaller extent – diferulic acid disappearance was substantially lower than NSP disappearance, indicated, however, that these components were indeed associated with a more resistant part of the cell wall. Possibly, technologies that specifically target phenolic acid associated (ester)-linkages, such as alkali treatments or enzymes having coumaroyl or feruloyl esterase activity can facilitate more extensive degradation of DDGS NSP. Furthermore, acetyl esterases may be useful because of the high substitution of recalcitrant maize GAX with acetyl groups[27].

Acid-extrusion tended to increase AID of NSP (6 percentage units, $P = 0.09$), only reaching statistical significance for AID of arabinosyl residues (11 percentage units, $P = 0.05$). At the mid colon, digestibility of NSP was greater (6 percentage units, $P < 0.01$) for the acid-extruded diet. Greater digestibility was mainly due to greater disappearance of arabinosyl, xylosyl, and glucosyl residues (Table 5.7), indicating that both arabinoxylan and cellulose degradability were affected by acid-extrusion. *In vitro* hydrothermal acid-treatment did not affect cellulose solubilization[192], but apparently, acid-extrusion opened the cell wall architecture facilitating accessibility of cellulose to microbial enzymes. Hindgut disappearance and ATTD of NSP and its constituent sugars did not differ between diets indicating that although acid-extrusion shifted fermentation of NSP to more proximal segments of the gastrointestinal tract, total extent of NSP degradation was not affected. Apparently, acid-extrusion affected mainly NSP structures that are not resistant to degradation by microbial enzymes in the pigs' gastrointestinal tract. Although, opening of the cell wall matrix facilitated more easy degradation of those structures, the most recalcitrant NSP structures (~35% of total) were still not affected. An increased passage rate in the upper gastrointestinal tract, caused by high concentrations of non-absorbed low molecular weight sugars or increased microbial activity, could have contributed to this

observation. Furthermore, factors as physicochemical properties and post-ileal nutrient flow may have affected digesta passage rate in the cecum and colon, and thus time available for fermentation in the large intestine[220].

To prevent formation of high quantities of small saccharides, future technologies to increase degradation of NSP from DDGS should be aimed at targeted degradation of recalcitrant structures only, without affecting relatively easy degradable NSP to prevent counteracting effects on passage rate, nutrient digestion and absorption. Enzyme technologies would, therefore, be preferred over chemical degradation, due to their specific activities. The fact that ATTD of NDF was greater in the acid-extruded diet ($P < 0.03$; Table 5.7) seems to be somewhat contradictory to the unchanged ATTD of NSP but might be explained by a shift of fiber fractions recovered in NDF and differences in particle size between the diets[192, 194]. As NDF is analyzed by gravimetric methods, including filtration, processing will affect the quantity and type of polysaccharides that are recovered in NDF, due to differences in physicochemical properties, in particular particle size, and solubility of NSP. Hence, evaluation of degradability of NDF fractions does not allow full evaluation of NSP degradation and seems rather futile when diets that fairly differ in type of NSP or in physicochemical properties, such as particle size, are compared.

In conclusion, acid-extrusion did not improve degradation of DDGS in growing pigs. Apparent ileal and total tract digestibility of CP and starch tended to be lower, resulting in reduced feed efficiency. Although acid-extrusion seemed to facilitate more rapid degradation of NSP and shifted fermentation to more proximal gastrointestinal segments, total extent of NSP degradation was not affected. More than 35 % of the NSP from DDGS remained undegraded, independent of technological processing. Coumaric acid and diferulic acid were found to be associated with a more resistant part of the fiber fraction. Possibly, enzyme technologies that specifically target ester-linked acetyl, feruloyl, or coumaroyl groups, might further disrupt the cell wall matrix to enable more extensive degradation of NSP.

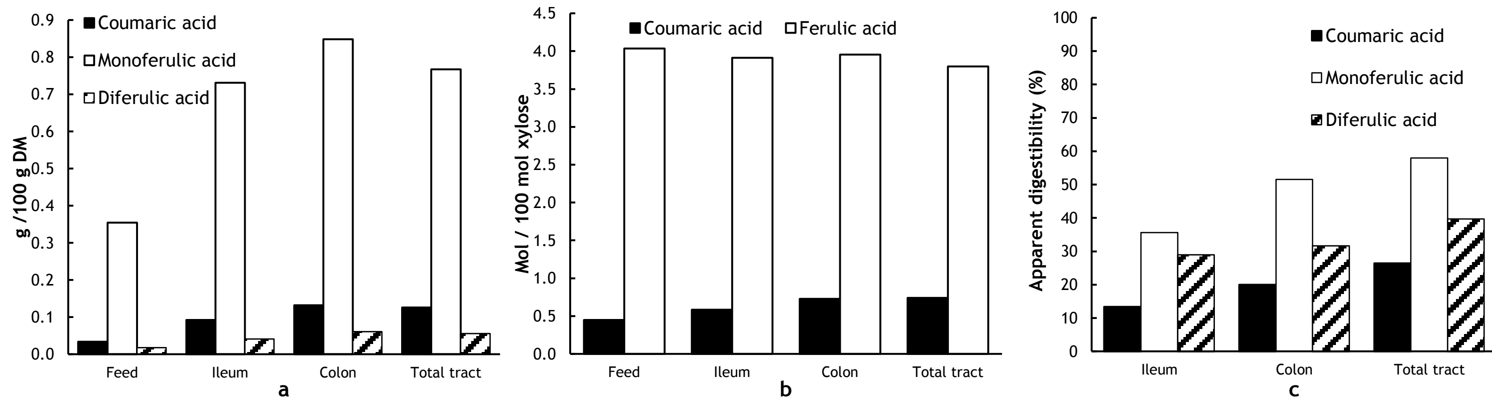

Figure 5.2. Content and degradability of coumaric acid, monoferulic acid, and diferulic acid from the unprocessed DDGS (control) diet observed in growing pigs. (A) Esterified coumaric acid, monoferulic acid, and diferulic acid content (g/ 100g DM) in feed, digesta an feces. (B) Coumaric:xylosyl and ferulic acid:xylosyl ratio (mol/ 100 mol xylosyl) in feed, digesta, and feces. (C) Apparent digestibility of coumaric acid, monoferulic acid, and diferulic acid (%) measured at the distal ileum (ileum), middle of the colon (colon), or in feces (total tract). Digesta or feces samples from 6 pens, with 4 pigs each, were pooled by weight before analyses (n=1).

Unfermented recalcitrant polysaccharide structures from (processed) rapeseed (Brassica napus) meal in pigs

Chapter 6

Degradability of rapeseed meal polysaccharides in pigs

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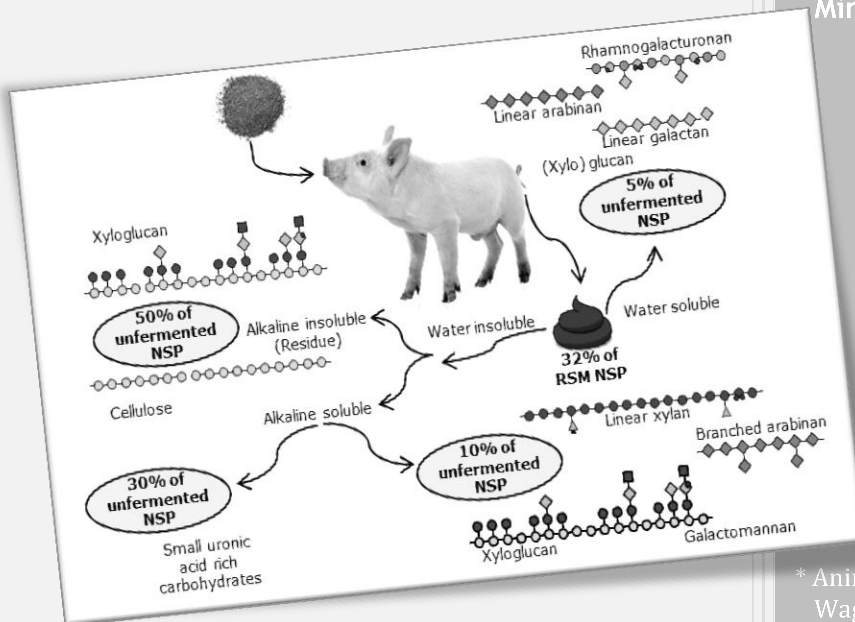

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ABSTRACT

Unprocessed and acid-extruded rapeseed meal (RSM) was fed to pigs as the only source of non-starch polysaccharides (NSP) and protein. Unfermented carbohydrate structures were analyzed. Acid-extrusion seemed to increase rigidness of the NSP-matrix *in vivo*, without affecting NSP-fermentability. Water-soluble NSP were almost completely fermented in the colon. Of the water-insoluble unfermented carbohydrates 46-68 % (wt/wt) was analyzed as the polysaccharides rhamnogalacturonan, (branched) arabinan, XXXG-type xyloglucan, linear xylan, galactomannan, and cellulose. A major fraction (35-54 %, wt/wt) of unfermented carbohydrates was unexpectedly released as small uronyl-rich carbohydrates (<14 kDa) during alkaline extraction using 6M NaOH. It follows that alkali-labile bonds hindered the complete fermentation of NSP in pigs and that fermentation may be improved by an alkaline pretreatment of RSM in the feed.

INTRODUCTION

Rapeseed meal (RSM) is a by-product from the production of rapeseed oil. After pressing and solvent-extraction of oil, the meal is dried by solvent-toasting and used as animal feed for its high protein content (around 38 %, wt/wt)[221]. Apart from protein, RSM is rich in non-starch polysaccharides (NSP; 16-22 %, wt/wt[222]), which potentially can be used as an energy source. The NSP present in RSM are represented by pectic polysaccharides (e.g homogalacturonan, rhamnogalacturonan, arabinan, and arabinogalactan), hemicelluloses (e.g. xyloglucan, glucuronoxylan, and galactomannan), and cellulose[203, 223, 224].

Animals lack the necessary digestive enzymes that can degrade NSP. Nevertheless, in pigs, in the terminal ileum, in the cecum, and in the colon, gut microbiota ferment these carbohydrates into short chain fatty acids, lactate, gases, and microbial biomass. The extent of NSP-fermentation varies depending on individual NSP-structures. In general, pectins and hemicelluloses are better fermented than cellulose[225].

So far, RSM digestion studies have focused on protein digestibility. When polysaccharide fermentability was described, mostly neutral detergent, acid detergent and/or crude fiber contents were analyzed[226, 227]. Or, in these digestion studies, the total NSP-content is calculated as dry matter minus the sum of crude protein, crude fat, ash, starch, and ethanol-soluble sugars[38], rather than based on analysis of each constituent carbohydrate present. In this way, fermentability of NSP from RSM was found to be around 58 %, which is rather low compared with other NSP-rich ingredients, such as sugar beet pulp from which around 85 % of the NSP is reported to be fermented by pigs[38]. So, more energy is potentially available from RSM, if all the NSP present are fermented.

Since the unfermented carbohydrate structures have not been studied in detail so far, the rate-limiting step in NSP-utilization from RSM by pigs is not known. Therefore, in the present study, digesta of different parts of the pigs' digestive tract are analyzed for the remaining unfermented carbohydrate structures. It has been shown in an *in vitro* assay, mimicking the upper gastro-intestinal tract, that an acid-treatment, combined with addition of commercial pectolytic enzymes, worked best in solubilizing carbohydrates from RSM[33]. Therefore, in this study, carbohydrate structures in digesta of pigs fed a diet containing untreated RSM are compared with a diet containing acid-treated RSM. Commercial pectolytic enzymes were not added in this study, since it is expected that enough enzymes are produced by the microbiota in the digestive tract[199]. The aim of this study is to find the rate-limiting step in NSP-fermentation. Therefore, residual carbohydrate structures are monitored during fermentation in pigs in detail. To be able to handle in-depth analysis of the residual carbohydrate structures, a limited amount of samples were aimed at, limiting the number of animals used.

MATERIAL AND METHODS

Chemicals

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

commercial cellulase preparation CellicCTec was kindly provided by Novozymes (Bagsvaerd, Denmark). m-Hydroxydiphenyl was sourced from Thermo Fisher Scientific (Waltham, MA, USA).

In vivo trial: animals and diets

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

Animals and pens. Twelve gilts (Topigs 20 × Talent, Van Haaren, Horssen, The Netherlands) with body weight 20.8±2.4 kg were divided over two pens. Each pen was equipped with two drinking nipples and one feeding trough, which was long enough to allow simultaneous eating of all pigs. Temperature was maintained between 16 °C and 23°C. The pigs were fed twice a day, during 24 or 25 days, 2.6 times their energy requirements for maintenance. All 12 pigs stayed in good health through-out the study. The feed conversion ratio of pigs fed unprocessed and acid-extruded RSM was 1.77 and 1.66 kg feed/kg body weight gain, respectively.

Experimental diets. The ingredient compositions of the diets are presented in Table 6.1. Rapeseed meal (Cargill, Antwerp, Belgium, 2011, supplied by Agrifirm, Apeldoorn, The Netherlands) was used as the only NSP- and protein-source in the diets. Diets were designed to meet the nutrient requirements of pigs according to CVB[38]. The first diet (Unprocessed RSM) contained 585 g/kg untreated RSM and the second diet contained 585 g/kg acid-extruded RSM (Acid-extruded RSM). Prior to extrusion, RSM, water (40 %, wt/wt), and maleic acid (2 %, wt/wt) were mixed using a paddle-mixer (Type F60, Halvor Forberg, Bygland, Norway). After 1 hour, acid-extrusion was performed in a double screw extruder (Baker-Perkins, Peterborough, UK) at 120˚C (product temperature at the die: 108˚C), feeding rate 33 kg/h, 250 rpm screw speed, and die size 6 mm. During a transition phase of 5 days, the feed was gradually changed from starter feed to the experimental feed. Pigs received diets in liquid form with a feed to water ratio of 1:3. An adaptation period of 14 days was followed by a collection period of 4 days, during which fecal samples were collected and pooled per pen.

Digesta collection. At termination of the study (days 24 and 25 of the trial) animals were anesthetized 4 h post-feeding experimental diets, and digesta samples were collected. Subsequently, each animal was euthanized. Digesta were collected from terminal ileum (last 100 cm from ileocecal valve) and cecum. The colon was separated in three equal parts (proximal, mid and distal colon) from which the middle 50 cm were used to collect digesta. Samples from ileum and colon were pooled per pen and immediately stored frozen (-20 °C). From ileal digesta and feces also individual samples were collected and analyzed. Digestibility of protein and starch and fermentability of NSP and constituent monosaccharides in the different intestinal compartments was calculated using the following formula:

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

Table 6.1. Ingredient composition (g/kg as-fed) of the diets containing unprocessed or acid-extruded rapeseed meal and analyzed content of starch, protein, fat, and NSP (%, wt/wt)) and molar composition of NSP (mol%).

	Unprocessed RSM diet	Acid-extruded RSM diet
Ingredient		
Rapeseed meal	585.5	585.5
Maize starch	269.4	269.4
Sugar	70	70
Soy oil	40	40
Mineral and vitamin premix[1]	10	10
Monocalcium phosphate	6	6
Limestone	5	5
Salt	4	4
Potassium bicarbonate	3	3
Sodium bicarbonate	3	3
L-Lysine HCL	3	3
L-Threonine	0.6	0.6
L-Tryptophan	0.3	0.3
Cr_2O_3	0.025	0.025
Analyzed nutrient composition[2]		
Starch	31	30
protein	20	19
Glucosinolates (μmol/g DM)	3.7	5.4
NSP	26	24
Molar composition of NSP (mol%)[3]		
Rha	1	1
Ara	20	20
Xyl	9	8
Man	5	3
Gal	7	7
Glc	31	30
UA	27	31

[1] Rapeseed meal is either unprocessed or acid-extruded before addition to the diet.

[2] Mineral and vitamin premix provided the following per kg feed: vitamin A: 6,000 IU; vitamin D3: 1,200 IU; vitamin E: 40 mg; vitamin K3: 1.5 mg; vitamin B1: 1 mg; vitamin B2: 3 mg; vitamin B6: 1 mg; vitamin B12: 15 μg; niacin: 20 mg; d-calcium pantothenate: 10 mg; choline chloride: 150 mg; folic acid: 1.2 mg; Fe: 80 mg ($FeSO_4 \cdot H_2O$); Cu: 15 mg ($CuSO_4 \cdot H_2O$); Mn: 30 mg (MnO); Zn: 50 mg ($ZnSO_4 \cdot H_2O$); Co: 0.2 mg ($CoSO_4 \cdot 7H_2O$); I: 0.7 mg (KI); Se: 0.2 mg (Na_2SeO_3).

[3] NSP as extracted from the feed. Mol%; molar composition presented as anhydrosugar moieties; Rha= rhamnosyl, Ara= arabinosyl, Xyl= xylosyl, Man= mannosyl, Gal= galactosyl, Glc= glucosyl, UA= uronyl.

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

Unprocessed and acid-extruded RSM, and their corresponding digesta from terminal ileum, cecum, proximal, mid and distal colon, and fecal samples were freeze-dried and milled using a ball mill (Retsch MM2000, Haan, Germany). The milled samples (~2.5g) were extracted three times with 45 mL demineralized water during 1 hour at 40°C. After each extraction, the solubilized material was separated from the insoluble residue by filtration over Whatman 595½ filters (Whatman, Kent, UK). Water-soluble fractions were combined, freeze-dried, and denoted as water-soluble solids (WSS). Water unextractable solids (WUS; ~0.75g) obtained were further extracted twice using 6M NaOH including 20mM $NaBH_4$ at 4 °C as described previously[228]. After each extraction, material was centrifuged (48,400*g*, 20 min, 4°C) and the supernatant was further separated from the insoluble residue by filtration on G2-glass filters. Alkali-soluble fractions were combined, neutralized using 6M HCl, dialyzed against demineralized water at 4˚C (Visking dialysis tubes, molecular mass cut-off 12-14kDa, pore diameter ca. 25Å, Serva, Heidelberg, Germany), freeze-dried, and denoted as alkali-soluble solids (ASS). The final residue was neutralized, washed with water, freeze-dried, and denoted as residue (RES).

Enzymatic fingerprinting

WSS-, ASS-, and RES-fractions from ileal digesta and fecal samples were incubated with polygalacturonase, rhamnogalacturonan hydrolase, β-galactosidase, endo-galactanase, endo-arabinanase, exo-arabinanase, a xyloglucan specific endo-glucanase, and endo-xylanase I, as described previously[224]. These pure enzymes were dosed at 0.1 µg enzyme-protein per mg substrate. Besides these pure enzymes, CellicCTec was used and dosed at 20 µg enzyme-protein per mg substrate. Incubations (5 mg/mL) were performed in 10 mM NaOAc buffer (pH 5.0) at 40˚C rotating 'head-over-tail' for 24 hours. All enzymes were inactivated by heating at 100˚C for 10 minutes. Digests were analyzed by HPSEC, HPAEC, and MALDI-TOF MS.

Analytical methods

Dry matter content was determined in duplicate by drying overnight in an oven at 103 °C. Protein content (N*5.3[229]) was determined in duplicate by the Dumas method[13, 230] on a Thermo Quest NA 2100 Nitrogen and Protein Analyzer (Interscience, Troy, NY, USA). Samples (~10 mg) were weighed into cups and directly analyzed. D-methionine was used for calibration. Total starch content was determined (in duplicate) enzymatically using the total starch assay procedure K-TSTA 04/2009 (Megazyme, Bray, Ireland). Non-starch polysaccharide extraction from the feed was performed as described elsewhere[199]. In short, the present starch was gelatinized and enzymatically degraded, after which NSP could be precipitated using acidified ethanol. Neutral carbohydrate content and composition was determined in duplicate according to Englyst and Cummings[49], as described previously[224]. Uronic acid content was determined in duplicate as described previously[224]. High Performance Size Exclusion Chromatography (HPSEC) as described previously[224], with the adaptation that the injection volume was 10 µL and column oven temperature was 55°C. Enzyme digests were analyzed without prior dilution. High Performance Anion Exchange Chromatography (HPAEC) was performed as described previously[224]. Matrix Assisted Laser-induced Desorption/Ionization Time-of-Flight Mass

Spectrometry (MALDI-TOF-MS) was performed using an UltraFlextreme workstation (Bruker Daltonics, Bremen, Germany) equipped with a Smartbeam II laser of 355nm as described previously[224]. Glycosidic linkage analysis was performed as described elsewhere[224]. Chromium (used as marker, added in the diets to enable calculation of nutrient digestibility) was analyzed by atomic absorption spectrometry after burning the samples at 250 °C and incineration at 550 °C, as described elsewhere[198]. Diets were additionally analyzed for contents of glucosinolates (ISO 9167-1).

RESULTS AND DISCUSSION

Characteristics of unprocessed and acid-extruded RSM

The RSM (Table 6.2; Unprocessed) was mainly composed of protein (33 % (N*5.3), wt/wt) and carbohydrates (27 %, wt/wt); including 25 % (wt/wt) NSP). Other compounds in RSM are expected to be lignin, fat, and ash[221]. RSM carbohydrates mainly contained glucosyl (40 mol%), arabinosyl (19 mol%), and uronyl residues (18 mol%) (Table 6.2). This molar carbohydrate composition indicated the presence of cellulose, xyloglucan, arabinan, and homogalacturonan, as was described before for *Brassica napus* meal[224]. The carbohydrate content in RSM is variable and can differ between cultivars[231] and can be affected by treatment during oil extraction[232]. The NSP-content of 25 % (wt/wt) found in this study is in line with values reported previously (16-22 %, wt/wt)[222]). Unexpectedly, the carbohydrate content was analyzed to be numerically higher and protein content numerically lower after acid-extrusion. This was not expected to be a result from acid-extrusion, since it was not seen in a previous study where RSM was treated similarly[224]. It can be noted that taking a small sample from this large batch (500 kg) for analysis may have affected the homogeneity, but the constituent monosaccharide composition was not affected by the processing performed (Table 6.2). The glucosinolate contents (calculated to be 5-9 μmol/ g DM RSM) of the RSM used in the current study was rather low[44].

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

	Unprocessed RSM	Acid-extruded RSM
Protein	33	30
Carbohydrate	27	35
of which water-soluble saccharides (DP≤4)[1]	7	12
of which water-soluble saccharides (DP>4)[1]	19	17
of which water unextractable[a]	24	71
Others (lignin, ash, fat)[2]	40	35
Molar composition of carbohydrates[3]		
Rha	1	1
Ara	19	19
Xyl	8	8
Man	4	4
Gal	10	10
Glc	40	40
UA	18	18

[1] % of water-soluble fructose, saccharose, raffinose, and stachyose (DP≤4) analyzed using HPAEC, other water-soluble saccharides (DP>4), and water unextractable carbohydrates from total carbohydrates.

[2] Calculated as dry matter minus protein and minus carbohydrates.

[3] Mol%; molar composition presented as anhydrosugar moieties; Rha= rhamnosyl, Ara= arabinosyl, Xyl= xylosyl, Man= mannosyl, Gal= galactosyl, Glc= glucosyl, UA= uronyl.

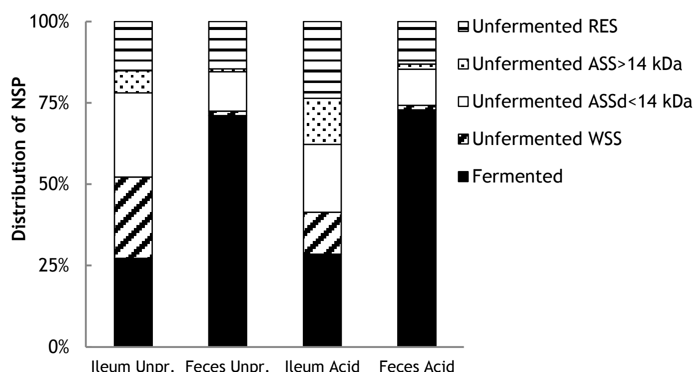

Figure 6.1. Distribution of NSP from rapeseed meal being digested or undigested. The undigested carbohydrates are further divided as water-solubles (WSS), calculated alkali-solubles <14kDa (ASSd), alkali-solubles >14kDa (ASS), and final residue (RES) of ileal digesta and feces of pigs fed unprocessed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

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

The digestibility and fermentability values were based on 6 animals per feed and were presented to be indicative rather than statistically analyzed (Table 6.3). In pigs fed a diet containing unprocessed and acid-extruded RSM, 22 % and 23 % of the NSP was fermented in the ileum, 56 % and 46 % of the NSP was cumulatively fermented up to the cecum, and 68 % and 72 % fermented total tract, respectively (Table 6.3). Ileal NSP-fermentability of other diets, containing similar NSP, e.g. based on chicory forage and sugar beet pulp, is reported to be between 26-29 %[233]. Total tract fermentability of RSM was expected to be 58 %[38].

Mass balance of carbohydrates from RSM in feed into digesta

The aim of this study was to analyze residual carbohydrate structures, remaining in the digesta after fermentation. These results were expected to indicate how a more complete fermentation of the carbohydrates from RSM could be achieved.

The ileal digesta mostly contained carbohydrates (52 %, wt/wt), next to proteins (15-19 %, wt/wt) (Table 6.4; Supplemental information Table 6.6), while for the fecal dry matter 30-33 % (wt/wt) was carbohydrates and 23 % (wt/wt) protein. Constituent monosaccharide compositions of the residual carbohydrates in digesta in ileum, cecum, colon, and feces are shown in Table 6.4. Figure 6.1 shows the distribution of carbohydrates originally present in the diet being fermented or unfermented. The unfermented carbohydrates were further fractionated into a water-soluble fraction (WSS), alkali-soluble fractions (ASS), and the remaining residue (RES). WSS contained 26 % (wt/wt) of the unfermented NSP in ileal digesta from pigs fed unprocessed RSM (Table 6.4), which was lower for ileal digesta of pigs fed acid-extruded RSM (14 %, wt/wt). From the feces, only 4-5 % of the unfermented NSP was water-soluble. So, nearly all water-soluble carbohydrates, as present in RSM itself (27 % of the carbohydrates present[224]) and solubilized by microbial enzymes, were fermented in the cecum and colon (Figure 6.1), which was also observed for soy NSP[234]. Unexpectedly, only 8-18 % (wt/wt) of the water unextractable carbohydrates in ileal digesta and feces from pigs fed unprocessed or acid-extruded RSM was

extracted in the corresponding ASS-fractions (Table 6.4). It was calculated from the NSP in the WUS-fractions minus the NSP collected in the ASS- and RES-fractions that 48-68 % (wt/wt) of the NSP in WUS was extracted with alkali. This implies that 40-54 % (wt/wt) of the NSP in WUS was not recovered in the ASS- and RES-fractions. This suggested that an important part of the water unextractable carbohydrates from the digesta and feces was released by alkali had a relatively low molecular mass (<14 kDa, cut-off value of the dialysis membrane). These alkali extracted carbohydrates are referred to as ASSd <14 kDa. A similar alkaline extraction of NSP from the unprocessed RSM added in the diet did not result in such a release of small carbohydrates[224]. For this phenomenon observed, three possible explanations are considered. First, in the fecal samples, small, partly-fermented RSM carbohydrates were present linked via ester-linkages. Ester-linkages to proteins and carbohydrates in RSM are reported to be present through sinapic, ferulic, and coumaric acid[235]. Alkaline extraction will break such bonds, thereby solubilizing these partly fermented carbohydrates. This explanation may indicate that the pigs' microbiota was able to degrade some of the polysaccharides present, but full degradation was hindered by ester-bonds. Second, 6M NaOH makes cellulose fibrils swell, as published for bamboo fibers[236]. Thereby, it releases low molecular weight non-covalently, e.g. by H-bonding, bound carbohydrate-structures. Third, small carbohydrates originating from RSM NSP can be bound to the microbial cell surface proteins mediated by a receptor, as is a known digestive mechanism for Bacteroidetes[237], which together with the phylum Firmicutes make up for >90 % of the microbiota in pigs' large intestine[238]. Again, alkali will then release these small partly fermented carbohydrates. We believe this third theory is less likely, because of the relative high amounts of released carbohydrates. Also, a similar extraction of a fecal sample from pigs fed a diet without RSM (samples from a study by Haenen et al.[239]) did not result in such a release of carbohydrates. This indicated that the first two explanations are more likely. Further research will be conducted to analyze the nature of these carbohydrates released. In a previous *in vivo* trial where broilers were fed RSM-rich diets, also an important part (39-52 %, wt/wt) of the water unextractable carbohydrates in the feces were extracted in alkali, but not recovered in the similarly prepared ASS- or RES-fractions from the excreta[224]. Apparently, in both broilers and growing pigs fermentation of RSM NSP was hindered by the ester-linkages or H-bonding present. After water and alkaline extraction, still 16 % (wt/wt) of the ileal carbohydrates and 44 % (wt/wt) of the fecal carbohydrates from pigs fed unprocessed RSM could not be extracted and remained in the residue (RES) (Table 6.4). In ileal digesta from pigs fed acid-extruded RSM, slightly more carbohydrates remained in the residue compared with unprocessed RSM. Combined with the decreased water-soluble carbohydrates, it was hypothesized that, *in vivo* the NSP-matrix became more rigid after acid-extrusion. In the feces the distribution of fractions is rather similar for pigs fed unprocessed RSM and acid-extruded RSM. Apparently, microbiota in the pigs' colon was not hindered by this more rigid matrix. Distribution of fermented and unfermented arabinosyl, xylosyl, glucosyl, and uronyl residues over water-soluble, alkali-soluble, and residual fraction is shown in Figure 6.2. The yield of constituent monosaccharides in the ASSd (<14 kDa) fraction was again calculated from the amount of each constituent monosaccharide present in WUS minus the amount recovered in both the ASS- and RES-fractions. Arabinosyl residues were mainly water- and alkali-soluble, probably

originating from arabinan (Figure 6.2A). Xylosyl residues were mainly alkali-soluble, probably originating from xyloglucan and xylan (Figure 6.2B). Glucosyl residues were both water-soluble, probably originating from glucans, and unextractable, probably originating from cellulose (Figure 6.2C). Uronyl residues were mainly alkali-soluble, probably originating from pectins (Figure 6.2D). The proportion of water-soluble arabinosyl residues in the ileal digesta decreased after acid-extrusion, in favor of the alkali-soluble fractions. The proportions of alkali-soluble xylosyl and uronyl residues in the same ileal digesta increased at the expense of the fermented fraction. The proportion of water-soluble glucosyl residues in the same ileal digesta decreased in favor of the residue. Again, this suggested that acid-extrusion increased rigidity of the NSP-matrix in ileal digesta. This may explain the decreased ileal fermentability of arabinan (arabinosyl), xyloglucan (xylosyl), and pectin (uronyl residues) after acid-extrusion, compared with pigs fed unprocessed RSM.

Characterization of recalcitrant water-soluble carbohydrate structures

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

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

Glycosidic linkage type analysis of ileal and fecal WSS-fractions

Carbohydrate structures in WSS-fractions were analyzed using glycosidic linkage type analysis (Table 6.5). It should be taken into account that in the protocol used some small saccharides got dialyzed out together with excess of reagents, possibly causing the high ratio between terminal and branched residues. Nevertheless, molar compositions analyzed by glycosidic linkage type analysis (Table 6.5) and neutral carbohydrate composition analysis (Table 6.4) were comparable. In the ileal WSS-fraction from pigs fed unprocessed RSM, arabinan was highly branched at the O2- and/or O3-position, as found for *Brassica campestris* meal[223], but not for *Brassica napus* meal[224]. After acid-extrusion of RSM, the water-soluble arabinan was less branched compared with those found in ileal digesta of pigs fed unprocessed RSM. Most likely acid-extrusion released some side-groups from arabinan. Also in the *in vitro* study[33], arabinosyl residues were most affected by acid treatment, since arabinosyl residues are most acid-labile. Galactosyl residues in the ileal WSS-fractions were found to be mainly terminal and 1,6-linked, probably originating from raffinose and stachyose and were not affected by acid-extrusion. Glucosyl residues were mainly present as terminal and 1,4-linked residues and some 1,4,6-linked residues, indicating

the presence of xyloglucan. Also smaller proportions of 1,2-, 1,3-, and 1,6- linked glucosyl residues were found, possibly originating from microbiota[240], since they were not found in RSM itself[223]. Relatively more glucosyl residues in the ileal WSS from pigs fed acid-extruded RSM were 1,4- and 1,4,6-linked compared with ileal WSS from pigs fed unprocessed RSM. Xyloglucan and cellulose can be tightly bound by hydrogen bonds[241] and most likely acid-extrusion disrupted some of these bonds and released xyloglucan into WSS. Such an acid-aided solubilization of xyloglucan was reported before for adzuki beans[242].

Despite the fact that in the fecal WSS-fraction of pigs fed unprocessed and acid-extruded RSM a relatively low amount of the carbohydrates was collected (Figure 1), the glycosidic linkages of the NSP present were still analyzed (Table 6.5). Mainly rhamnogalacturonan, arabinan, galactomannan, and xyloglucan were present, as were present in RSM itself[224]. Branchiness of the fecal water-soluble arabinan decreased compared with the ileal water-soluble arabinan, implying that microbial enzymes present in the colon had debranching activity. Rhamnogalacturonan was not expected to be water-soluble, because in RSM itself these structures remained in the RES[224]. Probably, the microbial enzymes present were able to solubilize rhamnogalacturonan.

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

	Unprocessed RSM						Acid-extruded RSM					
	Ileum[1]	Cecum	Prox. colon	Mid colon	Distal colon	Total tract[1]	Ileum[1]	Cecum	Prox. colon	Mid colon	Distal colon	Total tract[1]
Protein	60±9.05	65	70	70	73	70±1.24	69±2.32	65	69	71	73	70±1.58
Starch	98±0.13	99	99	99	100	100±0.01	98±0.36	98	99	100	100	100±0.03
NSP	22±4.89	56	69	73	70	68±0.78	23±7.09	46	67	72	76	72±1.92
Ara	21±10.19	74	82	85	85	85±0.61	12±10.21	63	83	88	88	85±1.59
Xyl	25±13.78	61	77	80	80	79±0.85	3±8.91	50	75	82	85	82±1.76
Glc	37±4.86	60	75	80	75	74±1.19	43±8.01	47	67	73	79	75±2.14
UA	18±4.26	42	55	59	54	51±0.64	10±5.56	28	53	58	64	58±3.47

[1] Ileal digesta and feces were analyzed for pigs individually (n=6 per treatment), therefore average± standard error were calculated. For the other colon compartments only pooled digesta samples were analyzed.

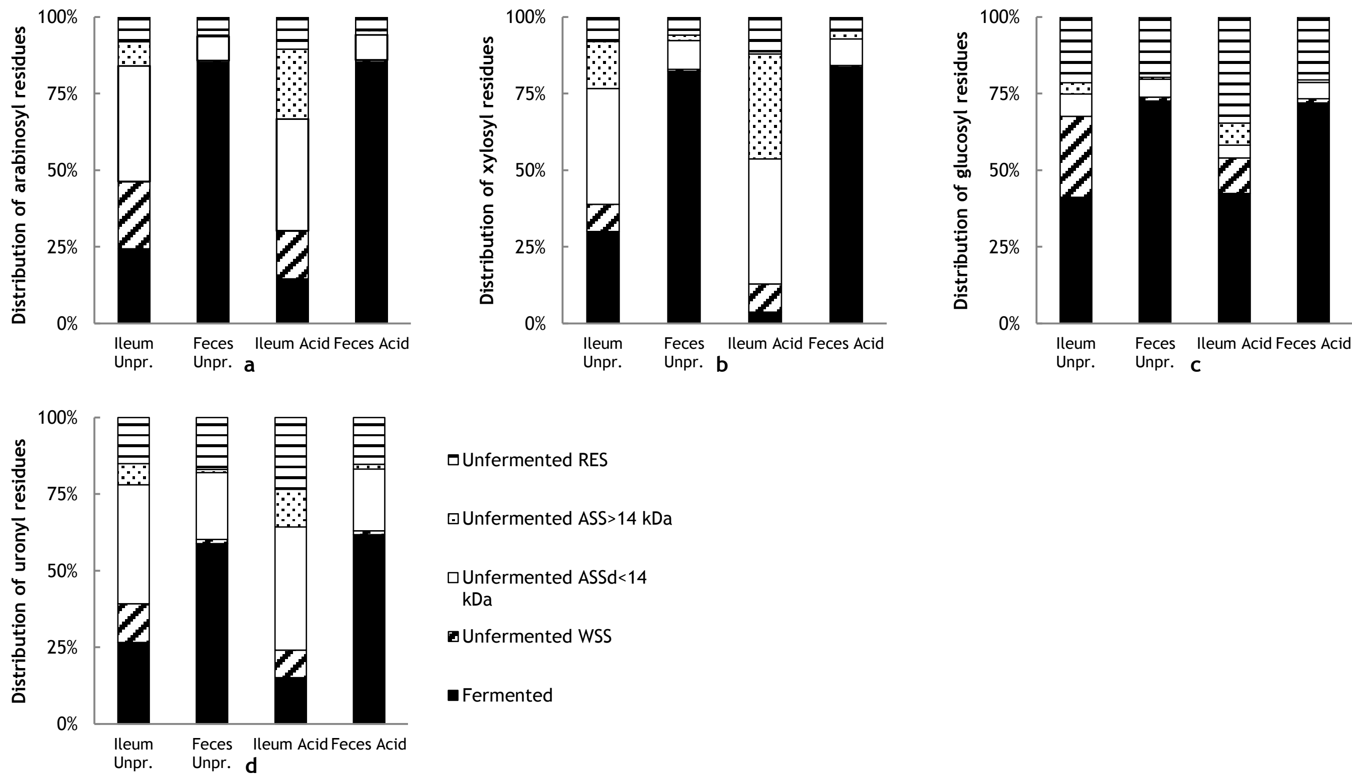

Figure 6.2. Distribution of arabinosyl (panel a), xylosyl (panel b), glucosyl (panel c), and uronyl (panel d) residues from rapeseed meal being digested or undigested. The undigested residues are further divided as water-solubles (WSS), calculated alkali-solubles <14kDa (ASSd), alkali-solubles >14kDa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

Table 6.4. Carbohydrate content (%, wt/wt), yield (%), and composition (mol%[1]) in digesta, fecal samples, and fractions thereof, obtained after in vivo digestion in pigs fed diets containing unprocessed or acid-extruded rapeseed meal (RSM).

	Rha	Ara	Xyl	Man	Gal	Glc	UA	Carbohydrates	
								Content[2]	Yield
Unprocessed RSM									
Ileum	1	19	8	4	15	28	25	52	100
WSS	1	19	3	4	23	39	11		26
ASS (>14 kDa)	1	24	19	3	11	20	22		7
ASSd (<14 kDa)	1	20	9	5	10	13	42		
Res	2	11	5	2	4	53	23		16
Cecum	3	13	9	4	9	39	23	36	
Proximal colon	3	15	8	4	11	34	25	33	
Mid colon	3	15	8	4	12	29	29	30	
Distal colon	4	15	8	5	11	30	27	34	
Feces	1	14	8	4	11	35	27	33	100
WSS	7	9	4	6	17	31	26		4
ASS (>14 kDa)	5	10	17	6	12	22	28		3
ASSd (<14 kDa)[3]	2	9	5	5	9	16	54		
Res	2	8	4	3	5	48	30		44
Acid-extruded RSM									
Ileum	1	21	10	4	9	22	23	52	100
WSS	2	23	6	6	14	34	15		14
ASS (>14 kDa)	1	29	19	3	10	18	18		15
ASSd (<14 kDa)[3]	1	21	10	4	11	7	46		
Res	2	10	4	2	4	56	22		25
Cecum	2	16	10	4	9	35	24	40	
Proximal colon	1	17	9	4	11	31	27	34	
Mid colon	3	14	7	4	11	29	32	32	
Distal colon	5	14	7	6	11	30	27	28	
Feces	4	5	8	4	11	30	28	30	100
WSS	9	8	4	6	17	30	26		5
ASS (>14 kDa)	4	18	15	5	13	18	27		5
ASSd (<14 kDa)[3]	2	0	5	4	9	4	26		
Res	4	7	3	2	5	48	31		46

[1] Molar composition presented as anhydrosugar moieties; Rha= rhamnosyl, Ara= arabinosyl, Xyl= xylosyl, Man= mannosyl, Gal= galactosyl, Glc= glucosyl, UA= uronyl.
[2] Total carbohydrates based on dry matter (%, wt/wt).
[3] Numbers for ASSd <14 kDa were calculated as WUS minus ASS minus RES.

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

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

[1] t: terminal
[2] Tr: trace amounts
[3] T/B: ratio terminally linked residues: branching points

Characterization of recalcitrant water unextractable carbohydrate structures

As discussed above, from the water unextractable carbohydrates, 8-18 % (wt/wt) was recovered as ASS and 16-44 % (wt/wt) was recovered in the residue. This leaves 40-54 % (wt/wt) of the water unextractable carbohydrates from ileal digesta and feces released by 6M NaOH, not recovered as ASS or RES. The molar constituent monosaccharide composition of these fractions (ASSd<14 kDa) were calculated (Table 6.4). All ASSd fractions (<14 kDa) were calculated to be composed of 42-54 mol% uronyl residues. This was around twice the molar proportion of uronyl residues in the recovered ASS-fractions (>14 kDa).

Glycosidic linkage type analysis of ileal and fecal ASS-fractions

Carbohydrate structures in the ASS-fractions (larger than 14kDa) were analyzed using glycosidic linkage type analysis (Table 6.5). Data obtained from glycosidic linkage type analysis of the fractions should be reviewed in a qualitative way instead of quantitative due to incomplete DMSO-solubility and the presence of uronic acids[228].

The results indicated the presence of a highly branched arabinan in ileal ASS of pigs fed unprocessed RSM, similar in structure to the corresponding water-soluble arabinan. Xyloglucan, originating from RSM, was found next to a branched xylan, of which indications have been found in RSM itself[223]. Probably this structure is accumulating in unfermented samples. In the fecal ASS obtained from the same pigs, still, branched arabinan, xyloglucan, and galactomannan was present. Next to that, the xylan analyzed in the feces was more linear than in the ileal digesta, indicating that microbial xylan-debranching enzymes were active in the colon. Ileal and fecal samples from pigs fed acid-extruded RSM showed similar glycosidic linkages in the ASS-fractions.

Enzymatic fingerprinting of ileal and fecal ASS-fractions

HPSEC-analysis of enzyme digests of the ASS-fraction of feces from pigs fed unprocessed RSM showed activity of arabinanases, galactanases, polygalacturonase, and cellulase towards the ASS-fraction, confirming the presence of arabinan, galactan, homogalacturonan, and cellulose (no further data not shown). In the cellulase-digest also xyloglucan-oligomers were formed, as analyzed by MALDI-TOF MS. These oligomers were XXXG or XSGG, XXLG or LSGG, XXFG and XLFG (data no shown; nomenclature according to Fry et al.[243]), indicating the presence of mainly XXXG-type xyloglucan with arabinosyl, galactosyl, and fucosyl residues, as was also indicated for *B. napus* meal[224]. All NSP-structures present in the various ASS-fractions analyzed are summarized in Supplemental information Table 6.7.

Unextractable carbohydrates in RES

The unextractable residual fraction (RES) mainly contained glucosyl (48-56 mol%) and uronyl residues (22-31 mol%; Table 6.4). Polymeric structures in the RES-fraction could not be accurately analyzed using glycosidic linkage type analysis due to their insolubility in DMSO. Also using enzymatic fingerprinting carbohydrate structures could not be elucidated, since the carbohydrates were inaccessible for the enzymes used to be degraded. It is hypothesized that the RES-fractions mainly contain cellulose and tightly bound pectins[244, 245].

CONCLUSION

In conclusion, the remaining recalcitrant carbohydrate structures detected in feces of pigs fed RSM-rich diets, were (branched) arabinan, linear galactan, XXXG-type xyloglucan, linear xylan, and cellulose. A major fraction (35-54 %, wt/wt) of these recalcitrant carbohydrates was unexpectedly released as small uronyl-rich carbohydrates (<14 kDa) during alkaline extraction using 6M NaOH. It follows that alkali-labile bonds hindered the complete fermentation of NSP in pigs and that fermentation may be improved by an alkaline pretreatment of RSM in the feed.

SUPPLEMENTAL INFORMATION

Table 6.6. Content (%, wt/wt), yield (%), and recovery (%) of dry matter, protein and total carbohydrate from water and alkaline extraction in digesta, fecal samples, and fractions thereof, obtained after in vivo digestion in pigs fed diets containing unprocessed or acid-extruded rapeseed meal (RSM).

	DM yield	Protein content	Yield
Unprocessed RSM			
Ileum	100	19	100
WSS	54	26	72
ASS (>14 kDa)	10	4	2
Res	81	8	7
Recovery	100		81
Feces	100	23	100
WSS	19	21	17
ASS (>14 kDa)	8	27	9
Res	42	11	20
Recovery	69		46
Acid-extruded RSM			
Ileum	100	15	100
WSS	38	20	50
ASS (>14 kDa)	18	5	6
Res	26	8	14
Recovery	82		70
Feces	100		100
WSS	19	23	20
ASS (>14 kDa)	11	23	14
Res	43	30	23
Recovery	73	12	57

Table 6.7 Summary of polysaccharides present in ileal digesta and feces from pigs fed unprocessed RSM (Unpr. RSM) and acid-extruded RSM (Acid RSM).

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

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

Chapter 7

Enzymes to improve rapeseed meal degradability in broilers

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ABSTRACT

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

INTRODUCTION

Rapeseed meal (RSM), a by-product from rapeseed oil production, is a suitable protein source for poultry feed, however, its nutritional value is limited by the high level of non-starch polysaccharides (NSP) that typically constitute 20 to 40 % of the meal[25, 246-248]. Non-starch polysaccharides can be partly fermented by the microbial community residing in the gastrointestinal tract, although in poultry, degradation is almost exclusively limited to the soluble fraction[73]. In RSM only 8 to 15 % of NSP is water-soluble, and NSP degradability values in chicken are only 3 to 6 %[21, 25, 249]. In addition, NSP may affect digestion of other nutrients, both directly due to physical hindrance and indirectly due to physiological changes in the gut, such as increased digesta viscosity[86].

Processing technologies and cell wall degrading enzymes, such as pectinases, might be used to modify cell wall architecture and improve NSP degradability, as recently reviewed by de Vries et al. (Chapter 2). Mechanical forces open the cell wall structure and reduce particle size, thereby increasing the surface area accessible for enzymes – i.e. both feed enzymes added to the diet and microbial enzymes present in the gastrointestinal tract. Hydrothermal treatment can break weak bonds between polysaccharides, but excessive heating may increase protein and free amino acid damage[99]. In addition, thermal treatments often increase digesta viscosity (Chapter 2), which can negatively affect nutrient digestion and absorption[86]. In a previous in vitro study[33], effects of wet milling – a technology that combines rigorous particle size reduction with moisturizing – and extrusion, combining hydrothermal treatment and shear, in combination with pectolytic enzymes were investigated. Although processing on its own did not improve NSP solubility in vitro, pectolytic enzymes were only effective when added to processed RSM, indicating that processing technologies are required to enable effective use of enzymes.

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

MATERIALS AND METHODS

Experimental design

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

Materials and diets

Rapeseed meal (*Brassica napus*, Cargill N.V., Antwerp, Belgium; 2011) with a geometric mean diameter (GMD) of 818 μm was obtained from a commercial feed mill (Agrifirm B.V., Utrecht, The Netherlands). Six diets, containing 35 % unprocessed or processed RSM, and two basal diets (with a and without pectolytic enzymes), were formulated to meet or exceed nutrient requirements of broilers[38] (Table 7.1). Basal diets were used to calculate RSM degradability using the difference method. Rapeseed meal was processed at Wageningen Feed processing Centre (Wageningen University, Wageningen, The Netherlands). Diets were fed as pellets; product temperature during pelleting was 65 to 70 °C.

Processing technologies

Wet-milling. Rapeseed meal was milled using a laboratory-scale refiner (Sprout-Waldron Inc., Muncy, PA) at a feed rate of 158 $kg \cdot h^{-1}$ using 480 liter water per h. Diameter of the discs was 30 cm with the distance between discs set at 0.07 mm and a rotation speed of 3000 rpm. Product temperature when leaving the machine was 32 ± 5.8 °C. Geometric mean diameter of the wet-milled RSM was 220 μm.

Extrusion-cooking. Water was added to the unprocessed products to reach a DM content of 75 % (wt/wt) and samples were mixed using a paddle-mixer (Type F60; Halvor Forberg, Bygland, Norway). Within 60 minutes after mixing, samples were extruded using a co-rotating double screw extruder (M.P.F.50; Baker Perkins Ltd., Peterborough, United Kingdom) with a length/diameter ratio of 25. The screw configuration was as follows: four 1.5D feed screw elements, one 1D single lead element, three 1D feed screw elements, one 1D single lead element, two 1D feed screw elements, two 4D 90 degree forwarding block paddles, one 1.5D feed screw elements, one 4D 90 degree forwarding block paddles, one 1.5D feed screw elements, two 4D 90 degree forwarding block paddles, and two 1.5D single lead elements, where D is one full revolution around the element. A die with two orifices (6 mm) was used; no die face cutter was used. Feeding rate was 36 $kg \cdot h^{-1}$, screw speed was 250 rpm. Barrel temperatures in the ten segments of the extruder were set at 30, 50, 72, 82, 90, 105, 115, 120, 120, and 120 °C. Product temperature at the die was 110 ± 1.6 °C, pressure at the die was 3.4 ± 0.05 MPa. Geometric mean diameter of the extruded RSM was 288 μm.

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

Cell wall degrading enzymes

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

Birds and experimental procedures

A total of 750 1-d-old female chicks (initial BW 40.7 ± 3.33 g; Ross 308, Aviagen Group, Newbridge, United Kingdom), obtained from a commercial hatchery (Broederij Lagerweij B.V., Lunteren, The Netherlands) were housed in 10 floor pens bedded with wood pellets (SOFTCELL, Agromed GmbH, Kremsmünster, Austria). All broilers were fed a starter diet with 20 % (wt/wt) RSM, containing 21 % (wt/wt) CP and 2743 kcal ME/kg. At d 14, 576 broilers were allocated to one of the 8 diets (Table 7.1 and 7.2) based on BW, and housed in metabolism cages (70 × 60 × 45 cm). In total, the experiment comprised 48 cages, with 6 cages per treatment. Each cage housed 12 broilers from d 14 to 25 and 11 broilers from d 26 to the end of the experiment. Average BW of the broilers at d 14 was 349.7 ± 37.7 g. Feed was available ad libitum and broilers had free access to water during the experiment. Ambient temperature was maintained 33 °C at d 1 and gradually decreased to 21 °C at d 25. Photoperiod was 23L:1D during the first two days and 16L:8D from d 3 onwards. Broilers were spray-vaccinated against Newcastle disease at d 15.

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

Analytical methods

The geometric mean diameter of unprocessed and processed RSM was analyzed by laser diffraction (Mastersizer 2000, Malvern Instruments Ltd., Malvern, United Kingdom). The water binding capacity (WBC) of diets and crop contents was analyzed in duplicate by soaking 1 gram of raw material or dried crop content in 25 mL of water for 24 h at room temperature. Samples were centrifuged at 3274 *g* for 20 min at room temperature and decanted. Water binding capacity was calculated as the weighed quantity of water retained per g of dry material.

Prior to chemical analyses, samples were milled in a mixer mill (MM 2000, Retsch GmbH, Haan, Germany) at amplitude of 80, during 1 min. All chemical analyses were performed in duplicate using standard laboratory methods[13, 171]. Diets, gastrointestinal contents, and excreta were analyzed for content of DM (AOAC 930.15), ash (AOAC 942.05), cobalt (by atomic absorption spectrophotometry, using a SpectrAA 300 atomic absorption spectrophotometer, Varian B.V., Middelburg, The Netherlands), ether extract (after hydrochloric acid hydrolysis, using Soxhlet apparatus and petroleum ether; AOAC 920.39), nitrogen (diets by Kjeldahl method: ISO 5983. Gastrointestinal contents and excreta by Dumas method: AOAC 990.03; using a Thermo Quest NA 2100 Nitrogen and Protein Analyser, Interscience B.V., Breda, The Netherlands), total starch (AOAC 996.11; using a commercial test kit, Megazyme international Ltd., Bray, Ireland), and total NSP measured as neutral sugars and uronic acids. Prior to neutral sugar and uronic acid analyses, NSP were extracted from diets[199]. Briefly, starch was gelatinized and enzymatically degraded, after which NSP were precipitated using acidified ethanol, to remove released mono- and disaccharides as well as soluble small saccharides naturally present in RSM (mainly sucrose and stachyose). Neutral sugar composition was analyzed by gas chromatography according to the method of Englyst and Cummings[49]. After pre-treatment with 72 % (wt/wt) H_2SO_4 for 1 h at 30 °C, samples were hydrolyzed with 1 M H_2SO_4 at 100 °C for 3 h. Constituent sugars were derivatized into alditol acetates and analyzed using a GC (Focus-GC, Thermo Scientific, Waltham, MA). Inositol was used as internal standard. Uronic acid content was analyzed according to the automated colorimetric m-hydroxydiphenyl assay[200] using an auto-analyzer (Skalar Analytical B.V., Breda, The Netherlands). Galacturonic acid was used for calibration. Non-starch polysaccharide content was calculated as the sum of neutral sugars and uronic acids minus glucosyl originating from remaining starch in the NSP extract (measured enzymatically as described above). Nitrogen (N) content of diets was corrected for differences between analytical methods (Kjeldahl vs. Dumas method) using: $N_{Dumas} = N_{Kjedahl} \times 0.9885 + 0.0103$[201]. Crude protein content was calculated as N × 6.25 (ISO 5983). Fecal nitrogen in the excreta was calculated as total N minus N in uric acid. Uric acid was analyzed enzymatic-colorimetric using a commercial test kit (10694, Human GmbH, Wiesbaden, Germany). Diets were additionally analyzed for content of neutral detergent fiber (NDF; AOAC 2002.04) and glucosinolates (ISO 9167-1), as well as phytate, total phosphorus, and phytate bound phosphorus (enzymatic-colorimetric; using a commercial test kit, K-PHYT 07/11, Megazyme international Ltd., Bray, Ireland).

Calculations and statistical analysis

Apparent total tract digestibility (ATTD) of RSM was calculated using the difference method[250], see Supplemental information Table 7.5 for ATTD of the basal diets. Small amounts of residual starch (2 to 3 g/100 g DM, data not shown) were present in excreta; therefore, ATTD of non-glucosyl polysaccharides (NGP; NSP – glucosyl) was calculated. Apparent total tract digestibility of starch, NSP, and NGP was calculated using the unprocessed control diet as the reference.

Data were analyzed by analysis of variance, with the GLM procedure of SAS (version 9.2, SAS Institute Inc., Cary, NC), using processing technology, enzyme addition, and their interaction as fixed effects. The effects of the blocking factor dissection day and its interaction with dietary treatment were tested, but found not to be significant and excluded from the model. Cage was the experimental unit. Model residuals were tested for homogeneity and normality, to verify model assumptions. Least square means (LSMeans) were compared using Tukey adjustments for multiple comparisons. Data are presented as LSMeans and pooled standard error of the mean (SEM) unless stated otherwise. Differences among means with $P < 0.05$ were accepted as representing statistically significant differences.

Table 7.1. Composition of basal and rapeseed meal diets (%, as-fed basis, unless indicated otherwise)

	Basal diets	Rapeseed meal diets
Ingredient		
Rapeseed meal	-	35.0
Maize	46.2	30.0
Maize starch	24.1	15.8
Wheat gluten meal	7.7	5.0
Fishmeal	6.2	4.0
Soy protein isolate	3.9	2.5
Soy oil	6.9	4.5
Limestone	1.08	0.70
Mineral and vitamin premix[1]	0.50	0.50
Monocalcium phosphate	0.69	0.45
Potassium bicarbonate	1.06	0.30
Salt	-	0.10
Sodium bicarbonate	0.17	0.11
L-Lysine HCL	0.46	0.30
D- Methionine	0.28	0.18
L-Threonine	0.12	0.08
L- Valine	0.15	0.10
L-Tryptophan	0.06	0.04
L-Isoleucine	0.12	0.08
L. Arginine	0.18	0.12
Co-EDTA	0.10	0.10
Cr_2O_3	0.025	0.025
Calculated nutrient composition[2]		
DE, kcal/kg	3,344	2,627
Calcium	0.72	0.76
Available phosphorus	0.28	0.31
Sodium	1.7	1.6
Digestible lysine	0.99	1.16
Digestible methionine + cysteine	0.84	0.95
Digestible threonine	0.56	0.78

[1] Provided per kilogram of diet: Vitamin A (retinyl acetate), 12.000 IU; cholecalciferol, 0.6 mg; vitamin E (DL-α-tocopherol), 50 mg; vitamin B_2 (riboflavin), 7.5 mg; vitamin B_6 (pyridoxine-HCl), 3.5 mg; vitamin B_1 (thiamin), 2.0 mg; vitamin K (menadione), 1.5 mg; vitamin B_{12} (cyanocobalamin), 20 µg; choline chloride, 460 mg; anti-oxidant (oxytrap PXN), 125 mg; niacin, 35 mg; pantothenic acid (d-calcium pantothenate), 12 mg; biotin, 0.2 mg; folic acid, 1 mg; Mn, 85 mg, as MnO; Fe, 80 mg, as $FeSO_4$; Zn, 60 mg, as $ZnSO_4$; Cu, 12 mg, as $CuSO_4$; I, 0.8 mg, as KI; Co, 0.4 mg, as $CoSO_4$; Se, 0.15 mg, as Na_2SeO_3.
[2] According to CVB[38].

Table 7.2. Analyzed chemical composition and water binding capacity (WBC) of basal diets and rapeseed meal (RSM) diets with (+) and without (-) addition of pectolytic enzymes (%, DM basis[1])

	Basal diet		Unprocessed RSM diet		Wet-milled RSM diet		Extruded RSM diet	
	-	+	-	+	-	+	-	+
DM, % as-fed	89.2	88.9	90	90.4	88	88.1	91.9	91.7
CP	21.1	20.7	27.9	28.7	28.3	27.8	28.8	28.1
Starch	57.3	54.3	35.1	33.9	35.4	38.8	34.1	33.6
Crude fat	10.3	10.3	9.2	9.2	9.5	9.2	9.4	9.1
Ash	4.6	4.7	5.5	5.4	5.5	5.6	5.5	5.4
Neutral detergent fiber	5.7	5.6	13.7	13.7	13.3	13.5	13.7	14.5
NSP[2]	6.8	5.6	17.6	17.2	18.0	18.0	18.0	16.3
Molar composition of NSP[3]								
Rhamnosyl	0	0	1	1	1	1	1	1
Arabinosyl	24	24	22	21	21	22	21	22
Xylosyl	41	39	16	16	15	17	15	16
Mannosyl	6	7	3	3	3	3	4	3
Galactosyl	6	6	7	6	6	7	7	7
Glucosyl	11	9	30	29	31	29	30	29
Uronyl	12	13	21	24	23	22	22	23
Phosphorus	0.63	0.53	0.57	0.53	0.65	0.65	0.59	0.55
Phytate bound phosphorous	0.17	0.23	0.32	0.28	0.34	0.30	0.34	0.31
Glucosinolates, µmol/g DM	0.7	1.0	1.2	1.4	0.9	0.9	1.4	1.2
WBC, g/g DM	1.5	1.6	1.7	1.8	2.1	2.0	2.4	2.3

[1] Unless indicated otherwise.
[2] Non-starch polysaccharides.
[3] Mol%; presented as anhydrous sugar moieties.

RESULTS AND DISCUSSION

Bird performance

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

The analyzed chemical composition of basal and RSM diets is presented in Table 7.2. Phytate and phytate bound phosphorus contents were in the range expected[38, 44] and differences between diets were small. The glucosinolate content (calculated to be < 3 μmol/ g DM RSM) of the RSM used in the current study was low[44]. Consequently, effects of processing on glucosinolates and myrosinase activity were minor. The WBC varied between RSM diets (1.7 to 2.4 g/g DM), with extruded RSM diets showing the highest WBC. Apparently, the combination of heat, pressure, and shear opened the RSM cell wall matrix, confirming previous observations[33].

Average daily gain and nitrogen retention did not differ between dietary treatments (Table 7.3). When corrected for DM content of the feed, also ADFI and feed conversion ratio (FCR) did not differ between dietary treatments.

Nutrient digestion and physicochemical properties of digesta

Apparent total tract digestibility of CP and crude fat were affected neither by processing technologies nor by enzyme addition (Table 7.4, Figure 7.1). Apparent total tract digestibility of DM mainly followed differences in NGP degradability as described below.

Physicochemical properties of the diet and digesta, as particle size, WBC, and viscosity, may affect gastrointestinal retention time[251-254], thereby potentially affecting nutrient digestibility in the small intestine. Addition of pectolytic enzymes decreased WBC of crop contents (Supplemental information Figure 7.4B), indicating that the feed matrix was affected to hold less water, possibly due to hydrolysis of RSM NSP. Dry matter content in the crop was affected by processing (Supplemental information Figure 7.4A), possibly reflecting differences in digesta viscosity resulting from processing, as found in vitro[33]. Dry matter content of small intestinal digesta tended to show the same trend (P = 0.058; Supplemental information Figure 7.4C).

Table 7.3. Growth performance of broilers fed diets containing unprocessed, wet-milled, or extruded rapeseed meal (RSM) with (+) or without (-) addition of pectolytic enzymes1

	Unprocessed RSM diet		Wet-milled RSM diet		Extruded RSM diet			Model *P* – value[2]		
	-	+	-	+	-	+	Pooled SEM	Processing (P)	Enzyme (E)	P × E
n[3]	5	6	6	6	5	6				
Initial mean BW, g	349.8	349.4	349.6	349.9	349.9	349.7	-	-	-	-
Final mean BW, g	1068	1059	1074	1072	1064	1070	-	-	-	-
Mortality, %	0	1.4	1.4	0	0	1.4	-	-	-	-
ADG, g/bird	64.1	62.4	63.5	64.7	63.7	63.3	3.20	0.788	0.794	0.505
ADFI, g DM/bird	81.3	81.1	82.2	81.5	79.3	80.0	2.29	0.060	0.933	0.718
FCR[4], g:g	1.27	1.31	1.30	1.26	1.24	1.26	0.059	0.357	0.744	0.246
N retention[5], g/bird per d	4.69	4.78	4.83	4.52	4.69	4.77	0.36	0.905	0.691	0.312
N retention[6], %	56.4	59.4	56.4	55.0	54.9	56.5	2.92	0.125	0.278	0.180

[1] Recorded from 14 to 25 d of age except for N retention which was recorded from 26 to 29 d of age.

[2] Effect of processing technology (P), addition of pectolytic enzymes (E), or their interaction (P × E).

[3] Number of replicate cages of 12 broilers each.

[4] Feed conversion ratio (g/g).

[5] Nitrogen retention (g/ bird per d).

[6] Nitrogen retention relative to nitrogen intake (%).

Table 7.4. Apparent total tract digestibility (ATTD) of DM and nutrients of diets containing unprocessed, wet-milled, or extruded rapeseed meal (RSM) with (+) or without (-) addition of pectolytic enzymes when fed to broilers

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

[1] Effect of processing technology (P), addition of pectolytic enzymes (E), or their interaction (P × E).

[2] Number of replicate cages of 11 broilers each.

[3] Non-starch polysaccharides.

[4] Non-glucosyl polysaccharides.

[a,b] Means within a row lacking a common superscript differ ($P < 0.05$).

Non-glucosyl polysaccharide degradation

Enzyme addition increased ATTD of NGP from unprocessed and processed RSM (9 to 20 % units, $P < 0.001$; Figure 1D). This coincided with an increase (4 to 7 g/g cobalt, $P < 0.001$) in NGP concentration in the ceca (Figure 7.2), indicating that either less NGP that had entered the ceca were fermented or that a higher quantity of NGP were solubilized and actually entered the ceca. The positive correlation (r = 0.53, $P = 0.001$) between the NGP concentration of ceca contents and the ATTD of NGP supports the latter explanation, which matches well with our expectations and previous in vitro results[33]. The monosaccharide profile from undegraded polysaccharides found in excreta (Figure 7.3) indicates that mainly arabinosyl residues (ATTD increased by 15 to 19 % units, $P < 0.001$, data not shown), and to a lesser extent uronyl residues (6 to 13 % units, $P = 0.006$, data not shown) were affected. Detailed analyses of residual carbohydrate structures in excreta, revealed that the pectolytic enzymes added to the diets mainly affect the degradation of branched water-soluble arabinan[255].

Despite improved NGP degradability, no differences in performance between birds fed RSM diets with or without pectolytic enzymes were observed. The expected improvement in growth as a result of increased NGP degradability in the ranges found is small (calculated to be < 1 g/d, assuming an energy requirement of 10 to 17 kcal ME/g[256, 257].

Apparent total tract digestibility of NGP was not affected by processing technologies (Table 7.4, Figure 7.2D). Regardless, wet-milling increased NGP concentration of ceca contents with 4 g/g cobalt ($P = 0.008$), compared with unprocessed RSM (Figure 7.2), indicating that more NGP have been solubilized and, therefore, could enter the ceca. These findings fit well with what one would expect based on the smaller particle size (GMD = 220 μm compared with 818 μm in unprocessed RSM) and the higher NGP solubilization of wet-milled RSM found in vitro[33]. A similar, but not significant, response was observed for extruded RSM diets, in line with the magnitude of particle size reduction in extruded RSM. Nevertheless, processing did not improve ATTD of NGP, indicating that although polysaccharides were solubilized as a result of particle size reduction, structures could still not be degraded by the cecal microbiota. Particle size reduction, at magnitudes used in the present study, is expected to increase extractability of NSP by increasing surface area, rather than degrading arabinans or their side-chains[134]. Whilst the pectolytic enzymes used in the current study reduced branchiness of arabinans, wet-milling and – to a smaller extent extrusion – probably yielded highly-branched polysaccharide fragments. Unpublished results from our research group[255] indicate that branched arabinans, although solubilized, remain undegraded when broilers are fed RSM diets without additional pectolytic enzymes.

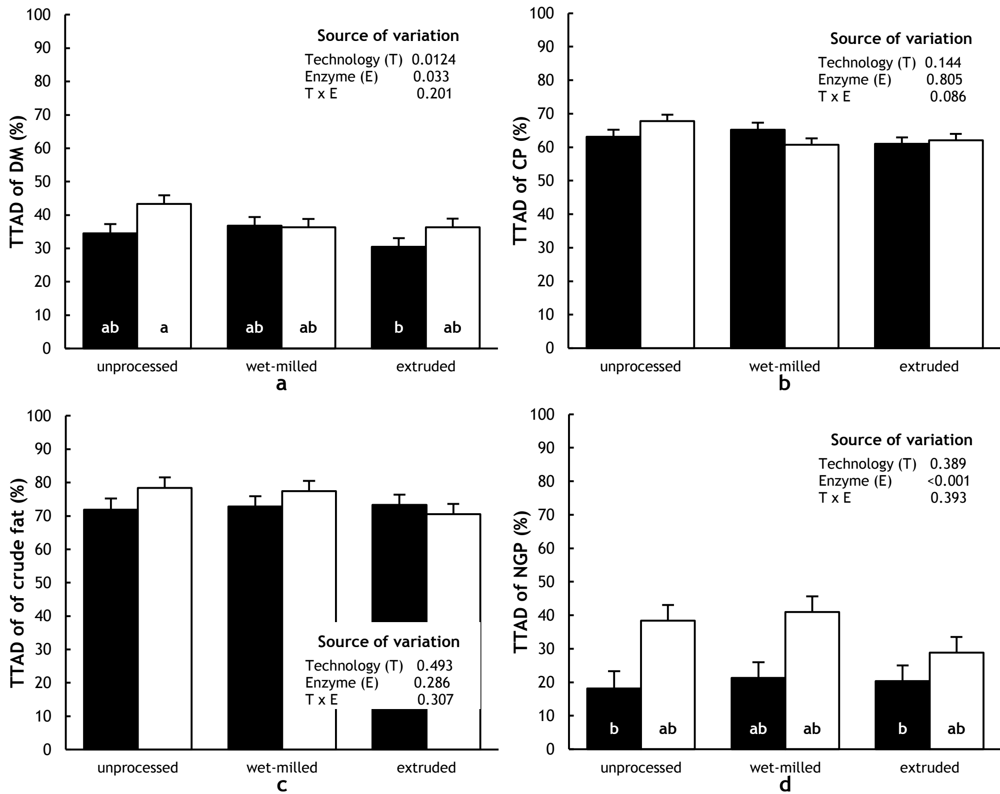

Figure 7.1. Apparent total tract digestibility (ATTD) of DM (A), CP (B), crude fat (C), and non-glucosyl polysaccharides (NGP; D) from unprocessed, wet-milled, or extruded rapeseed meal (RSM) with (white bars) and without (black bars) addition of pectolytic enzymes when fed to broilers. Error bars indicate SEM. Means lacking a common data label (a,b) differ ($P < 0.05$).

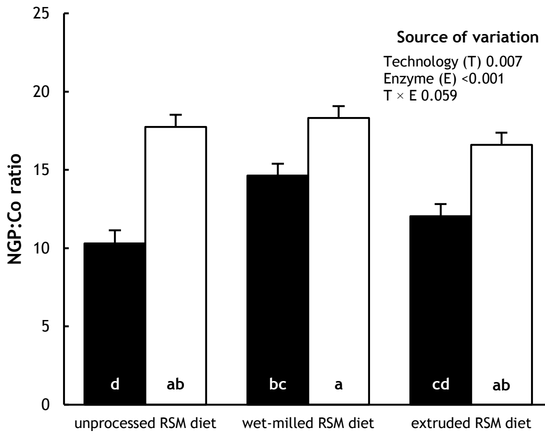

Figure 7.2. Non-glucosyl polysaccharide content expressed relative to Co-EDTA marker in the ceca contents from broilers fed diets containing unprocessed, wet-milled, or extruded rapeseed meal (RSM) with (white bars) and without (black bars) addition of pectolytic enzymes. Error bars indicate SD of total non-glucosyl polysaccharides.

No interaction between processing technologies and enzyme addition on NGP degradation was found. In vitro, processing of RSM was required to enable enzymes to effectively improve NSP solubilization[33], suggesting that the cell wall architecture is limiting enzyme accessibility in unprocessed RSM. In the current study enzymes were also effective in unprocessed RSM diets, indicating that, in vivo, the processing technologies studied did not facilitate accessibility of NSP to pectolytic enzymes added to the diet. Possibly, physicochemical modifications that occur during the digestion process in the bird, sufficiently opened the cell wall architecture to enable effective accessibility of NSP to pectolytic enzymes. Alternatively, NSP might have been better accessible to pectolytic enzymes in the current RSM batch, which had a relatively high content of soluble NSP (3.7 g/ 100g DM) compared with the RSM batch used previously (2.6 g/ 100 g DM[33]). Apparent total tract digestibility of NSP in the current study was higher than values reported previously for broilers and laying hens (~ 11 to 17 % units)[21, 25, 249, 258]. The high variation in NSP content as well as in composition between various batches of RSM[248, 249] can have considerable effects for its degradation in the bird[249]. In agreement with what one would expect based on ceca physiology[253, 259, 260], fermentative degradation of NSP in poultry is almost exclusively limited to the soluble fraction[73]. Therefore, the relative high content of soluble NSP in the RSM batch studied (3.7 compared with ~ 2 g/100 g DM found in above mentioned studies) may, in part, be responsible for the high extent of NSP degradation found. In addition, the long adaptation period to RSM in the current study, may contribute to these findings.

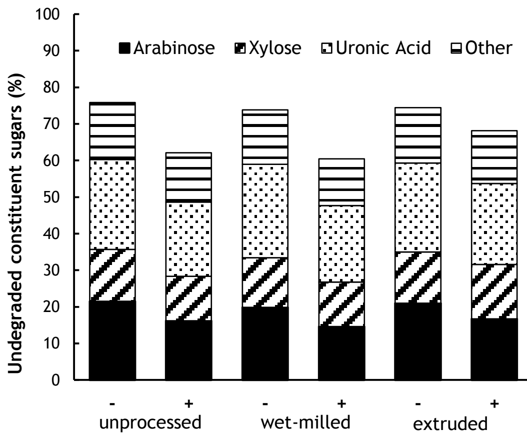

Figure 7.3. Undegraded constituent sugars in excreta (% of non-glucosyl polysaccharides consumed) from broilers fed diets containing unprocessed, wet-milled, or extruded rapeseed meal, with (+) and without (-) addition of pectolytic enzymes.

In conclusion, the pectolytic enzymes used, effectively modified the cell wall structure of RSM, resulting in increased NGP degradability by 9 to 20 % units ($P < 0.001$), independent of prior technological processing of RSM. This coincided with an increase in NGP concentration in the ceca of 4 to 7 g/g cobalt ($P < 0.001$), indicating that more NGP were solubilized such that they could enter the ceca and become available for fermentation. Particle size reduction facilitated solubilization of polysaccharides from RSM, increasing the concentration of NGP found in the ceca by 4 g/g cobalt ($P = 0.008$). Without help of additional pectolytic enzymes, these solubilized structures could, however, still not be degraded by the cecal microbiota. Feed intake, ADG, and FCR were not affected. No interaction between processing technologies and enzyme addition was found. Apparently, the processing technologies studied were not facilitating accessibility of NSP to pectolytic enzymes added to the feed in vivo.

SUPPLEMENTAL INFORMATION

Table 7.5. Mean growth performance[1] and apparent total tract digestibility (ATTD) of DM and nutrients of basal diets with (+) or without (-) addition of pectolytic enzymes when fed to broilers.

	Basal diet (-)	Basal diet (+)	Pooled SEM
n[2]	6	5	
Performance parameters			
Initial mean BW, g	349.2	351.0	-
Final mean BW, g	1012	1029	-
Mortality, %	1.4	2.8	-
ADG, g/bird	58.1	60.2	2.04
ADFI, g DM/bird	89.1	88.4	2.38
FCR[3], g:g	1.37	1.31	0.06
ATTD, %			
DM	83.8	84.4	0.84
CP	80.3	81.4	0.96
Crude fat	94.2	94.5	1.09
NSP[4]	66.7	65.8	1.43
NGP[5]	67.1	68.3	2.58

[1] Recorded from 14 to 25 d of age, unless indicated otherwise.
[2] Number of replicate cages of 12 (growth performance) or 11 (ATTD) broilers each.
[3] Feed conversion ratio (g/g).
[4] Non-starch polysaccharides.
[5] Non-glucosyl polysaccharides.

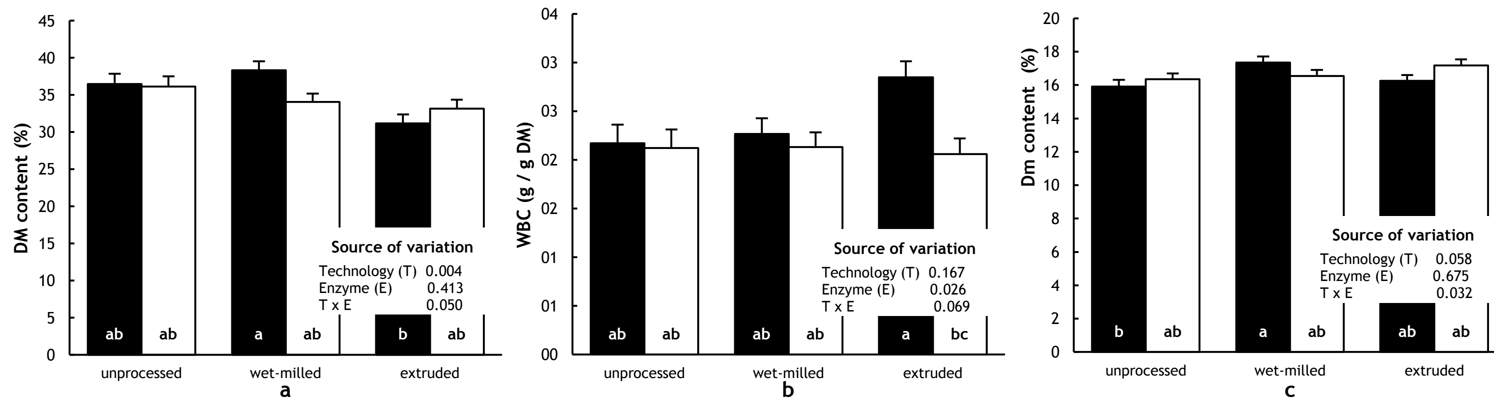

Figure 7.4. Dry matter (DM) content (panel a) and water binding capacity (WBC, panel b) of crop contents and DM content (panel c) of ileum contents from broilers fed diets containing unprocessed, wet-milled, or extruded rapeseed meal, with (white bars) or without (black bars) addition of pectolytic enzymes. Error bars indicate SEM. Means lacking a common data label (a,b) differ ($P < 0.05$).

Separation of digesta fractions complicates estimation of ileal digestibility using marker methods with Cr_2O_3 and Co-EDTA in broiler chickens

Chapter 8

Marker methods to evaluate fiber-rich diets in broilers

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ABSTRACT

Marker methodologies to measure ileal and total tract digestibilities of diets varying in content and degradability of dietary fiber in broiler chickens were evaluated. Chromium sesquioxide (Cr_2O_3) and cobalt-ethylenediamine tetraacetic acid (Co-EDTA) were used as markers of solid and soluble fractions, respectively, and compared with digestibility values obtained with the total collection method. Groups of broilers (n=17, 11 broilers/group) were assigned to a low-fiber diet or one of two high-fiber diets, the latter two containing 35 % rapeseed meal (RSM). Pectolytic enzymes were added to one RSM diet to improve degradability of the fiber-fraction. Excreta were quantitatively collected for 96 h and contents from ileum and ceca were collected at slaughter at 29, 30, or 31 d of age. Chromium recovery in excreta ranged between 86 and 95 %, whereas cobalt recovery was considerably lower (66 to 70 %). Chromium:cobalt ratio was higher in ileal digesta than in feed. Hardly any chromium was found in the ceca, indicating that separation of the marker and specific digesta fractions occurs. Estimates of apparent total tract digestibility (ATTD) were lower when calculated using the marker method compared with the total collection method, particularly in high-fiber diets. Using Cr_2O_3 as a marker, differences were relatively small and effects due to enzyme addition were generally similar. Using Co-EDTA as a marker, ATTD of all components were lower compared with values obtained using the collection method (3 to 45 % units, $P < 0.001$), likely related to the low Co recovery. When estimating apparent ileal digestibility (AID), separation of marker and digesta resulted in unrealistic high estimates for the digestibility of non-glucosyl polysaccharides (54 to 66 %), exceeding ATTD values by 16 to 42 % units. Moreover, the effect of pectolytic enzyme addition on the AID of non-glucosyl polysaccharides was in opposite direction when compared with total collection. The data illustrate that fractionation of digesta, particularly in high-fiber diets, complicates accurate AID measurements in broilers, regardless the choice of markers used.

INTRODUCTION

Digestibility measurements are a crucial tool in the evaluation of the nutritive value of feedstuffs. The marker method, where digestibility is estimated from the ratio between an indigestible marker and the nutrient of interest in feed and digesta or excreta, is commonly used as alternative for the laborious total collection method[203, 261]. Ileal digestibility measurements in broilers are important in feedstuff evaluation, especially when crude protein and amino acid digestibility are of interest, due to the contribution of urine and microbial protein to the protein levels found in the excreta and the influence of the cecal microbiota on protein metabolism[262, 263]. As ileostomisation and cecetomy are invasive, elaborate, and only possible in the adult bird, ileal sampling using the marker method is the method of choice to assess ileal digestibility in young broilers[262-265] .

Chromium sesquioxide (Cr_2O_3), is widely used as marker in digestibility studies with poultry[261]. Chromic sesquioxide is a water-insoluble marker, that may be slightly soluble in alkaline and acidic conditions[266]. Chromic sesquioxide does, however, not flow at the same rate as large fiber particles and is hardly found in the ceca[253, 267, 268] – the major site of fiber degradation in the chicken[269] –, where only soluble digesta is found[92, 253, 259, 260]. This challenges the suitability of Cr_2O_3 as a marker for digestibility evaluation in fiber-rich diets. Indeed, several studies indicate that care should be taken when Cr_2O_3 is used to estimate digestibility of fiber-rich diets[23, 68, 270]. In these studies negative or unrealistic high digestibility values for specific fiber fractions were reported and apparent ileal digestibility (AID) sometimes exceeded apparent total tract digestibility (ATTD). In particular when digestibility of the fiber-fraction itself is the subject of investigation, limitations of the marker method using Cr_2O_3 become clear, but inevitably, separation of digesta fractions would go at the expense of the accuracy of AID of all nutrients. Especially when dietary treatments differentially affect physicochemical properties (e.g. solubility, viscosity, and water binding capacity) of the digesta, digesta flow and reflux may be differently affected for the various treatments[252, 271, 272]. The use of solid phase markers like Cr_2O_3, that potentially separate from the feed component of interest, will in these cases not only lead to misestimation of the level of digestibility, but also dietary treatment effects on digestibility will be inextricably confounded with effects on marker and digesta flow. Instead, markers that are associated with the soluble fraction of digesta, such as cobalt-ethylenediamine tetraacetic acid (Co-EDTA)[273] may be better suited to evaluate degradation of fiber-fractions in poultry.

The objective of this study was to evaluate marker methodologies to measure ileal and total tract digestibilities of diets varying in dietary fiber content and physicochemical properties as well as degradability of the fiber-fraction in broiler chickens. Ileal and total tract digestibility of a low-fiber diet and two high-fiber diets were estimated using Cr_2O_3 or Co-EDTA as markers for the solid and soluble phases, respectively, and compared with digestibility values obtained with the total collection method. Cr and Co can be analyzed in the same assay and thus are potentially good markers for a dual phase marker system. Pectolytic enzymes were added to one of the high-fiber diets to improve degradability of the fiber-fraction. It is hypothesized that fiber content and characteristics will influence digesta and marker flow, thereby affecting the accuracy of digestibility values obtained with the marker methods.

MATERIALS AND METHODS

Experimental design.

The experiment described in the present manuscript was part of a larger study published by de Vries et al. (Chapter 7). A low-fiber diet based on maize, maize starch, wheat gluten meal, fish meal, and soy protein isolate, and two high-fiber diets, the latter two containing 35 % rapeseed meal (RSM), were compared. Rapeseed meal was included either untreated (RSM diet), or after treatment with pectolytic enzymes (RSM+ diet) to improve fiber degradability (14 % units, Chapter 7). Ileal and total tract digestibility of DM, nutrients, and the fiber fraction – measured as non-starch polysaccharides (NSP) – were estimated with the marker method, using either Cr2O3 or Co-EDTA as a marker, and compared with values obtained with the total collection method.

Materials, diets, experimental procedures, analytical methods

The experiment was conducted at research farm 'De Haar' of Wageningen University. All experimental procedures were approved by the Animal Care and Use Committee (DEC) of Wageningen University. Enzyme use was approved by the Dutch Ministry of Agriculture, Nature, and Food Quality. Materials and experimental procedures are described in detail elsewhere (Chapter 7[274]), and briefly summarized below.

Diets were formulated to meet or exceed nutrient requirements of broilers (CVB, 2007; see Chapter 7, for details on diet ingredients and chemical composition) and fed as pellets. The lower fiber diet contained 21% crude protein, 57% starch, 10% crude fat, and 7% NSP, whereas the RSM diets contained 28% crude protein 35% starch, 9% crude fat, and 18% NSP. Chromic sesquioxide and Co-EDTA were included as markers in the feed at a level of 0.25 and 1.0 g/kg (wt/wt, as-fed basis), respectively. The enzyme treatment comprised a combination of commercial enzymes with mainly pectolytic and some hemicellulolytic activities, as described elsewhere (Chapter 7).

Female broilers (average BW at d 14: 349.7 ± 37.7 g; Ross 308, Aviagen Group, Newbridge, United Kingdom) were fed the experimental diets from 14 days of age. Birds were housed in metabolism cages with 12 (d 14 to 25) or 11 (d 26 to end) birds per cage. Feed was available ad libitum and broilers had free access to water during the experiment. After 10 d of adaptation to the experimental diets, excreta were collected quantitatively for 96 h. At the end of the experiment (d 30, 31, or 32) animals were euthanized by intravenous injection of T61and contents from the ileum (last 50 cm before the ileocecal junction), and ceca were collected by gentle finger-stripping. Broilers were fasted from 6 to 3 h before euthanasia and subsequently allowed to consume feed for 3 h until euthanasia, to ensure presence of sufficient chyme in the various sections of the gastrointestinal tract. Before removal, the different segments of the gastrointestinal tract were isolated using tie-wraps, to prevent mixing of digesta from different segments. Feed intake was recorded per cage throughout the experiment. Gastrointestinal contents and excreta were pooled per cage, immediately frozen (-20 °C) and subsequently freeze-dried.

Contents of DM, nitrogen (N), crude fat, starch, NSP, and uric acid were analyzed as described elsewhere (Chapter 7). Chromium (Cr) and cobalt (Co) content were analyzed after ashing and acid hydrolysis as described by Williams et al.[198], using a

SpectrAA 300 atomic absorption spectrophotometer (Varian B.V., Middelburg, The Netherlands). Coefficient of variation (CV) of Cr analyses was 2.7 %; that of Co analyses 1.4 %. Crude protein content was calculated as N × 6.25 (ISO 5983)[171]. Fecal nitrogen in the excreta was calculated as total N minus N in uric acid.

Calculations and statistical analysis

Apparent ileal and total tract digestibility values were calculated using Cr_2O_3 or Co-EDTA as a marker[203]. Apparent total tract digestibility values were calculated based on the quantitative collection method. Marker recovery in excreta was calculated as the total amount of Cr or Co excreted relative to the total amount of Cr or Co consumed during the 96 h collection period. Small amounts of residual starch (2 to 3 g/100 g DM, data not shown) were present in excreta; therefore, ATTD of non-glucosyl polysaccharides (NGP; NSP – glucosyl) was calculated.

Data were analyzed by analysis of variance, with the GLM procedure of SAS (version 9.2, SAS Institute Inc., Cary, NC), using diet as fixed effect. Contrasts were used to compare the low-fiber diet with RSM diets (low-fiber diet vs. RSM and RSM^+ diet) and enzyme addition with no enzyme addition (RSM diet vs. RSM^+ diet). Differences between the ATTD values obtained by total collection, by Cr_2O_3, or by Co-EDTA were analyzed by analysis of variance, using method, diet and its interaction as fixed effects. Contrasts were used to compare the total collection method with the marker method using Cr_2O_3 or a Co-EDTA marker. Cage was the experimental unit in all statistical analyses. Model residuals were tested for homogeneity and normality, to verify model assumptions. Data are presented as LSMeans and pooled standard error of the mean (SEM) unless stated otherwise. Differences among means with $P < 0.05$ were accepted as representing statistically significant differences.

RESULTS

Analyzed chemical compositions of diets, bird performance, nutrient digestibility and physicochemical properties of digesta are discussed in detail by de Vries et al. (Chapter 7).

Apparent ileal and total tract digestibility

Apparent ileal and total tract digestibility of DM, CP, and crude fat were highest for the low-fiber diet in all cases (Tables 8.1 and 8.2). Total tract digestibility of DM and NGP was higher ($P < 0.05$) in birds fed the RSM^+ diet compared with birds fed the RSM diet. Apparent total tract digestibility of crude fat was numerically higher in broilers fed the RSM^+ diet compared with those fed the RSM diet, but this difference was only found to be significant ($P = 0.039$) when estimated using the total collection method. Similarly, ATTD of CP only tended ($P = 0.059$) to be higher in broilers fed the RSM^+ diet compared with those fed the RSM diet, when estimated using the total collection method. Apparent ileal digestibility of both DM and NGP were lower in birds fed the RSM^+ diet compared with those fed the RSM diet, when calculated using Cr_2O_3 as a marker. Using Co-EDTA, only AID of NGP tended to be lower ($P = 0.060$).

When compared with the total collection method, Cr_2O_3-based estimates of ATTD of most nutrients were slightly lower when estimated using Cr_2O_3, the greatest

differences found for NGP (3 to 10 % units, $P = 0.006$). Using Co-EDTA as a marker, ATTD of all components were substantially lower compared with values obtained with the total collection method ($P < 0.001$). Similar to the Cr_2O_3-method, the greatest discrepancy was observed when estimating ATTD of NGP (30 to 35 % units, $P < 0.001$). Also AID values were lower when Co-EDTA was used as a marker instead of Cr_2O_3. Differences between AID obtained with Cr_2O_3 or Co-EDTA were small for starch (2 % units), greater for CP (8 to 10 % units) and DM (9 to 14 % units), and greatest for NSP (17 to 38 % units) and NGP (11 to 30 % units).

Marker concentrations in ileum, ceca, and excreta

Analyzed marker concentrations in the diets were 193 ± 5.8 mg Cr and 161 ± 9.1 mg Co per kg (wt/wt ± SD, as-fed basis), which was close to expected based on added marker concentrations (171 mg Cr and 168 mg Co per kg).

Dry matter content of ileal and cecal digesta was lower in broilers fed the low-fiber diet compared with those fed RSM diets ($P < 0.001$, Table 8.3). Also DM content of excreta was lower for broilers fed the low-fiber diet compared with those fed RSM diets (36 to 44 g/kg, $P < 0.001$). Chromium and Co concentrations were lower in excreta than in ileal digesta. This difference was smaller for the RSM^+ diet compared with the low-fiber diet and untreated RSM diet. The Cr:Co ratio in the diet was 1.2, and increased to values ranging between 1.6 (RSM^+ diet) and 2.1 (low-fiber diet; Figure 1). In excreta, the differences between Cr and Co concentrations were smaller, but still present, resulting in Cr:Co ranging between 1.5 and 1.7. Cobalt concentration in cecal digesta was considerably higher than Cr concentration, resulting in a low Cr:Co (0.04 to 0.08). In ileal digesta and excreta, Cr:Co was higher for broilers fed the low-fiber diet compared with those fed RSM diets ($P < 0.001$), whereas in cecal digesta Cr:Co was lower for broilers fed the low-fiber diet ($P = 0.005$). Recovery of Cr in excreta ranged between 86 and 95 %, with no differences between diets. Recovery of Co was considerably lower than Cr recovery, and tended to be lower ($P = 0.074$) in broilers fed the low-fiber diet (66 %) compared with broilers fed RSM diets (68 to 70 %).

Table 8.1. Apparent total tract digestibility (ATTD, %) of DM[1], CP, crude fat, non-starch polysaccharides (NSP), and non-glucosyl polysaccharides (NGP) in broilers fed a low-fiber diet, rapeseed meal diet (RSM), or RSM diet with addition of pectolytic enzymes (RSM+), as well as degradable NSP content of the diet (g/kg DM), calculated using the total collection method or marker method with Cr_2O_3 and Co-EDTA as a marker.

	Low-fiber diet	RSM diet	RSM+ diet	Pooled SEM	Model P-value[2]	
					Fiber	Enzyme
n[3]	6	5	6			
Total collection						
DM	83.8[x]	66.5[x]	70.1[x]	0.64	<0.001	0.005
CP	80.3	71.5	74.4	0.71	<0.001	0.059
Crude fat	94.2	88.2	90.2	0.58	<0.001	0.039
NSP	14.4	19.8	35.8	1.60	0.010	0.010
NGP	34.7	24.2	37.9	1.23	0.212	0.001
Degradable NGP[4]	20.8	28.8	45.2	1.05	<0.001	<0.001
Cr_2O_3						
DM	83.0[x]	63.6[x]	65.1[y]	0.45	<0.001	0.015
CP[b]	79.3	69.0	69.9	0.80	<0.001	0.446
Crude fat	93.9	87.2	88.4	0.63	<0.001	0.119
NSP[c]	10.3	12.6	25.4	1.28	0.031	0.013
NGP[b]	31.3	17.2	27.6	1.29	0.015	0.018
Co-EDTA						
DM[a]	70.6[y]	43.5[y]	47.8[z]	0.98	<0.001	0.036
CP[a]	70.0	59.5	62.0	0.83	<0.001	0.162
Crude fat[a]	91.2	83.2	85.3	0.89	<0.001	0.129
NSP[a]	-30.5	-14.0	5.7	1.81	0.001	0.023
NGP[a]	0.2	-7.8	8.6	1.42	0.966	0.007

[1] Not corrected for DM originating from urine found excreta.
[2] Analysis of variance with contrast analysis comparing the low-fiber diet vs. RSM and RSM+ diets (Fiber) or RSM diet vs. RSM+ diet (Enzyme).
[3] Number of replicate cages of 11 broilers each.
[4] Calculated degradable NGP content of the diet (g/kg), based on ATTD as estimated using the total collection method.
[a,b,c] Superscripts indicate that method (Cr_2O_3 or Co-EDTA) differs significantly ([a] $P < 0.001$, [b] $P < 0.01$, [c] $P < 0.05$) from total collection method. When an interaction between method and diet was found (DM, $P < 0.001$), means within a column without a common superscript (x,y,z) differ ($P < 0.05$).

Table 8.2. Apparent ileal digestibility (AID, %) of DM, CP, starch, non-starch polysaccharides (NSP), and non-glucosyl polysaccharides (NGP) in broilers fed a low-fiber diet, rapeseed meal diet (RSM), or RSM diet with addition of commercial pectolytic enzymes (RSM+), as well as ileal degradable NSP content of the diet (g/kg DM), calculated using Cr_2O_3 and Co-EDTA as a marker.

	Low-fiber diet	RSM diet	RSM+ diet	Pooled SEM	Model *P*-value[1]	
					Fiber	Enzyme
n[2]	6	5	6			
Cr_2O_3						
DM	88.2	74.8	71.0	0.77	<0.001	0.019
CP	89.2	80.7	78.4	1.03	<0.001	0.345
Starch	98.8	95.3	96.1	0.66	<0.001	0.285
NSP	52.1	69.1	54.0	1.39	<0.040	0.011
NGP	63.0	66.2	53.6	1.65	<0.502	0.042
Degradable NGP[3]	37.8	79.0	63.9	1.32	<0.001	0.005
Co-EDTA						
DM	78.8	61.0	60.2	0.69	<0.001	0.527
CP	80.5	70.8	70.3	1.07	0.001	0.873
Starch	97.8	93.1	94.6	0.67	<0.001	0.129
NSP	14.2	49.1	36.9	1.66	<0.001	0.095
NGP	33.4	55.5	36.2	2.37	0.135	0.060

[1] Analysis of variance with contrast analysis comparing the low-fiber diet vs. RSM and RSM+ diets (Fiber) or RSM diet vs. RSM+ diet (Enzyme).

[2] Number of replicate cages of 11 broilers each.

[3] Calculated ileal degradable NGP content of the diet (g/kg), based on AID as estimated using Cr_2O_3 as a marker.

Table 8.3. Dry matter, chromium, and cobalt concentration[1] in ileum, ceca, and excreta as well as marker recovery in excreta from broilers fed a low-fiber diet, rapeseed meal diet (RSM), or RSM diet with addition of commercial pectolytic enzymes (RSM+).

	Low-fiber diet	RSM diet	RSM+ diet	Pooled SEM	Model *P*-value[2]	
					Fiber	Enzyme
n[3]	6	5	6			
Ileum						
DM, g/kg fresh	61	159	164	2.2	<0.001	0.681
Chromium	1.80	0.85	0.73	0.197	<0.001	0.188
Cobalt	0.84	0.46	0.45	0.113	<0.001	0.721
Ceca						
DM, g/kg fresh	112	165	192	1.8	<0.001	0.013
Chromium	0.29	0.30	0.23	0.153	0.642	0.243
Cobalt	7.39	3.60	3.37	0.417	<0.001	0.479
Excreta						
DM, g/kg fresh	206	242	250	2.3	0.003	0.587
Chromium	1.24	0.58	0.61	0.133	<0.001	0.315
Cobalt	0.72	0.37	0.41	0.076	<0.001	0.078
Marker recovery, %						
Cr_2O_3[4]	95.4	92.0	86.1	1.49	0.129	0.244
Co-EDTA[5]	65.6	70.3	68.3	1.13	0.074	0.422

[1] Concentration in g/kg DM, unless stated otherwise.
[2] Analysis of variance with contrast analysis comparing the low-fiber diet vs. RSM and RSM+ diets (Fiber) or RSM diet vs. RSM+ diet (Enzyme).
[3] Number of replicate cages of 11 broilers each.
[4] Marker recovery in excreta expressed as total amount of chromium (Cr_2O_3) or cobalt (Co-EDTA) excreted relative to total amount of chromium or cobalt consumed during a 96 h period.

DISCUSSION

The results of the present study illustrate the problems arising when the marker method is used to estimate the digestibility of feed components in fiber-rich diets. Apparent total tract digestibility was estimated using the total collection method as well as the marker method using either Cr_2O_3 or Co-EDTA as a marker. The total collection method is generally considered as the 'golden standard', but also the use of the marker method, especially Cr_2O_3, TiO_2, or acid insoluble ash as a marker, is widely applied in digestibility studies with poultry[261]. Differences between Cr_2O_3 and the total collection method were within the range reported previously[261] and effects on ATTD due to enzyme addition were generally similar. Nonetheless, particularly in the high-fiber diets, digestibility estimates using Cr_2O_3 were lower than using total collection, reaching statistical significance for ATTD of DM, CP, and NGP. Discrepancies between ATTD values obtained with Cr_2O_3 and total collection method for high-fiber feedstuffs were also reported by Han et al.[275] (reviewed by Sales and Janssens[261]). Using Co-EDTA, differences were greater, as could be expected from the low recovery of this marker in excreta. The lower precision of the digestibility values obtained with the marker method reduced the power to detect differences between dietary treatments compared with the total collection method. Consequently, treatment effects of enzyme addition to the RSM diet found for ATTD of crude fat and CP using the total collection method were not detected using the marker method.

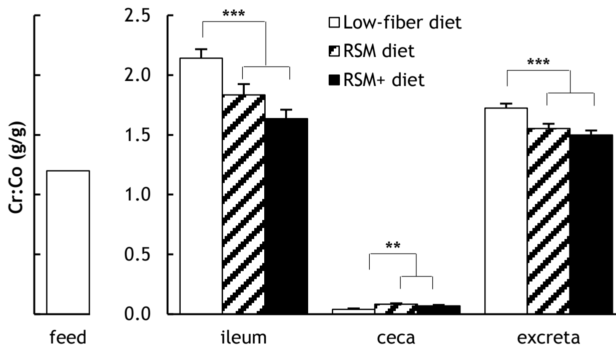

Figure 8.1. Chromium:cobalt ratio (Cr:Co) in feed, ileum, ceca, and excreta from broilers fed a low-fiber diet, rapeseed meal diet (RSM) or RSM diet with addition of commercial pectolytic enzymes (RSM+). Error bars represent SEM. Asterisks indicate significant differences (** $P = 0.005$; *** $P < 0.001$).

When estimating AID in broilers, quantitative digesta collection is not possible, and results obtained with marker methods can only be validated against values obtained with other markers or ATTD. Remarkably, AID values were higher than ATTD, particularly for NGP (16 to 42 % units). These unrealistically high digestibility values for NGP clearly indicate that the marker method yields erroneous results when used to estimate ileal digestibility, especially in fiber-rich diets. Furthermore, treatment effects found for enzyme addition to the RSM diet were in opposite directions compared with the differences found for ATTD. Enzyme addition increased ATTD of NGP, whereas AID of NGP was found to be lower for the RSM+ diet compared with the RSM diet. Higher AID compared with ATTD can be partly ascribed to inclusion of endogenous losses (Ravindran et al., 1999), which is primarily of importance for DM and CP. Still, these results clearly indicate that essential prerequisites to use the marker method are violated by the separation of the marker from specific digesta fractions, leading to erroneous estimates of digestibility values in fiber-rich diets; for NGP as well as for other nutrients.

Separation of the markers and specific digesta fractions is illustrated by the limited amounts of Cr_2O_3 that entered the ceca (Figure 8.1), whereas co, originating from Co-EDTA, was found abundantly in the ceca. Our findings confirm previous observations of a.o. Clemens et al.[92], Fenna and Boag[260], Björnhag and Sperber[259], and Vergara et al.[253] in chickens and other avian species that ceca access is restricted to mainly fluids and some small particles (< 0.2 mm), which are selectively transported into the ceca by antiperistaltic movements.

The data of the current study show, however, that separation of the marker and specific digesta fractions also complicates digestibility measurements anterior to the ileocecal junction. Reflux of solid material up into the ileum and selective transport of small particles and soluble fractions into the ceca likely concentrated Cr in ileal digesta[92, 252, 276] (Table 8.3, Figure 8.1). Undoubtedly, assumptions of unidirectional prop-flow – as required for traditional digestibility calculations using the marker method[203] – are violated, particularly, but not exclusively, in high fiber-diets. Chromium:cobalt ratios throughout the gastrointestinal tract were differently affected

in birds fed the low-fiber diet compared with RSM diets, indicating that fiber content of the diet affects marker flow, which was also found by van der Klis and van Voorst[268]. This implies that effects on digestibility found for dietary treatments that affect digesta flow and reflux, for example through fiber content or physicochemical properties of the fiber fraction[252, 268, 271], are inextricably confounded with effects on digesta flow.

The observation that Cr_2O_3 does not follow the soluble digesta fraction down to the ceca implies that inclusion of a soluble marker is crucial in digestibility studies where fermentation is of interest. As digesta flow into and out of the ceca cannot be considered to be a unidirectional prop-flow, standard digestibility calculations using a soluble marker will still be of limited use. The ratio between the soluble marker and the nutrient of interest provides, however, more insight. As shown by de Vries et al. (Chapter 7), Co:NGP ratios in the ceca correlate well with ATTD of NGP. In addition, simultaneous use of a particulate phase marker and a soluble marker in a dual-phase marker system, combined with mathematical modeling of digesta flow pathways can be useful[277].

Although Co-EDTA may have added value when fiber fermentation is of interest, Cr_2O_3 appears preferable above Co-EDTA for ATTD measurements as the recovery of Co-EDTA was lower. Cobalt, even when complexed with EDTA, can be absorbed from the gastrointestinal tract[278-281] and secreted back into the intestinal lumen[279]. Microorganisms residing in the ceca of the bird utilize Co for vitamin B_{12}-synthesis[282, 283], possibly facilitating uptake of Co by the host in the form of cobalamins. This conflicts with the requirements for a nutritional marker, which should be inert and neither absorbed nor metabolized within the gastrointestinal tract[203]. Figures on the use of Co-EDTA in poultry are scarce, but fecal recovery of this marker has been reported to range between 40 and 100 % in several other species[160, 273, 284, 285]. Köhler et al.[286] reported higher ileal recovery of Co-EDTA in pigs compared with Cr_2O_3 for insoluble fiber- and pectin-rich diets, but lower Co-EDTA recovery for a semisynthetic diet. In the present experiment Co(II)-EDTA was used[273], which has a much lower stability constant compared with Co(III)-EDTA as sometimes used in literature[273, 287, 288], thereby potentially influencing absorption of Co in the animal. Nonetheless, in poultry, absorbed Co should still be recovered in excreta, as urine and feces are simultaneously excreted. When absorbed, Co can accumulate in body tissue, as liver (over 160 μg /g dry tissue), kidney (over 140 μ/g dry tissue), and bones[280, 289, 290]. The quantity of Co not recovered in excreta during the 14d feeding period (~30 % * 1.490 kg * 0.16 mg/kg = ~75 mg per bird), however, seems be too large to be fully explained by body accumulation and incorporation into microbial biomass, although not enough information on maximum Co concentrations in body tissue as muscle and bone is available to make indicative calculations. Possibly, high Cr concentrations in excreta as well as ileal digesta, interfered with the Co measurement, resulting in an underestimation of Co concentrations in these samples[256].

In conclusion, the results of this study show that care should be taken when the marker method is used to estimate digestibility, particularly in fiber-rich diets. Separation of marker and specific digesta fractions occurs, and especially when degradation of fiber fractions is the matter of interest, the marker method has limitations. Estimation of ATTD using Cr_2O_3 as a marker results in digestibility values that are somewhat lower than those obtained by total collection, particularly in fiber-rich diets, but the effects of pectolytic enzyme addition on NGP digestion were mostly similar. The unrealistically high estimates of AID of NGP observed in our study emphasize the inhomogeneity of digesta samples taken from the ileum, magnified at high dietary fiber contents. Moreover, the effect of pectolytic enzyme addition on the AID of non-glucosyl polysaccharides was in opposite direction when compared with total collection. The data illustrate that fractionation of digesta, particularly in high-fiber diets, complicates accurate AID measurements in broilers, regardless the choice of markers used. It is recommended to add a soluble marker when fiber degradation is of interest, even though it does not allow quantifying fermentative degradation of nutrients.

β-Glucan and resistant starch alter the fermentation of fiber from canola meal and maize dried distillers grain with solubles in growing pigs

Chapter 9

Interaction between fiber-content and digestive utilization of the diet

To be submitted

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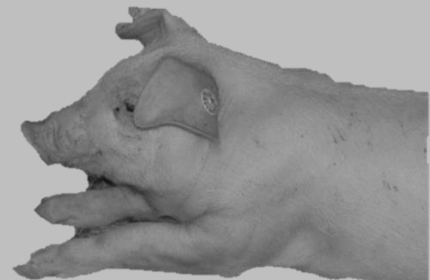

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ABSTRACT

To test the hypothesis that the presence of relatively easy fermentable fiber interacts with the degradation of recalcitrant fiber fractions in the diet, the effects of adding two fermentable fiber sources that differ in their rate of fermentation and ability to modulate physicochemical digesta properties, were tested in a 3 × 2 factorial arrangement. β-glucan (6 % wt/wt, as-fed basis) or resistant starch (RS; 40 % wt/wt, as-fed basis) were added to semi-synthetic diets, with a single fiber-rich feed ingredient as main protein and only fiber source. Feed ingredients contained either pectic polysaccharides, xyloglucan and cellulose (canola meal, CM) or (glucurono) arabinoxylan and cellulose (dried distillers grain with solubles, DDGS) as major fiber structures. Diets were fed to 6 ileal cannulated growing pigs (initial BW 28 ± 1.4 kg), in a 6 × 6 Latin square to evaluate apparent ileal digestibility (AID) apparent total tract digestibility (ATTD), and digesta transit time were evaluated. β-Glucan increased ($P < 0.001$) ATTD of non-glucosyl polysaccharides (NGP) from CM by 6 % units, but did not affect ATTD of NGP from DDGS. In contrast, resistant starch reduced ($P < 0.001$) ATTD of NGP from CM and DDGS by more than 10 % units. Furthermore, β-glucan and RS reduced AID of crude protein and starch, associated with a reduced retention time of digesta in the upper gastrointestinal tract by 50 (CM, $P < 0.01$) and 30 min (DDGS, $P < 0.04$). In conclusion, the addition of β-glucan and RS alter digestion and fermentation of nutrients and fiber from other sources in the diet.

INTRODUCTION

Fiber-rich by-products from food or biofuel industries are increasingly used as feed ingredients for pig diets. The presence of some specific fiber types can have distinct effects on digestive processes, e.g., through their modulating effects on physicochemical properties and gastrointestinal transit of digesta, and on microbial colonization in the gastrointestinal tract[291]. Hence, the presence of these fibers may not only affect the extent of fiber fermentation, but also the digestibility of other nutrients such as protein and starch. For example, the presence of fermentable fiber may increase digesta mass entering the hindgut, reducing digesta transit time in the large intestine, potentially decreasing fiber fermentation[84, 96, 292, 293]. The effects of soluble, readily fermentable fiber, such as β-glucan, on fecal bulk and hindgut retention time are, however, expected to be minor[294]. Furthermore, viscous fiber, can increase (gastric) retention time[82, 295], although effects differ between fiber sources and levels[82, 296], prolonging the time available for nutrient degradation in the stomach. In contrast, increased digesta viscosity may reduce nutrient digestibility in the small intestine, due to reduced enzyme accessibility or reduced retention time[81, 97]. Hence, viscous fermentable fiber sources have been reported to affect protein, starch, and fat digestibility, both positively and negatively[77-81].

In the present study, effects of fermentable fiber sources were tested in semi-synthetic diets with a single fiber-rich feed ingredient as main protein and fiber source. Canola meal (CM) and dried distillers grain with solubles (DDGS) were chosen as model feedstuffs, representing fiber fractions rich in pectic polysaccharides, xyloglucan, and cellulose (CM) or (glucurono) arabinoxylans and cellulose (DDGS). Both fiber fractions are rather recalcitrant and only partly fermented by the pigs' microbiota (Chapter 5 and 6), but the microbial populations involved in their degradation might differ. β-Glucan and resistant starch, differing in rate of fermentation and ability to modulate physicochemical digesta properties, were selected as additional fiber sources. By choosing the added fiber sources to consist solely of glucosyl-polysaccharides, degradation of these added fiber sources could be separated from degradation of other (non-glucosyl) polysaccharides in the diets, allowing evaluation of individual fiber fractions.

It was hypothesized that addition of relatively easy fermentable fiber will alter degradation of recalcitrant fiber from CM and DDGS. As β-glucan is fermented already extensively before the terminal ileum, it was hypothesized that its action would mainly lie in stimulation of bacterial activity, whereas the action of RS was hypothesized to be based more on reduced digesta retention time in the hindgut due to increased fecal bulk. In addition, it was expected that β-glucan will increase digesta viscosity thereby delaying gastric emptying and potentially increasing enzymatic digestion of protein and fat, whereas RS was hypothesized to have minor effect on enzymatic digestion in the upper gastrointestinal tract.

Table 9.1 Composition (g/kg, as-fed basis unless indicated otherwise) and physicochemical properties of experimental diets containing either 50 % canola meal or maize dried distillers grain with solubles (DDGS), with or without inclusion of β-glucan or resistant starch (RS)[1]

	Canola meal			DDGS		
	Control	β-glucan	RS	Control	β-glucan	RS
Ingredient						
Canola meal[2]	500	500	500	-	-	-
Maize DDGS[3]	-	-	-	500	500	500
Maize starch[4]	413	235	-	433.5	255.5	-
Barley β-glucan extract[5]	-	200	-	-	200	-
Retrograded tapioca[6]	-	-	413	-	-	433.5
Caseinate[7]	22	-	22	22	-	22
Canola oil	30	30	30	-	-	-
Sodium bicarbonate	7	7	7	7	7	7
Potassium bicarbonate	-	-	-	5	5	5
Limestone	5	5	5	5	5	5
Dicalcium phosphate	5	5	5	5	5	5
Salt	4	4	4	4	4	4
Mineral and vitamin premix[8]	10	10	10	10	10	10
L-Lysine HCL	2	2	2	4	4	4
DL-Methionine	-	-	-	0.7	0.7	0.7
L-Threonine	-	-	-	1	1	1
L-Tryptophan	-	-	-	0.8	0.8	0.8
TiO_2	2	2	2	2	2	2
Analyzed chemical composition[9]						
DM, g/kg as-fed	912	914	920	925	923	929
CP	230	212	213	177	173	166
Crude fat	52	55	55	53	54	49
Total glucosyl	528	518	553	579	594	608
Starch[2]	394	277	399	430	316	437
β-glucan	1	58	1	3	63	4
Residual glc[3]	133	184	153	145	216	168
Non-glucosyl polysaccharides	71	100	67	105	116	99
Rhamnosyl	1	2	2	1	1	0
Arabinosyl	23	33	21	31	36	30
Xylosyl	9	24	9	44	49	41
Mannosyl	4	6	3	9	12	9
Galactosyl	8	9	8	8	8	8
Uronyl	25	27	24	10	10	10
Water binding capacity (g/g DM)	2.32	3.58	2.38	1.85	2.52	1.90
In vitro viscosity (mPa·s)	1.28	60.0	1.37	1.63	11.8	1.62

[1] Maize starch (control diet) was either substituted for β-glucan (~60 g/kg, as-fed basis) or RS (retrograded tapioca; ~400 g/kg, as-fed basis).
[2] *Brassica napus* meal. For composition see Table 9.2.
[3] High protein maize DDGS. For composition see Table 9.2.
[4] Native maize starch.
[5] Barley β-glucan concentrate extracted from barley flour (CDC Rattan hullless barley, Tomtene Seed Farm, Birch Hill, Saskatchewan, Canada). 100g contains 4 g moisture, 3 g ash, 6 g CP, 24 g starch, and 49 g NSP of which 27 g β-glucan. For more information on composition see Table 9.2 and 9.3.
[6] C*Actistar™ 11700 (Cargill, Haubourdin, France). For information on composition see Table 9.2 and 9.3.
[7] Emser 14 (DMV International, Veghel, The Netherlands).
[8] Provided per kilogram of diet: Cu, 50 mg, as $CuSO4 \bullet H_2O$; Fe, 75 mg, as $FeSO_4 \cdot H_2O$; I, 0.5 mg, as KI; Mn, 25 mg, as MnO; Se, 0.3 mg, as Na_2SeO_3; Zn, 125 mg, as $ZnSO_4 \cdot H_2O$; Vitamin A (retinyl acetate), 2.5 mg; vitamin D (cholecalciferol), 19 µg; vitamin E (DL-α-tocopherol), 33 mg; vitamin B1 (thiamin), 2.5 mg; vitamin B2 (riboflavin), 5 mg; vitamin B6 (pyridoxine-HCl), 1.5 mg; vitamin K3 (menadione), 4 mg; vitamin B12 (cyanocobalamin), 15 µg; niacin, 37.5 mg; pantothenic acid (d-calcium pantothenate), 15 mg; biotin, 250 mg; folic acid, 2.5 mg.
[9] Canola meal diets provided per kilogram of diet: 18.7 MJ GE, 15.2 MJ DE, 2.5, 10.6, 7.7, 6.7, and 2.2 g apparent ileal digestible phosphorus, lysine, methionine+cysteine, threonine, and tryptophan, respectively. Dried distillers grain with solubles diets provided per kilogram of diet: 18.5 MJ GE, 15.3 MJ DE, 3.5, 10.5, 7.1, 6.4, and 2.1 g apparent ileal digestible phosphorus, lysine, methionine+cysteine, threonine, and tryptophan, respectively. Calculated based on data from Centraal Veevoeder Bureau[38] and Widmer et al.[297].

MATERIALS AND METHODS

Materials and Diets. Effects of the presence of β-glucan and RS in diets on nutrient and fiber degradability of CM (*Brassica napus*) and DDGS were tested in a 3 × 2 factorial arrangement. Two basal diets, containing either 50 % CM or DDGS and ~40 % maize starch were formulated to meet or exceed nutrient requirements of growing pigs[38]. Maize starch was (partly) substituted for either a viscous (β-glucan) or non-viscous fermentable fiber source (RS), resulting in 6 dietary treatments (Table 9.1). The β-glucan concentrate (Agri-Food Discovery Place, University of Alberta, Edmonton, Canada) was extracted from barley (*Hordeum vulgare L.*) flour (CDC Rattan hullless barley, Tomtene Seed Farm, Birch Hill, Saskatchewan, Canada); the RS consisted of retrograded starch (RS3; C*ActistarTM 11700, Cargill, Haubourdin, France) obtained by crystallizing hydrolyzed tapioca (maltodextrins), originating from cassava (*Manihot esculenta*) root[298].

Animals and Experimental Procedures. The animal experiment was conducted at the Swine Research and Technology Centre at the University of Alberta (Edmonton, AB, Canada). The animal protocol was approved by the Animal Care Committee of the University of Alberta and followed the guidelines established by the Canadian Council on Animal Care[299]. A total of 10 crossbred barrows (initial body weight, 28 ± 1.4 kg ± SD; Duroc × Large White/Landrace; Hypor, Inc., Regina, SK, Canada) were moved 1 week before surgery into individual metabolism pens (1.2 × 1.2 m). Each pen was equipped with a single-space feeder and a low-pressure bowl drinker. Pigs were surgically modified with a T-cannula at the distal ileum. Starting 10 d post-surgery, six pigs were randomly assigned in 6 periods to each of the six experimental diets according to a 6 × 6 Latin square design. The remaining animals served as reserve animals and were used to replace experimental animals when required. In total, four pigs had to be replaced during the experiment, due to problems with their cannula. Replacement was done such that a minimum of two observations (two periods) was realized for each animal and each diet was tested at least in six periods, resulting in a total of 46 observations in 10 animals over 7 periods. Diets were fed as mash and mixed with water (1 : 3) in the feed through. The daily feed allowance was 2.6 times energy requirements for maintenance (460 kJ digestible energy/kg of $BW^{0.75}$, based on the calculated ME content of the control CM or DDGS diet) in two equal meals at 8.00 h and 15.00 h. All animals consumed their complete allowance during the whole experiment. Pigs had free access to water. After a 5 d gradual transition from starter to experimental diets, the experiment, consisting of 7 sequential experimental periods, started. Each 14 d experimental period consisted of a 9 d adaption to the diets followed by 2 d collection of feces, 2 d collection of ileal digesta for digestibility measurements, and 1 d collection of ileal digesta for the measurement of retention time. Pigs were weighed weekly during the experiment after consuming their morning meal. Feces were collected from 08.00 to 17.00 h using bags attached to rings glued around the anus. Bags were collected within 1 h after defecation and immediately frozen (-20 °C). Ileal digesta for digestibility measurements were collected from 08.00 to 17.00 h into plastic bags (10 cm in length and 4 cm in diameter). The bags were removed when filled approximately 70 % with digesta, or after a maximum of 1 h, and immediately frozen (-20 °C). For retention time measurements, on day 14 of each

experimental collection, 3.4 g Cr as Cr_2O_3 (solid phase marker) and 3.4 g Co as Co-EDTA (soluble phase marker) were mixed into the morning meal. Digesta were collected at 45, 90, 180, 270, 360, 540 and 720 min after feed consumption (5). At each time-point, cannulas were opened and digesta were collected into plastic bags during 5 min. If no sample was obtained after 5 min bags were left on until digesta was collected or until the next collection time point. The actual time of collection was recorded for each sample.

Digesta and feces were thawed, pooled per observation, subsampled, and immediately frozen (-20 °C). Digesta and feces samples for digestibility measurements were freeze-dried.

Table 9.2 Analyzed chemical compositions (g/kg, as-fed basis) of canola meal, maize dried distillers grain with solubles and fiber sources

	Canola meal	DDGS	β-glucan	RS
CP[1]	320	254	62	n.a.[2]
Total carbohydrates (CHO)	257	356	725	898
Starch	30	45	238	825
β-glucan	0.5	4.3	267	n.a.[2]
Sugar composition of CHO (Mol%[3])				
Rhamnosyl	2	0	0	0
Arabinosyl	20	19	7	0
Xylosyl	8	25	11	0
Mannosyl	3	5	2	0
Galactosyl	11	5	0	0
Glucosyl	39	42	78	99
Uronyl	16	5	1	1

[1] Crude protein content was calculated from the N content using a protein conversion factor of 5.3 for canola meal[229], 5.7 for β-glucan[181], and 5.9 for DDGS[43].
[2] Not analyzed.
[3] Mol%; presented as anhydrous sugar moieties.

Analytical methods. Water binding capacity (WBC) of diets was analyzed in duplicate by soaking 1 g of material in 25 mL of water for 24 h at room temperature. Samples were centrifuged (3274 g for 20 min) and decanted. Water binding capacity was calculated as the weighed quantity of water retained per gram of dry material. Before viscosity analyses, diets were diluted with distilled water (1:2, wt/wt), incubated for 3 h at 37 °C, centrifuged (12000 *g* for 10 min), and decanted. Digesta samples were thawed, centrifuged (12000 *g* for 10 min), and decanted. *In vitro* apparent viscosity of supernatants was analyzed before and after heating (80 °C, 10 min) in duplicate using a rheometer (UDS 200; Paar Physica, Glenn, VA, USA) at a shear rate of 1.29/s, 12.9/s and 129/s and a temperature of 20 °C[79].

Before chemical analyses, individual freeze-dried samples were milled in a centrifugal mill using a 0.5 mm screen (model ZM1, Retsch; Brinkman Instruments, Rexdale, ON, Canada). All chemical analyses were performed in duplicate using standard laboratory methods[13, 171]. Diets, digesta, and feces were analyzed for content of DM (AOAC 930.15), titanium (AOAC 540.91), nitrogen (AOAC 990.03; using a Thermo Quest NA 2100 Nitrogen and Protein Analyser, Interscience B.V., Breda, The Netherlands), ether extract after hydrochloric acid hydrolysis (AOAC 920.39; using Soxhlet apparatus and petroleum ether), free glucose and total starch (AOAC 996.11,

DMSO format; using a commercial test kit, Megazyme International Ltd., Ireland), mixed linked β-glucan (using a commercial test kit, Megazyme International Ltd., Ireland), and total carbohydrates measured as neutral sugars and uronic acids after removal of small soluble saccharides by precipitation in acidified ethanol[50]. Before analyses of neutral sugar and uronic acid contents in diets, NSP were extracted from diets[199]. In addition, total glucosyl was measured in complete diets, before NSP extraction. Neutral sugar composition was analyzed according to the method of Englyst and Cummings[49]. After pre-treatment with 72% (wt/wt) H_2SO_4 for 1 h at 30 °C, samples were hydrolyzed with 1 M H_2SO_4 at 100 °C for 3 h. Constituent monosaccharides were derivatized into their corresponding alditol acetates and analyzed using gas chromatography (Focus-GC, Thermo Scientific, Waltham, MA). Inositol was used as an internal standard. Uronic acid content was analyzed according to the automated colorimetric m-hydroxydiphenyl assay[200], using an auto-analyzer (Skalar Analytical B.V., Breda, The Netherlands). Galacturonic acid was used for calibration. Residual glucosyl was calculated as total glucosyl minus glucosyl originating from starch and β-glucan. Crude protein content was calculated as N (%, wt/wt) × 6.25 (ISO 5983) unless indicated otherwise.

Digesta samples needed for retention time measurements were thawed, dried (70 °C for 16 h, 103 °C for 4 h; AOAC 930.15), incinerated (550 °C for 3 h; AOAC 942.05), and analyzed for content chromium and cobalt after acid hydrolysis[198, 273], using a SpectrAA 300 atomic absorption spectrophotometer (Varian B.V., Middelburg, The Netherlands).

Statistical Methods. Apparent ileal digestibility (AID) and apparent total tract digestibility (ATTD) were calculated according to the marker method, using TiO_2 as a marker[203]. Mean retention time (MRT) of digesta until the terminal ileum was calculated using the Cr_2O_3 (solid phase) or Co-EDTA (soluble phase) marker[292].

Data were analyzed using a generalized linear mixed model with beta-distributed error for the response variable and a logit link function (PROC GLIMMIX, SAS version 9.2, SAS Institute Inc., Cary, NC). A normal distributed error and identity link was assumed for AID of non-glucosyl polysaccharide, a gamma distributed error and log link for ileal viscosity and MRT. Pig within period was the experimental unit. Feed ingredient (CM or DDGS), fiber source (β-glucan or RS), their interaction and period were included as fixed effects. Pigs was regarded the random subject effect, period was modeled as R-side effect to account for repeated observations within pig. Degrees of freedom were approximated using the Kenward-Rogers method. Model assumptions and goodness of fit of the model and covariance structure were evaluated through the distribution of residuals and the ratio of the obtained generalized Chi-square to the degrees of freedom. Preliminary analyses identified best fit of a heterogeneous autoregressive covariance (ARH(1)) structure for AID and ATTD of residual glucosyl and NGP, MRT, and ileal viscosity, and a compound symmetry (CS) structure for all other parameters. Significance of differences was tested using type III pseudo-likelihood ratio statistics. Contrasts were used to compare β-glucan- and RS-diets with control diets. Data are presented as back-transformed means and pooled SEM unless stated otherwise. Differences among means with $P < 0.05$ were considered statistically significant.

RESULTS

Diets. Analyzed chemical composition of feed ingredients and diets are presented in 9.1 to 9.3. Starch analysis of the diets repeatedly revealed lower contents than expected, despite the boiling in DMSO (Table 9.3). Therefore, glucosyl and starch contents of all starch-containing ingredients were analyzed and the starch content of the diets was calculated based on analyzed values of ingredients. Water binding capacity and *in vitro* viscosity were greater in the β-glucan diets than in the control and RS diets (Table 9.4).

Ileal and apparent total tract digestibility. Apparent ileal and total tract digestibility of CP, crude fat, and starch of the CM diets were greater compared with the DDGS diets (Table 9.4). Apparent ileal degradability of NGP of the CM diets was negative and lower compared with the DDGS diets, whereas ATTD was greater. β-Glucan reduced AID and ATTD of DM, CP, and starch in the CM as well as the DDGS diet. Apparent ileal and total tract digestibility of crude fat were only significantly reduced by β-glucan in the DDGS diet. β-Glucan increased AID of NGP in the CM diet and ATTD of NGP in both diets, particularly so in the CM diet. Resistant starch reduced AID and ATTD of DM, starch, and NGP. Apparent ileal digestibility of CP was lower in the RS diet compared with the control diet for DDGS ($P < 0.01$) but not for CM ($P = 0.66$; P I × S = 0.06). Resistant starch increased AID of crude fat in the CM but not in the DDGS diet. Resistant starch did not affect ATTD of crude fat in the CM diet, whereas it reduced ATTD of crude fat in the DDGS diet.

Digesta kinetics. Mean retention time (MRT, min) in the stomach and small intestine of the soluble phase was greater for DDGS diets compared with CM diets whereas MRT of the solid phase did not differ (Table 9.5). β-Glucan and RS both reduced MRT of the solid phase in the CM and the DDGS diets by nearly 50 ($P < 0.01$) and 30 min ($P < 0.04$), respectively. Viscosity of digesta in the ileum was lower in DDGS diets compared with CM diets (Table 9.5). β-Glucan increased viscosity measured in heated supernatant ($P < 0.01$), whereas RS had no effect.

Table 9.3 Analyzed and calculated sugar composition (% (wt/wt), as-fed basis) of experimental diets[1] and starch-containing feed ingredients

	Canola meal diets			DDGS diets			Feed ingredients		
	Control	β-glucan	RS	Control	β-glucan	RS	Barley β-glucan	Tapioca	Maize starch
Total glucosyl	48.1	47.4	50.8	53.5	54.9	56.5	58.8	88.4	86.7
Free glucose	n.a.[2]	n.a.[2]	n.a.[2]	n.a.[2]	n.a.[2]	n.a.[2]	1.17	n.a.[2]	0.02
Starch[3]	36.0	25.3	36.7	39.8	29.2	40.6	23.8	82.5	71.2
β-glucan	0.1	5.8	0.1	0.3	6.3	0.4	26.7	n.a.[2]	n.a.[2]
Residual glucosyl[4]	12	17	14	13	20	16	-	-	-
Total sugar in NSP extract[5]	9.6	18.2	9.5	14.1	20.5	13.5	n.a.[2]	n.a.[2]	n.a.[2]
Glucosyl	3.1	9.0	3.3	4.4	9.8	4.3	n.a.[2]	n.a.[2]	n.a.[2]
of which starch	0.04	0.29	0.10	0.13	0.17	0.12	n.a.[2]	n.a.[2]	n.a.[2]
NSP glucosyl removed[6]	6	9	7	4	11	7	-	-	-

[1] Diets contained either 50 % canola meal or maize dried distillers grain with solubles (DDGS). Maize starch (control diet) was either substituted for β-glucan (~60 g/kg, as-fed basis) or resistant starch (RS, retrograded tapioca; ~400 g/kg, as-fed basis).

[2] Not analyzed.

[3] Starch content of feed ingredients were analyzed enzymatically (AOAC 996.11, DMSO format; using a commercial test kit, Megazyme international Ltd., Ireland). Starch content of diets was calculated based on analyzed glucosyl contents (total glucosyl minus free glucose) of starch sources and analyzed starch contents of other feed ingredients. Maltodextrins are included in starch content.

[4] Residual glucosyl in diets calculated as total glucosyl minus glucosyl from calculated starch and β-glucan.

[5] Non-starch polysaccharides (NSP) were extracted from diets as described by Jonathan et al.[199].

[6] Amount of glucosyl from NSP fraction washed out during NSP-extraction procedure, calculated based on expected NSP-glucosyl content of diets (residual glucosyl minus free glucose) and analyzed glucosyl content in NSP extracts.

Table 9.4 Apparent ileal and total tract digestibility (%) of DM and nutrients in growing pigs fed diets containing either 50 % canola meal or maize dried distillers grain with solubles (DDGS), with or without with or without inclusion of β-glucan or resistant starch (RS)[1,2]

	Canola meal			DDGS			Pooled	*P* – value[3]			
									F		
	Control	β-glucan	RS	Control	β-glucan	RS	SEM	I	β-glucan	RS	I x F
n[4]	10 (7)	7 (7)	7 (6)	8 (7)	7 (6)	7 (6)					
Ileum											
DM	69.5	59.6	46.5	71.4	61.8	46.3	1.69	0.014	<0.001	<0.001	0.137
CP	74.7	71.2	75.1	72.8	68.0	70.3	1.98	<0.001	<0.001	<0.001	0.057
Crude fat	82.1[b]	80.4[b]	84.7[a]	77.0[x]	71.5[y]	75.5[x]	0.28	<0.001	<0.001	<0.001	0.044
Total glucosyl	93.1[a]	82.8[b]	50.3[c]	91.2[x]	82.3[y]	52.7[z]	2.09	0.226	<0.001	<0.001	0.033
Starch	99.6[a]	98.9[b]	54.7[c]	97.6[x]	95.8[y]	51.5[z]	1.83	<0.001	0.003	<0.001	<0.001
β-glucan	n.a.[5]	23.7	n.a.[5]	n.a.[5]	24.9	n.a.[5]	0.81				
Residual glucosyl[6]	77.8[a]	82.5[a]	32.1[b]	77.5[x]	82.0[x]	53.5[y]	7.60	0.013	0.056	<0.001	0.004
NGP[7]	-26.5[b]	-8.7[a]	-33.5[b]	19.3[x]	5.0[x]	3.4[x]	12.97	<0.001	0.762	0.051	0.040
Arabinosyl	-0.5	7.7	-1.5	18.5	5.8	12.7	10.32	0.006	0.615	0.464	0.064
Xylosyl	-5.6	14.6	-26.6	27.1	8.0	15.9	7.25	0.045	0.835	0.079	0.074
Uronyl	-50.1[a]	-57.5[a]	-54.6[a]	-35.9[x]	-58.6[y]	-86.9[z]	20.08	0.127	0.027	<0.001	<0.001
Total tract											
DM	83.4[a]	80.9[b]	80.5[b]	85.1[x]	81.9[y]	79.9[z]	1.09	0.038	<0.001	0.011	0.021
CP	82.6	76.4	73.8	82.0	73.0	68.4	2.63	<0.001	<0.001	0.001	0.106
Crude fat	78.3[a]	75.9[b]	77.0[ab]	72.6[x]	66.9[y]	66.6[y]	1.85	<0.001	<0.001	0.538	0.031
Total glucosyl	98.0	97.6	97.1	96.8	96.8	96.0	0.61	<0.001	0.399	0.014	0.654
Starch	99.9[a]	99.8[b]	99.8[b]	99.9[x]	99.8[y]	99.7[z]	0.06	0.033	<0.001	0.243	0.044
β-glucan	n.a.[5]	99.9	n.a.[5]	n.a.[5]	99.9	n.a.[5]	0.003				
Residual glucosyl[6]	92.2	93.5	91.4	87.4	91.2	86.1	2.13	<0.001	0.027	0.024	0.501
NGP[7]	70.3	76.1	59.7	51.4	52.3	34.5	4.42	<0.001	0.040	<0.001	0.134
Arabinosyl	86.6	88.1	80.0	54.7	60.0	44.2	4.90	<0.001	0.129	<0.001	0.914
Xylosyl	84.5	91.2	75.8	43.3	49.3	27.9	5.76	<0.001	0.004	<0.001	0.378
Uronyl	50.9	54.6	45.6	54.7	49.4	40.7	6.03	0.271	0.730	<0.001	0.099

[1] Maize starch (control diet) was either substituted for β-glucan (~6 % as-fed basis) or resistant starch (RS, retrograded tapioca; ~40 % as-fed basis).
[2] Data are back transformed least square means and pooled standard errors, except for digestibilities of β-glucan which are presented as raw means and pooled standard errors.
[3] Model established P- values for fixed effects of feed ingredient (I, CM vs. DDGS), fiber source (β-glucan or RS vs. control) and the interaction between feed ingredient and fiber source (I x F). When an interaction between ingredient and fiber source was found, superscripts ([a,b,c] CM or [x,y,z] DDGS) indicate differences between diets within an ingredient ($P < 0.05$).
[4] Number of replicate observations. Values between parentheses indicate the number of pigs in which replicate observations were made. In total, 46 observations were realized in 10 animals over 7 periods.
[5] Not analyzed.
[6] Residual glucosyl calculated as total glucosyl minus glucosyl from starch and β-glucan.
[7] Non-glucosyl polysaccharides; monosaccharides represent anhydrous sugar moieties.

Table 9.5. Mean retention time (MRT, min) in the stomach and small intestine, cumulative chromium:cobalt ratios, and intestinal viscosity measured at the terminal ileum in growing pigs fed diets containing either 50 % canola meal (CM) or maize dried distillers grain with solubles (DDGS), with or without with or without inclusion of β-glucan or resistant starch (RS)[1]

	Canola meal			DDGS			Pooled	*P* – value[3]			
									F		
	Control	β-glucan	RS	Control	β-glucan	RS	SEM	I	β-glucan	RS	I x F
n[4]	10 (7)	7 (7)	7 (6)	8 (7)	7 (6)	7 (6)					
MRT, solid phase[5]	378	341	337	373	314	350	4.9	0.355	0.005	0.035	0.200
MRT, soluble phase[6]	267	286	288	335	335	295	6.5	0.025	0.153	0.561	0.444
Viscosity[9]	1.65	1.63	1.62	1.33	1.65	1.45	0.04	0.047	0.081	0.529	0.276
Viscosity (heated)[10]	3.24	21.50	3.53	2.73	235.7	2.90	0.56	<0.001	<0.001	0.476	<0.001

[1] Maize starch (control diet) was either substituted for β-glucan (~6 % as-fed basis) or resistant starch (RS, retrograded tapioca; ~40 % as-fed basis).
[2] Data are back transformed least square means and pooled standard errors.
[3] Model established *P*- values for fixed effects of ingredient (I, CM vs. DDGS), fiber source (β-glucan or RS vs. control) and the interaction between feed ingredient and fiber source (I x F).
[4] Number of replicate observations. Values between parentheses indicate the number of pigs in which replicate observations were made. In total, 46 observations were realized in 10 animals over 7 periods.
[5] Mean retention time (min) in the stomach and small intestine measured using a solid phase marker (Cr_2O_3).
[6] Mean retention time (min) in the stomach and small intestine measured using a soluble phase marker (Co-EDTA).
[9] Viscosity (mPa·s) measured in digesta supernatant at shear rate of 129/s.
[10] Viscosity (mPa·s) measured in digesta supernatant after heating (80 °C, 10 min) at shear rate of 129/s.

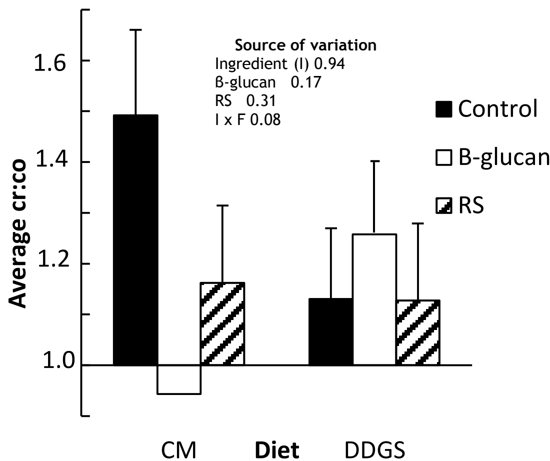


Figure 9.1. Average chromium:cobalt ratio in ileal digesta collected during 12 h after feeding a pulse dose of Cr_2O_3 and Co-EDTA markers. Digesta samples were collected at 45, 90, 180, 270, 360, 540 and 720 min after feed consumption. Baseline (1.0) represents Cr:Co in feed. Model established *P*- values for fixed effects of ingredient (I, CM vs. DDGS), fiber source (β-glucan or RS vs. control) and the interaction between feed ingredient and fiber source (I x F) are presented in the figure. Error bars indicate SEM.

DISCUSSION

The results of the present study confirm our hypothesis that addition of β-glucan and RS to fiber-rich diets affects digesta properties, nutrient flow, and gastrointestinal transit time and interacts with degradation of recalcitrant fiber fractions from CM and DDGS.

Nutrient digestibility of CM and DDGS diets

Crude protein from CM was more digested than CP from DDGS, as expected[33] (Chapter 4). Canola oil was included as crude fat source in CM diets whereas crude fat in the DDGS diets originated completely from the DDGS, explaining the lower crude fat digestibility found for DDGS diets. Similarly, the presence of, presumably resistant, starch in DDGS (4.5 %, Table 9.2), can explain the lower AID of starch in the DDGS diets compared with the CM diets.

Fiber degradability

Non-starch polysaccharides (NSP) can be measured as the sum of constituent carbohydrates released by acid hydrolysis of samples after removal small soluble saccharides by ethanol[49]. In low-starch feed ingredients, as well as in digesta and feces, which contain typically negligible amounts of starch, the content of neutral carbohydrates and uronic acids can be measured directly and NSP content is calculated by subtraction of starch from the total carbohydrate content. Because of the high starch content of the diets, their polysaccharide content is generally measured in NSP extracts after removal of starch[50]. Calculations based on analyzed NSP contents of individual feed ingredients (Table 9.2), indicate however, that some soluble NSP may be removed along with starch resulting in underestimation of NSP measured in NSP extracts of diets. For the current diets, especially NSP-glucosyl was

underestimated (4 to 11%, wt/wt; Table 9.3), whereas underestimation of NGP was relatively low (0 to 2%, wt/wt; data not shown). Hence, degradation of various glucosyl-polysaccharide fractions and non-glucosyl-polysaccharides was evaluated separately in the current study. Residual glucosyl, calculated as total glucosyl minus glucosyl from starch and β-glucan, approximates the sum of free glucose and NSP-glucosyl. Besides allaying the analytical constraints associated with the measurement of NSP-glucosyl, the current approach also benefits from the separate analysis of glucosyl- and non-glucosyl-polysaccharides. This allows the particular evaluation of the effects of the additional fermentable fiber sources β-glucan and RS, both constituted of glucosyl-units only, on the (non-glucosyl) fiber fractions from DDGS and CM.

Apparent ileal digestibility of NGP in the DDGS control diet was as expected (Chapter 5), whereas that of CM was lower than observed previously (Chapter 6) and found to be negative. Negative AID of NSP, particularly insoluble NSP fractions as cellulose and arabinoxylan, is reported more often and has been ascribed to the presence of endogenous and microbial material in ileal digesta and potential separation of marker and digesta[89-91, 96, 98, 128, 210]. Considering the contribution of endogenous and microbial material to ileal digesta (< 15%, wt/wt DM basis[114, 209, 300, 301]) and the NGP contents of these components, this would typically explain less than 1% of NGP. Hence, collection of ileal digesta through the T-cannulas may have resulted in selective recovery of specific digesta fractions (see e.g. Köhler et al.[286]). Although not quantitatively collected, the ratio between chromium and cobalt (Cr:Co) in ileal digesta samples collected after pigs received a pulse-dose of Cr_2O_3 and Co-EDTA, provides some insight in the individual recoveries of solid and soluble digesta fractions. The Cr:Co supplied was 1.0, and deviations from this baseline indicate selective collection of specific fractions at that time-point. Averaged over 7 time-points in a period of 12 h from feeding, the Cr:Co of digesta is expected to approximate to the baseline of 1.0, assuming recovery of soluble and the solid digesta fractions is complete within 12 h[292]. However, the average Cr:Co of > 1 in ileal digesta indicates an overrepresentation of solid material in the digesta samples (Figure 9.1), especially for the control CM diets. The discrepancy between the different diets might be related to physicochemical properties of the diets, such as solubility and water binding capacity of fiber fractions, which are certainly influenced by the feed ingredients and additional fiber sources. Differences found for AID of NGP might therefore reflect differences in physicochemical properties between diets, rather than actual degradation of fiber fractions.

Total tract degradation of residual glucosyl and NGP from CM were greater than those from DDGS, as expected[38, 42] (Chapter 5 and 6).

Effect of added fiber on nutrient and fiber degradability

β-Glucan. *In vitro* viscosity of the CM diet with β-glucan was considerably greater compared with the control CM diet, whereas β-glucan had less effect on *in vitro* viscosity in the DDGS diet. Enzymes in DDGS remaining from the bioethanol production process or interactions between β-glucan and polysaccharides in CM, might explain this observation. Approximately 25% of the added β-glucan was degraded before the end of the ileum, both in the CM and the DDGS diet. This is lower than reported for barley β-glucan previously[12, 150, 161]. Possibly the abundance of

other, recalcitrant, polysaccharides in the diets of the present study, led to a lower degradation of β-glucan compared to diets with less, and more easily degradable, NSP. Apparent total tract digestibility of β-glucan was nearly complete, as expected[12, 35, 150, 161]. β-Glucan had minor effects on ileal viscosity measured in unheated digesta samples ($P < 0.08$). However, β-glucan may insolubilize during freezing (T. Vasanthan, personal communication) and freezing digesta samples may have masked effects of β-glucan addition on digesta viscosity, measured in the unheated supernatant. Viscosity analysis after heating of ileal, thereby solubilizing β-glucan, show indeed greater viscosity in β-glucan diets compared with control diets (18 and 233 mPa·s for CM and DDGS diets respectively, $P < 0.01$). The reduced AID of CP (~4 % units), crude fat (~2 % units), and starch (1 to 2 % units) indicate either increased microbial matter in digesta[91] or that β-glucan interferes with enzymatic digestion in the upper gastrointestinal tract. This can be explained by reduced enzyme accessibility[98] or by a reduction in digesta retention time in the stomach and small intestine[81], as indicated by the reduced MRT of solid digesta (Table 9.6). In contrast, some previous studies reported that viscous fiber increased gastric retention time[302]. Those results were observed, however, with carboxymethylcellulose, a non-fermentable viscous fiber source, which exerts high viscosity throughout the upper gastrointestinal tract and does not affect microbial activity[291]. In addition, in the present study only cumulative retention time in the stomach and small intestine was evaluated, whereas β-glucan is expected to have most effect on gastric retention time[302] and small intestinal retention time might be reduced[81]. Apparent total tract digestibility of NGP was higher in β-glucan diets compared to the control diets, mainly because of increased degradation of xylosyl-polysaccharides was increased (Table 9.4). Apart from β-glucan, the barley β-glucan extract contained 23 % (wt/wt, as-fed basis) other NSP. These polysaccharides, that accounted for ~30 % of the NGP in the CM β-glucan diet and for ~10 % of the NGP in the DDGS β-glucan diet, were presumably rather soluble, due to the nature of the extraction process. Assuming an ATTD of ~ 70 % for these polysaccharides[12, 150, 161], it can be calculated that the degradation of NGP originating from DDGS itself were unaffected. The increased ATTD of NGP in the DDGS β-glucan diet can, thus, be attributed to a better degradability of barley NGP compared with those from DDGS, which is as expected based on the structural characteristics of the fiber-fractions (Chapter 2). Degradability of NGP from CM, however, can be calculated to be increased when β-glucan is added to the diet, even after correction for the presence of barley NGP. The ATTD of constituent sugar moieties indicates that mainly degradation of xylosyl-polysaccharides, which may originate majorly from xyloglucan, was increased (Table 9.4). Possibly, β-glucan affected total retention time, thereby allowing more time for fermentation and degradation of the rather recalcitrant xyloglucan matrix. Alternatively, the presence of β-glucan specifically stimulated colonization and activity of microbiota that possess activity towards CM xyloglucan.

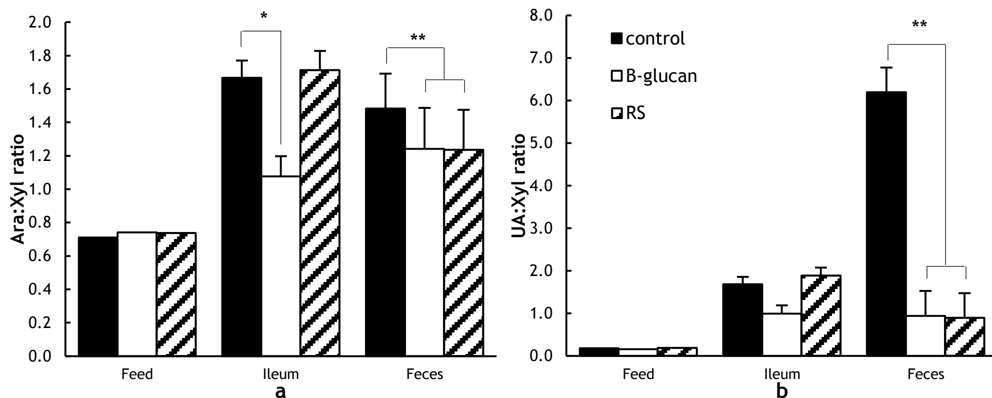


Figure 9.2. Mean molar arabinosyl:xylosyl ratio (Ara:Xyl, panel a) and uronyl:xylosyl ratio (UA:Xyl, panel b) in control, β-glucan, or resistant (RS) diets (n=1) as well as in ileal digesta and feces from growing pigs fed diets containing 40 % (wt/wt) DDGS as the only NSP source, with or without inclusion of β-glucan or RS. Error bars indicate SEM. Asterisks indicate significant differences (**P = 0.05; ***P < 0.01).

Resistant starch. *In vitro* viscosity of the diets and ileal digesta were not affected by RS addition. However, viscosity analyses were performed on supernatants of digesta such that insoluble polysaccharides, as RS, were excluded. Native tapioca has a higher *in vitro* viscosity compared with native corn starch, and although retrogradation may reduce viscosity, ozone retrograded tapioca still has a 2- to 3-fold higher viscosity than native corn starch[303]. Hence, it cannot be excluded that RS increased viscosity of diets and digesta, although not detected with the analytical procedures applied. The AID of starch of ~50 % in RS diets indicates that indeed the retrograded tapioca was not well degraded in the small intestine. Although substantial degradation of starch occurred in the large intestine, ATTD of starch was reduced in RS diets. Furthermore, RS reduced AID of CP in both the CM and DDGS diet, whereas it reduced AID of crude fat in the CM diet. The increased AID of crude fat might be related to the selective recovery of ileal digesta, as the dietary crude fat content is low (~ 5%, wt/wt, DM basis).

Resistant starch reduced ATTD of NGP, both in CM (11 % units, $P < 0.01$) and the DDGS diet (17 % units $P < 0.01$). Apparently the large amount of RS that entered the hindgut undegraded was preferentially degraded by the microbiota over CM and DDGS fiber. Preferential fermentation of RS over other polysaccharides was also suggested for a wheat- and barley-based diet, although not quantitatively assessed[199]. The greater amount of DM disappearance in the hindgut indicates that microbial activity and thus fermentation was stimulated in RS diets, as expected[304]. The possibility that RS reduced hindgut retention time, thereby reducing the time available for fermentation of recalcitrant NGP from CM and DDGS, cannot be excluded although the extensive fermentation of DM (mainly from RS), seems to contradicts this. The ATTD of constituent sugar moieties indicates that arabinosyl-, xylosyl-, as well as uronyl- containing polysaccharides were affected by RS (Table 9.4). For DDGS, the ratio of arabinosyl (Ara:Xyl) and uronyl (UA:Xyl) to xylosyl residues is indicative for the degree of substitution and thus related to the structure of the GAX present in the feed or of the undegraded GAX remaining in digesta and feces (Chapter 5). The

lower Ara:Xyl as well as UA:Xyl in feces from pigs fed the RS diets compared with pigs fed the control diets (Figure 9.2), indicates that especially degradation of relatively linear xylan-fragments was impaired. Despite the increased fermentation activity and presumed higher microbial losses, the amount of CP that disappeared in the hindgut was lower in the RS diets than in the control diets, suggesting reduced absorption of ammonium from the colon or increased influx of urea into the large intestine, shifting N excretion from urine to feces[305]. Opposed to results found for other RS sources[306], pigs fed a retrograded tapioca diet had, with lower digestible energy intake (239 kcal /d), similar weight gain and feed efficiency compared with pigs fed a digestible starch diet[304].

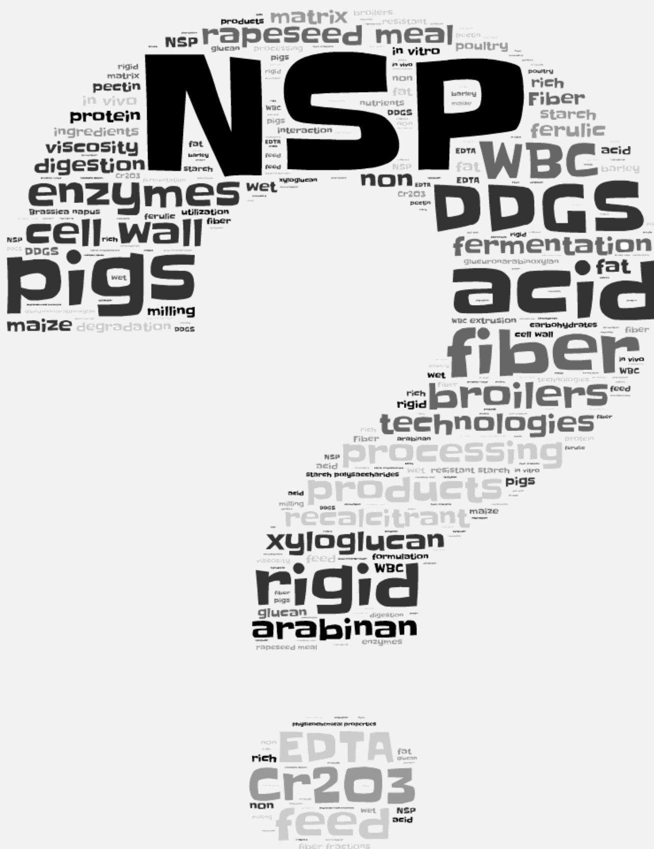
In conclusion, addition of β-glucan and RS clearly alters digestion and fermentation of nutrients and fibers from other sources in the diet. Addition of 6% (wt/wt, as-fed basis) β-glucan to a CM diet increased degradation of NGP from CM by 6 % units, whereas it had no effect on the degradation of NGP from DDGS. Addition of 40 % (wt/wt, as-fed basis) RS reduced degradation of NGP from CM and DDGS by more than 10 % units. Furthermore, AID of CP and starch was reduced when β-glucan or RS were added to the diet, corresponding to a reduced retention time of digesta in the upper gastrointestinal tract.

‘Fiber research in nutrition is still in its early days’

(Dr. David Mela, 2013[15])

Chapter 10

General Discussion



MAIN MESSAGES

- Degradation of fiber fractions strongly depends on structural arrangements of polysaccharides in the cell wall. In growing pigs, NSP not associated with lignin, seem to be well fermented. In broilers, only a part of the soluble NSP is degraded, resulting in typically low degradability values.
- Fiber fractions from DDGS and RSM consist of complex polysaccharides deriving from a rigid cell wall matrix present in the original maize and rapeseed. Recalcitrance of NSP structures is possibly aggravated by the extensive process conditions undergone by these by-products.
- Common, gravimetric, fiber analysis methods such as CF, NDF, or ADF, represent a variable part of the fiber-fraction and are not suited to compare samples that have different physicochemical properties, e.g. solubility or particle size. When fiber structure is studied in detail, or when degradation of fiber in the animal is evaluated, Instead, enzymatic-chemical methods (Englyst[49] method) are preferred when degradation of fiber fractions in the animal are evaluated, whereas gravimetric-chemical[47] methods may be used for routine evaluation of feed ingredients and diets.
- Although both processing and enzyme technologies can be effective in solubilizing NSP from DDGS and RSM, *in vivo* research demonstrates the limited potential to improve the degradation, and thus feeding value, of recalcitrant fiber fractions. Future research should aim at targeted degradation of recalcitrant NSP structures only, while minimizing the effects on relatively easy degradable NSP and other nutrients. Enzyme technologies are preferred over chemical or physical degradation, due to their specific activities.
- The presence of specific fiber fractions in the diet interacts with the degradation of NSP and other nutrients. Feed formulation practices could be optimized if these interactions are included.

For many years, dietary fibers have been regarded as a more or less meaningless feed component and their chemical and functional diversity have long been ignored in the nutrition of monogastric animals. The increased use of fiber-rich feedstuffs in pig and poultry diets has, however, instigated the interest in the nutritional aspects of fiber and their potential effects on the animal.

The aim of this thesis was to improve digestive utilization of fiber-rich feedstuffs for pigs and poultry, by modification of recalcitrant fiber structures. Potential feed technologies to open up cell wall architecture were identified through a literature review (Chapter 2) and effects of selected technologies on modification of cell wall polysaccharides and their degradation were studied *in vitro* (Chapter 3 and 4) and *in vivo* (Chapter 5, 6, 7). To identify structures limiting the ease of degradation of non-starch polysaccharides (NSP), carbohydrate structures resistant to fermentation in pigs and poultry were characterized (Chapter 5, 6, 7). One of the most notable facts identified, was that the analytical methodologies commonly used in digestibility studies are not always appropriate for studying fiber degradation. Working with extreme diets containing up to 50 % of fiber-rich feedstuffs and focusing on the behavior of specific fiber fractions, concerns associated with generally accepted methods were identified, such as the separation of the marker from digesta when measuring ileal digestibility (Chapter 8). In Chapters 3 to 8, fiber degradation was studied in single feedstuffs or in diets where the feedstuff of interest was included as the only NSP source, thereby ignoring possible interactions between fiber sources when included simultaneously in the diet. Hence, in Chapter 9 it was investigated how degradation of fiber-rich feedstuffs is altered by the inclusion of different fermentable fiber sources.

In this final Chapter the results of the studies described in this thesis are discussed. Finally, implications of the results for current feed evaluation practices are addressed.

FIBERS IN MONOGASTRIC NUTRITION AND THEIR FATE IN THE GASTROINTESTINAL TRACT

Fiber-rich feedstuffs

Feed costs constitute the largest variable costs in pig and poultry production (50-70 %). Over the past decade, costs of raw materials for animal feed have increased, mainly due to increased demands for cereals and oil seeds by emerging markets and increased use of these products for energy production or human consumption[85]. Hence, the use of by-products from other industries, such as the biofuel industry, as substitute for higher priced feed ingredients in animal diets has become increasingly attractive. Globally, availability of these products is expected to further increase in the next decade[56] and their relevance for animal nutrition may be expected to persist in the future.

Chapter 4 to 9 focused on two fiber-rich feedstuffs that are widely used in pig and poultry diets and differ in the type of fiber present: maize dried distillers grain with solubles (DDGS) and rapeseed meal (RSM). As described in Chapter 1, these products are by-products from the biofuel industry and were exposed to extensive treatment conditions during the biofuel production process.

Fiber degradability

The quantity and type of NSP, as well as their physicochemical properties vary considerably between feedstuffs. Degradation of NSP in the animal depends to a large degree on the original cell wall matrix in which the polysaccharides are embedded (Chapter 2). Generally, NSP that are more easily solubilized will be better degraded as they are not firmly anchored in the cell wall. Likewise, NSP firmly bound to lignin are usually more recalcitrant[35]. However, as the degradation of NSP is also affected by physical characteristics of the feed matrix, e.g. viscosity, which are in turn partly determined by the physicochemical properties of the NSP fraction[307]. In chickens, NSP degradation is much lower than in pigs and almost exclusively limited to the soluble fraction. This follows logically from the digestive physiology of the chicken, where the solid fraction of digesta will never reach the main site of fermentation: the ceca (Chapter 8).

Unlike the rather detailed information available on composition of fiber from common crops (e.g. cereal grains and legumes, and fractions thereof) and its degradation in the animal and even more so in humans, very little is known about the composition and degradation of NSP from DDGS and RSM. Fiber fractions in these products originate from cell walls present in the original feedstock, but due to prior processing specific cell wall components will concentrate and fiber properties may be modified. Hence, not only will these by-products contain more fiber, also the composition of these fiber fractions can be substantially more complex than those found in the original feedstock.

Table 10.1 Comparison of content of non-glucosyl polysaccharides (NGP, % (wt/wt, as-fed basis)), as well as apparent total tract digestibility (ATTD, %) of NGP and its constituent sugars in growing pigs of diets used in Chapter 5 and Chapter 9. Diets contained 40 % (wt/wt, as-fed basis; Chapter 5) or 50 % (chapter 9) maize distillers grain with solubles (DDGS).

	Chapter 5	Chapter 9
Feed		
NGP	14	11
Ara:Xyl[1]	0.55	0.71
UA:Xyl[2]	0.26	0.18
ATTD		
NGP[3]	62	51
Arabinosyl	61	51
Xylosyl	55	43
Uronyl	80	55

[1] Molar ratio of arabinosyl:xylosyl.
[2] Molar ratio of uronyl:xylosyl.
[3] Non-glucosyl polysaccharides; monosaccharides represent anhydrous sugar moieties.

DDGS. The fiber fraction of maize DDGS was found to consist of complex, highly substituted glucuronoarabinoxylans (GAX) that are cross-linked to or associated with cellulose and lignin within the cell wall matrix (Chapter 4 and 5). In pigs, total tract degradation of NGP from DDGS was found to be between 51 (Chapter 9) and 62 % (Chapter 5, Table 10.1). Although, these values are in the expected range, it was observed that quite some variation between sources is present. The DDGS used in Chapter 9 had a lower NGP content than the batch used in Chapter 5, but the GAX present had more arabinosyl substitutions, which may explain the lower NGP

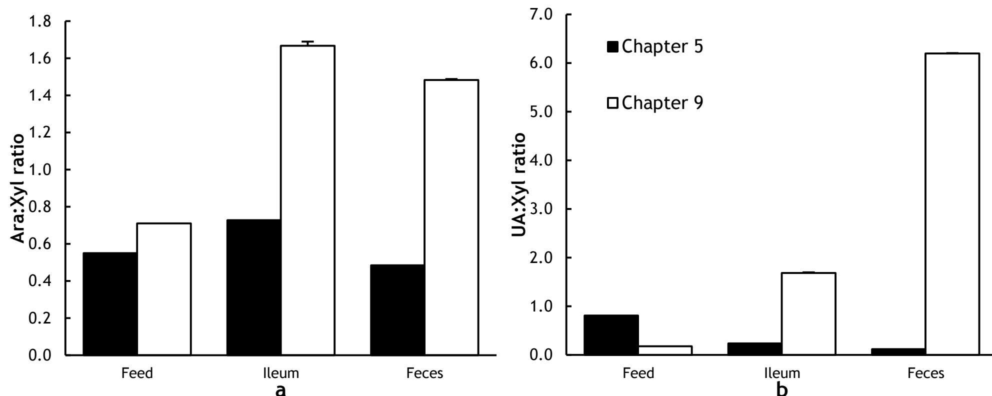


Figure 10.1. Comparison of mean molar arabinosyl:xylosyl ratio (Ara:Xyl, panel a) and uronyl:xylosyl ratio (UA:Xyl, panel b) in diets (n=1) as well as in ileal digesta and feces from growing pigs of diets used in Chapter 5 and Chapter 9. Diets contained 40 % (wt/wt, as-fed basis; Chapter 5) or 50 % (chapter 9) maize distillers grain with solubles (DDGS).

digestibility found in Chapter 9. This variability between sources may result from differences in processing methods and conditions, such as the ratio of solids and solubles. At least 35 % of NSP from DDGS remained undegraded. These unfermented polysaccharides were likely rich in densely substituted xylans with large oligomeric side chains, and cellulose. The results of Chapter 5 indicate that coumaric acid and ferulic acid associated (ester)-linkages contribute to the recalcitrance of DDGS fiber to fermentation in the pig. The considerable differences found for degradation of some of the constituent sugars of NGP, especially uronyl (Table 10.1), indicates that there is substantial variation in degradation of specific fiber structures. The reason for this variability between the two experiments remains unclear. Not only were two different batches of DDGS used in the experiments of Chapter 5 and 9, also individual variation between pigs and the microbial population in their gastrointestinal tract can be of influence. Furthermore, the time available for microbes to adapt to the specific fiber structures might play a role. In Chapter 5 pigs were adapted to the DDGS diet for. 14 d, whereas in Chapter 9 adaptation lasted only 9 d and pigs were alternately switched from RSM to DDGS diets, according to the Latin square design. Little information on degradability of fiber from DDGS in poultry is available. In broilers, ATTD of hemicellulose (NDF-ADF) from DDGS is ~29 % (calculated from Liu et al.[308]).‡

RSM. The fiber fraction of RSM consists of pectic polysaccharides, xyloglucan, and cellulose. The main pectic polysaccharides are homogalacturonan – of which 60 % of the galacturonic acid residues are methyl-esterified -, arabinan, and arabinogalactan type II[224]. Results of chemical characterization of RSM polysaccharides[224] and their unfermented residues in digesta and feces from pigs (Chapter 6) and broilers[255], indicate that these pectic polysaccharides and xyloglucan are linked within a cellulose-lignin network via ester-linkages or H-bonds forming a rigid cell wall matrix.

‡ Apparent total tract digestibility of NSP is expected to be lower than that of hemicellulose as 1. degradation of cellulose, which has a low degradability in chicken, is not included, and 2. feed polysaccharides that are solubilized during digestion are not recovered in NDF (Chapter 1, Figure 1.1), resulting in an overestimation of degradability. Indeed, DDGS is expected to be poorly fermented in chicken, due to the low solubility of the fiber-fraction.

This rigid matrix was found to hinder the complete degradation of NSP from RSM. In pigs, total tract degradation of NSP from RSM was ~70 % (Chapter 6 and 9). Nearly 50 % of the unfermented carbohydrate structures in feces were tightly bound pectins (e.g. rhamnogalacturonan and arabinan), xyloglucan, and cellulose. The other half consisted of smaller uronyl-rich carbohydrates, presumably ester-linked or H-bound. In broilers, total tract degradation of NSP from RSM was ~24 % (Chapter 7). Apart from the carbohydrate structures that are unfermented excreted in pigs, a substantial amount (~20 % of unfermented NSP) of water soluble carbohydrates were found in broiler excreta[255]. This fraction contained small oligosaccharides (fructose, raffinose, and stachyose), branched arabinan, linear galactan, homogalacturonan, and (xylo-) glucan. The solubility of these carbohydrates indicates that their degradation is not so much limited by their physical entanglement in the cell wall matrix, but rather by a lack of appropriate enzyme activities. Apparently, opposed to pigs, the cecal microbiota in broilers do not produce the required enzymes to degrade these structures or time is limiting their full operation.

Interactions between fiber and other nutrients

In current feed formulation systems interactions between feed ingredients are assumed to be absent. The results of Chapter 9 clearly show the interactive effects between the presence of specific fiber types and digestive utilization of the diet. Also in Chapter 5, digestion of other nutrients in the diet was affected by the fiber fractions present. This implies that the nutritive value of the diet can be assessed more accurately if these interactions are accounted for in feed evaluation. Although the effects of fiber inclusion on the digestive utilization of the diet are complex, specific properties can be ascribed to certain fiber types. Viscous, fermentable fiber, such as β-glucan, may decrease enzymatic digestion of other nutrients (Chapter 9), as opposed to the enhancing effects observed using viscous non-fermentable fibers, like carboxymethylcellulose[82, 295]. β-Glucan, a rapidly fermentable fiber source, enhances the degradation of xyloglucan from RSM but does not seem to affect the recalcitrant fiber fraction of DDGS. In contrast, resistant starch (RS), a more slowly, but-well fermentable fiber, decreased degradation of fiber-fractions from DDGS as well as RSM.

Scope for improvement

The increased use of fiber-rich feedstuffs in pig and poultry diets requires an optimal utilization of these ingredients. Hence, the animal feed industry explores opportunities to improve degradability of these feedstuffs and maximize their inclusion levels in pig and poultry diets. Processing and enzyme technologies can modify the physicochemical characteristics of the fiber-fraction from feed ingredients, thereby affecting their degradability. The fiber fraction, largely contributes to the unutilized portion of fiber-rich feedstuffs as DDGS and RSM, due to its high levels and low degradability. In growing pigs, over 30 % of NSP from RSM and 35 % from DDGS remain undegraded, accounting for up to 10 % of the total amount of unused gross energy of these products. Increased degradability of these fiber fractions will provide additional energy to the animal. In addition, alleviation of adverse effects on digestion and absorption of other nutrients may further enhance digestive utilization of the diets. How much improvement can be made depends on animal related factors (e.g. species, age), but also on the fiber type considered. Obviously, the extent of

improvement depends on the original degradability of the fiber and rigidness of the cell wall matrix, but also the utilization of additionally degraded polysaccharides may differ between fibers[309, 310].

Furthermore evaluation of the interactive effects between the presence of fiber and the digestive utilization of the diet will allow more accurate estimations of the nutritive value of the fiber fraction.

METHODS TO EVALUATE FIBER DEGRADATION

Analytical methods

In animal nutrition, fiber contents of feedstuffs and diets are traditionally characterized by gravimetric methods as CF, and the detergent fiber system[46]. A major drawback of the use of these methods is, however, that they represent a variable part of the NSP-fraction[26, 51, 52, 86] (Chapter 1, Textbox 1.1 and Figure 1.1), hampering the comparison between diets or feedstuffs that differ in type of NSP or in their physicochemical properties. When evaluating effects of processing or enzyme technologies on fiber degradation, comparison of these disparate fractions, as obtained with common gravimetric methods, is not very helpful. Instead, one could make calculations using the fiber fraction of the unprocessed diet as the reference, thereby ascribing differences in chemical composition, such as solubility of polysaccharides, as well as effects on its degradation, to the technology concerned (as applied in Chapters 3 to 7). Although this procedure may be useful to indicate whether the technology of interest alters degradation of the fiber fraction, information about specific NSP structures is lacking. Furthermore, also digestive processes in the animal modify physicochemical properties of ingested feed, such that comparison between gravimetrically characterized fiber fractions before and after digestion is dubious.

For scientific purposes, a more appropriate alternative, is the use of enzymatic-chemical methods, as the Englyst[50] or the Uppsala[48] procedures[35]. These methods measure polysaccharides as their monomeric sugar moieties after removal of small saccharides by precipitation in ethanol. Not only do these methods provide detailed information on the chemical composition of the fiber fraction, they also have advantage that they are not sensitive to physical factors such as solubility and particle size of samples, which makes them more appropriate to evaluate fiber degradability in the animal. Generally, separate evaluation of glucosyl- and non-glucosyl-polysaccharides (NGP) will allow more accurate assessment of fiber fractions, as glucosyl originates from NSP as well as from starch, and several corrections for analyzed glucosyl values are required to identify glucosyl originating from NSP (Chapter 9). These sophisticated methods, are, however, not suitable for routine analysis as they are expensive, both in terms of labor and equipment, and are less robust to analytical errors due to the multiple subsequent analytical steps, as well as the small amount of sample material. Furthermore, the small amount of sample material hampers representative sampling in inhomogeneous feed materials. The enzymatic-gravimetric procedure[47] is less sensitive to solubility of polysaccharides compared to the detergent and CF methods, but is still sensitive to particle size of the sample (Figure 1.1). When the filtration step could be optimized or replaced by e.g.

centrifugation, the enzymatic-gravimetric DF procedure can be a potential adequate and robust method for routine analyses of fiber fractions.

Methods to study degradation of fiber-rich feedstuffs

In vitro methods. *In vitro* degradation studies were used to identify potential processing and enzyme technologies to improve digestive utilization of fiber (Chapter 2, 3, and Pustjens et al.[33]). Although advanced dynamic models that realistically simulate gastro-intestinal conditions exist, rapid *in vitro* methods that allow ranking between ingredients based on their fermentability, were of interest and might be better suited due to the fiber-rich products[311]. The procedure used was based on the approach of Sappok et al.[312], consisting of simulation of digestion in the upper gastrointestinal tract (adapted Boisen methodology[17]) followed by fermentation (gas production method[170]). In this procedure, solubilized material is eliminated prior to *in vitro* fermentation, simultaneous with the removal of abundant Boisen incubation fluids. Separation of solids is not only a convenient way to prepare the incubated substrates for fermentation, but also removes solubilized degradation products from starch and protein that will influence fermentation kinetics, whereas they will be absorbed in the *in vivo* situation. This approach assumes, however, that all solubilized polysaccharides during Boisen incubation, will be readily degraded by microbiota and absorbed in the animal. Indeed in pigs, easily solubilizable cell wall polysaccharides were well degraded (Chapter 2 and 6) and it can be assumed that all NGP solubilized during Boisen incubation will be degraded *in vivo* (Figure 10.2, Chapter 6). As polysaccharides that are recalcitrant to fermentation are of concern, separation of solids and solubles is a valid approach when one wants to evaluate degradation in the gastrointestinal tract of the pig. In broilers, polysaccharides that are not solubilized during Boisen incubation will likely not be degraded in the broiler (Chapter 8). Hence, neglecting the soluble polysaccharides, the only fraction potentially degraded, is less sensible.

If *in vitro* methods are to be used to evaluate effects of fiber-modifying technologies, as was the objective in this thesis, appropriate read-out parameters should be chosen. Selection of technologies was partly based on their effect on solubility of polysaccharides before and after Boisen incubation. However, differences in solubilization of polysaccharides observed *in vitro*, do not necessarily correspond to differences in degradability in the pig, as illustrated by the comparison of *in vitro* and *in vivo* studies with DDGS and RSM (Figure 10.2). Although *in vitro* solubilization of RSM NGP was higher compared with NGP from DDGS, the difference in ATTD between RSM and DDGS in pigs was much smaller. These data demonstrate that contrasts in *in vitro* solubilization may be elucidated *in vivo*, as soluble polysaccharides are readily fermented in the pig anyway. Instead, read-out parameters of *in vitro* studies should be able to discriminate between technologies that affect recalcitrant fiber fractions from those that only affect well-fermentable fibers. The total extent of fiber fermentation, as measured by the quantitative disappearance of DM or specific fractions (e.g. NSP) during Boisen incubation and subsequent *in vitro* fermentation might be a good indicator. In addition fermentation kinetics may be useful to evaluate

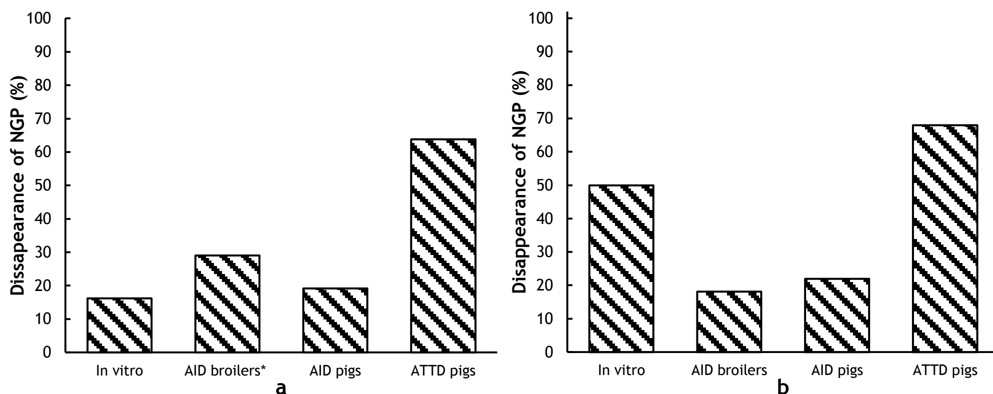


Figure 10.2. In vitro solubilization of non-glucosyl polysaccharides (NGP) from maize dried distillers grain with solubles (DDGS, panel a) and rapeseed meal (RSM, panel b) and during enzymatic digestion simulation (Boisen procedure) and in vivo apparent ileal digestibility (AID) or total tract (ATTD) in broilers and growing pigs. Data from: Chapter 4 to 7, Pustjens et al.[33], and Liu et al.[308]. *Apparent total tract digestibility (ATTD) of hemicellulose (NDF-ADF); ATTD of NGP is expected to be lower (see text).

differences in rate of fermentation of easily solubilizable NSP. The end time-point for *in vitro* fermentation is a point of consideration. At 48h or beyond, nearly all structures that can potentially be degraded with the enzyme spectrum produced by the microbiota *in vitro* will be degraded, diminishing differences in fermentability between various polysaccharides. Instead, 24 h is suggested to be a more relevant and better discriminating time-point[189].

In broilers, ATTD of NGP from RSM is less than half of the NGP solubilized *in vitro* (Figure 10.2), implying that solubilization in itself is not the only prerequisite to degradation in the chicken. It follows that also in broilers, technologies that improve *in vitro* solubilization of polysaccharides do not necessarily correspond to improved fiber degradability *in vivo* (Chapter 7) and optimization of technologies based on their solubilizing effects on polysaccharides seems oversimplified. Hence, *in vitro* methods to study fiber degradation in poultry should focus on detailed structural characterization of solubilized polysaccharides, possibly combined with enzymatic fingerprinting techniques and analyses of physicochemical characteristics, such as viscosity and WBC. Reduction of the amount of Boisen incubation fluid could be considered to amplify contrasts in viscosity. However, to allow successful translation of *in vitro* findings to the *in vivo* situation more information about the limiting factors in degradation of fiber fractions in poultry is needed

In vivo methods. Often, fiber-rich diets have pronounced effects on physical characteristics of the feed matrix, thereby affecting a.o. digesta flow and transit time[307]. These factors may indirectly affect fermentation of fiber, as well as digestion and absorption of other nutrients. In chickens, soluble and solid digesta fractions are separated and retrograde digesta flows occur. These processes are dependent on physicochemical properties of the feed, and hence may complicate digestion studies, particularly, but not exclusively, in high-fiber diets. One of such an example is the separation of marker and digesta when measuring ileal digestibility in chickens (Chapter 8). But also in pigs the influence of fiber on digestibility measurements

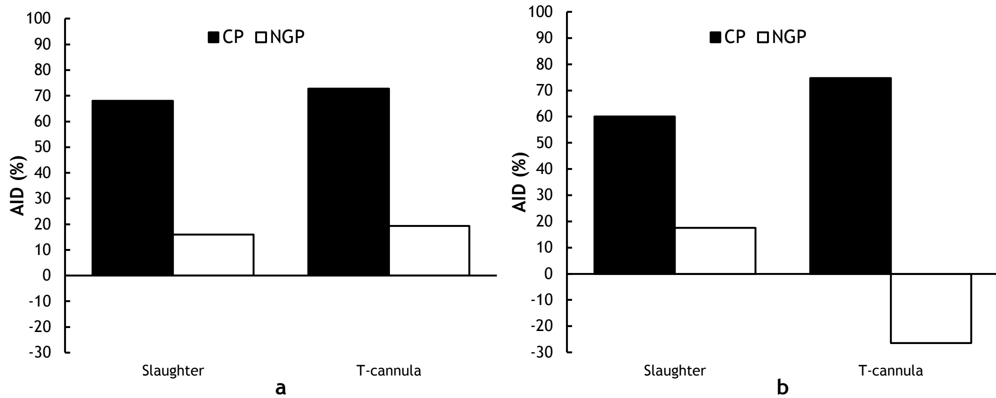


Figure 10.3 Comparison of apparent ileal digestibility (AID) of crude protein (CP) and non-glucosyl polysaccharides (NGP) from maize dried distillers grain with solubles (DDGS, panel a) and rapeseed meal (RSM, panel b) as measured with the slaughter or cannulation (T-cannula) method. Data from: Chapter 5,6, and 9.

should be considered. This is illustrated by the comparison of AID of NGP from RSM obtained by either the slaughter (Chapter 6) or the cannulation (Chapter 9) method (Figure 10.3). The reason for this discrepancy remains unclear. Different batches of DDGS and RSM were used in Chapter 5 and 6 versus Chapter 9, but the chemical composition of these batches was comparable. The discrepancy between the ileal digestibility values found between the studies, seem too large to be explained by batch variation. Possibly, spot-sampling at one single time-point after a meal with the slaughter method resulted in biased digestibility estimates. Alternatively, biased estimates can result from the T-cannula technique, due to selective sampling of digesta fractions. The latter is supported by the different recoveries of solid and soluble phase markers (Chapter 9). Remarkably, the discrepancy between methods was not found for DDGS. In addition, the results of Chapter 9 indicated that the problem of selective recovery of solid digesta was higher for rapeseed meal than for DDGS diets. Possibly, the higher solubility and water binding capacity of RSM NSP compared with those from DDGS (Table 9.1; Figure 10.2), contributes to this observation.

IMPROVING DIGESTIVE UTILIZATION OF FIBER-RICH FEEDSTUFFS BY TECHNOLOGY: SENSE OR NONSENSE?

With the increased use of fiber-rich feedstuffs in pig and poultry diets, also the interest to improve degradability of these products and enhance their digestive utilization has increased. Evidence was found in literature that processing technologies (Chapter 2) as well as enzyme technologies (Text box 10.1) could be used to modify fiber fractions, with consequential effects on degradation in the animal. It was suggested that especially combinations between processing and enzyme technologies seemed promising, due to reinforcing effects of technological pretreatment on enzyme accessibility and counteracting activity of enzymes towards adverse effects of processing, such as increased viscosity.

Technologies to improve NSP degradability

Based on the literature review in Chapter 2, it was concluded that common feed processing technologies may improve degradability of easily solubilizable NSP, but might not be sufficient to affect rather recalcitrant NSP fractions, such as those found in DDGS and RSM. This conclusion was supported by i*n vitro* studies in DDGS (Chapter 4) and RSM (text box 10.1 and Pustjens et al.[33]) where only hydrothermal acid treatment (DDGS) or technological pretreatment combined with cell wall degrading enzymes (RSM) improved *in vitro* degradation of NSP. *In vivo*, hydrothermal acid treatment did not improve degradability of NSP from DDGS for pigs (Chapter 5), despite the increased solubility of the fiber fraction. Although acid treatment shifted fermentation to more proximal gastrointestinal segments, total extent of NSP degradation was not affected. Apparently, acid-extrusion accelerated degradation of NSP structures that are not resistant to degradation by microbial enzymes in the pigs' gastrointestinal tract, whereas the most recalcitrant NSP structures were still not affected. Furthermore, acid treatment reduced feed intake, digestibility of CP and starch and tended to reduce digestibility of crude fat. Although investigation of the mechanisms involved was beyond the scope of this thesis, it was suggested that acid-treatment might have resulted in 'over processing' of the relatively easy degradable NSP, thereby indirectly affecting digestive and absorptive processed through alterations in e.g. digesta passage rate, osmolality, or microbial activity. Hence, it was concluded that, especially for pigs, technologies should target degradation of recalcitrant NSP structures

Textbox 10.1

MISMATCH OF ENZYME ACTIVITIES

A plethora of literature describing the effects of cell wall degrading enzymes on NSP degradation exists, as reviewed by several authors[1-5]. In pigs, improvement in ATTD of fiber fractions after addition of cell wall degrading enzymes ranges between -6 and 18 % units[6-12]. The effects are generally higher in piglets compared with older animals[16]. The effects in poultry are generally more pronounced compared with pigs, and NSP digestibility in broilers can be improved over 20 % units depending on the quantity and type of enzymes and the NSP source[18-23]. The effects in adult birds, which have a larger capacity to ferment solubilized NSP, can be even higher[22, 24, 25]. Nevertheless, also in poultry, several studies failed to prove positive effects of cell wall degrading enzymes on digestibility of fiber fractions [22, 24, 25, 28-30]. The contradictory results of enzyme addition on the digestive utilization of pig and poultry diets have been attributed to a mismatch between enzyme activities and substrate structures and a lack of knowledge regarding side activities of enzyme preparations and optimal concentrations[32].

only, while minimizing the effects on relatively easily degradable NSP and other nutrients. This requires detailed knowledge of the recalcitrant NSP structures and their entanglement within the cell wall architecture.

In broilers, a combination of commercial enzymes with mainly pectolytic activities improved degradation of NSP from RSM (Chapter 7). Addition of pectolytic enzymes facilitated degradation of branched water-soluble arabinans, which could not be degraded by the cecal microbiota without help of these additional enzymes[255]. Particle size reduction, through wet milling and extrusion, facilitated solubilization of NSP, but solubilized structures could still not be degraded by the cecal microbiota. In this context, enzyme technologies seem to be more effective, due to their targeted activity to specific structures, in this case the side-chains of soluble arabinans. The fact that even rather extensive technological processes could not achieve the same result (Chapter 7 and Pustjens et al.[255]) indicated that the scope for improvement of NSP degradability of RSM by processing technologies is rather low. In addition, it can be speculated that thermal treatments may even adversely affect degradability of recalcitrant fiber fractions, due to cross-link formation with lignin, which seems to be confirmed by our findings that acid-extrusion increased rigidness of the NSP-matrix in RSM (Chapter 6 and Pustjens et al.[255]). The lack of interaction between technological processing and enzyme addition indicates that, also, the technological processes used seem to be of little use to improve enzyme accessibility *in vivo*. It can, however, not be excluded that technological pretreatment might be beneficial when other enzymes – that act at different sites of the polysaccharides – or lower doses of enzymes are used.

Textbox 10.2

ENZYMES ATTACKING DDGS FIBER

So far, enzyme technologies have been of limited success to improve degradability of DDGS fiber. Continuous efforts in the field of lignocellulosic biomass degradation to find new enzymes, targeting specific NSP structures and to produce enzyme mixtures at a commercial scale, offer perspectives for development of feed enzyme technologies. For DDGS, enzyme technologies that specifically target ester-linked acetyl, feroloyl, or coumaroyl groups were suggested (Chapter 5). Hence, Ultraflo L (Novozymes, Bagsvaerd, Denmark), an enzyme mixture with a.o. endo-1,4-β-glucanase, endo-1,4-β-xylanase, α-L-arabinofuranosidase, and feruloyl esterase activities, could be of interest. This enzyme mixture was tested *in vitro* in combination with another commercial enzyme preparation (Chapter 4). Addition of Ultraflo L to DDGS did not improve solubilization of NSP, regardless the physical or chemical pretreatment used. However, the same enzyme mixture, successfully removed feruloyl groups from maize fiber oligosaccharides that were solubilized by rather extensive hydrothermal acid (11 % DM, H_2SO_4, 140 °C, 30 min.) and enzymatic pretreatments[27]. Apparently, the cell wall matrix was hindering enzyme accessibility of specific NSP structures in the in vitro study, and the technological pretreatments used did not alleviate these physical constraints. Nonetheless, if *in vivo*, physical, chemical, and microbial degradation will open the cell wall matrix and facilitate accessibility of NSP, fiber degradation in the animal may still be enhanced by this feruloyl esterase containing enzyme mixture.

Future strategies

The results of the studies described in this thesis indicate that the greatest prospect for improvement of digestive utilization of fiber fractions from products as DDGS and RSM lies in the degradation of highly resistant, complex, structures. Unlike the modification of relatively easily degradable fiber fractions, modification of fiber-structures that resist fermentation in animals such as the pig is far from easy. The

risk for 'over processing' of relatively easily degradable NSP and other feed components makes the challenge even bigger. Characterization of residual structures in fractions resistant to chemical detergents and unfermented residues in excreta of the animal can assist to identify potential structures to be targeted to improve degradation of the fiber fraction. In DDGS as well as RSM, ester-linkages or H-bonds seemed to be involved in the recalcitrance of the fiber fraction to degradation in the animal (Chapter 5, 6, and Pustjens et al.[255]), presumably due to anchorage of NSP in the rigid cellulose-lignin matrix. Hence, technologies that degrade such linkages, as alkali treatments or esterases could be of interest for future research (Text box 10.2). Results of a preliminary *in vitro* study indicated, however, that hydrothermal alkali treatment (alkali-extrusion) still did not improve fiber degradation of RSM (Text box 10.3). Esterases may have more potential, and additionally may reduce the risk for formation of large quantities of small saccharides and protein damage, due to their specific activities.

Further opportunities to improve degradability of fiber fractions may be found in the degradation of lignin itself, or other lignin-associated linkages. Several chemical and physicochemical pretreatments to modify lignin fractions exist[313], but processing conditions are generally to rigorous for application in animal feed. Recent advances in degradation of lignocellulosic materials using fungi[314] may bring us one step closer to this goal. In broilers, the cecal microbiota lack the capacity to degrade highly branched structures. Enzyme technologies that target these structures, such as debranching and exo-acting enzymes[315], may be of interest to enhance NSP degradation in poultry.

Textbox 10.3

EXTRUSION WITH ACID AND ALKALI TO IMPROVE RAPESEED MEAL DEGRADABILITY

Aim. Test effect of extrusion under neutral, acidic, or alkali conditions on *in vitro* degradability of rapeseed meal (RSM).

Methods. RSM was used either unprocessed (control) or extruded (Table1 10.2) with a co-rotating double screw extruder, using the settings described in Chapter 7 except for barrel temperature, which were set at 35, 55, 75, 90, 95, 125, 140, 155, 175 °C. Conditions for the extrusion process were chosen rather excessive, reaching the limits of what can be achieved with RSM. Digestion in the upper gastrointestinal tract (adapted Boisen methodology[17]), and subsequent, large intestinal fermentation (gas production) were simulated in duplicate as described in Chapter 3. Samples were analyzed for content of DM and CP. Fiber content was approximated by subtracting CP from DM. Data were analyzed by analysis of variance, using treatment as fixed effect. Contrasts were used to compare extrusion, acid-extrusion, or alkali-extrusion to the unprocessed control.

Table 10.2 Conditions used for the various extrusion treatments.

	Chemical additive	Moisture (%)	Product temperature (°C)	Pressure (psi)
Extrusion	-	30	139	380
Acid-extrusion	maleic acid (2 %, wt/wt DM)	30	138	380
Alkali-extrusion	NaOH (2 %, wt/wt DM)	30	128	360

Results. Extrusion processing did not affect DM and fiber disappearance during Boisen incubation, but protein digestion was 2-3 % units higher than the unprocessed control for all extrusion treatments (no further data presented), the increase being similar to results found for extrusion under milder conditions (20 % moisture and product temperature of 119 °C)[33]. Extrusion processing did not affect total degradation of DM, protein, and fiber during the complete digestion and subsequent fermentation procedure did not differ (Fig. 10.4).

Conclusion

Extrusion processing at relatively high product temperatures did not improve in vitro degradability of DM and fiber from RSM, regardless the chemical additive used.

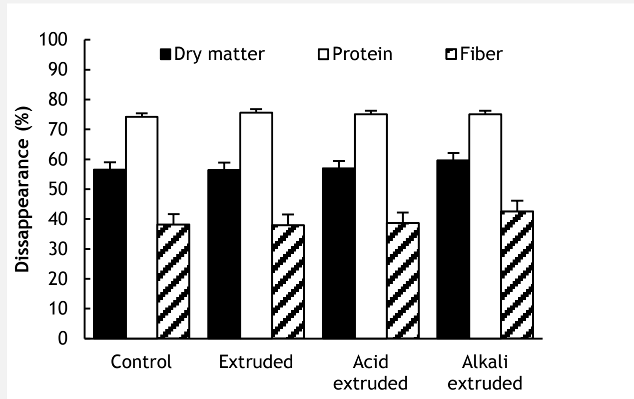


Figure 10.4 Total losses of dry matter, protein, and fiber from unprocessed (control) or extruded RSM during *in vitro* digestion and fermentation. Extrusion was performed with or without addition of maleic acid (acid-extrusion) or NaOH (alkali-extrusion). Data are presented as LSMeans (n=2). Error bars represent SE. Total losses of DM, protein, and fiber of extruded, acid-extruded, or alkali-extruded RSM did not significantly differ from unprocessed RSM ($P > 0.1$).

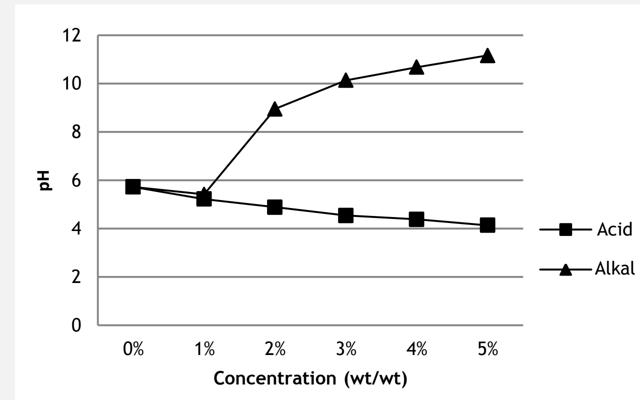


Figure 10.5 pH of rapeseed meal after addition of various concentrations of maleic acid (acid) and NaOH (alkali).

Buffering capacity of RSM. To select the concentration of acid and alkali to be used, a small-scale test using various concentrations of maleic acid and NaOH was performed (Figure 10.5). The range of concentrations tested was between 1 and 5 % (wt/wt); higher concentrations are expected to be irrelevant because of high amounts of acid or alkali that should be added to the feed. The pH of untreated RSM was 5.7. Addition of 1 % acid or alkali had minor effects on pH. Addition of 2 % alkali resulted in a 3.2 unit increase in pH, whereas 2 % acid decreased pH by 0.8 units only, indicating buffering capacity by RSM protein.

GENERAL CONCLUSIONS AND RECOMMENDATIONS

Fiber-rich by-products. The rapid evolvement of processing technologies in biofuel production leads to continuous changes in diversity and composition of by-products that can be potentially used as ingredients for animal diets. Animal nutritionists should be aware of the variable quality of these products and possibly implement rapid screening assays to monitor quality of these raw materials. By-products are of significant importance in the economic viability of biofuel production[316]. Sale of distillers grain, for example, contributes 15 to 20 % to the total revenues of bioethanol production[317]. Hence, biofuel production plants could benefit from thorough quality assessment of their by-products and focus on reduction of their variability. Possibilities to optimize process conditions based on quality requirements of by-products alongside those for the main product should be investigated. Technologies that are not cost-effective when incorporated in feed processing practices, e.g. due to excessive energy requirements for drying, might be used to produce higher valued by-products when incorporated in the biofuel production processes.

Fiber degradability. Basically, degradation of NSP in the animal is restricted by physical entanglement of polysaccharides in the cell wall matrix, limited retention time in the gastrointestinal tract, and absence of appropriate enzyme activities as determined by the microbial colonization and activity in the gastrointestinal tract. The three are interrelated and it is difficult to identify the limiting factors in the degradation of various fiber fractions. In pigs, physical inaccessibility seems to be only the case for tightly bound NSP linked within a rigid polymer-lignin matrix. The observation that some structurally complex NSP that are well degraded in sows, are less well degraded in growing pigs indicates that retention time can be a limiting factor in degradation of these polysaccharides[67]. Hypothetically, the fiber fraction from DDGS that resists degradation in growing pigs may benefit from increased retention time, as cell wall polysaccharides found in maize are rather complex but not highly lignified. This corresponds to the higher degradation of maize fiber in adult sows compared to growing pigs[67]. The recalcitrant fiber fraction from RSM, however, is lignified to a higher extent and it can be speculated that degradation of RSM fiber may not benefit, or not as much, from longer digesta retention time[67]. In the chicken, solubilization is a prerequisite for fermentation, but even if solubilized, a substantial part of NSP will still remain undegraded. This can be due to the short time available for fermentation, but the observation that additional enzymes can facilitate degradation of NSP structures within the transit time, indicates a lack of appropriate enzyme activities.

Analytical methods. When fiber structure is studied in detail, or when degradation of fiber in the animal is evaluated, enzymatic-chemical methods as the Englyst[49] method, are preferred, to avoid confounding effects of physical factors, such as solubility of polysaccharides and particle size of samples. For routine analysis of feed ingredients and diets, however, a gravimetric-chemical[47] method is more appropriate, but effects of filtration procedures should not be underestimated.

In vitro methods. For pigs, total DM or fiber degradation during enzymatic digestion followed by in vitro fermentation (after separation of solubles) seems to be a good parameter to discriminate between ingredients based on their fermentability. For poultry, polysaccharides that are solubilized are of interest, rather than those that remain unaffected during in vitro digestion. However, as not all solubilized polysaccharides are degraded detailed structural characterization of in vitro solubilized polysaccharides may be required. It should be noted that although in vitro methods may be used to discriminate between fiber sources based on their degradability, e.g. to rank differently processed feed ingredients, compensatory mechanisms in the animal, such as altered retention time in the gastrointestinal tract, are not accounted for.

Technologies. Within the limits of processing conditions that can be used for animal feed applications, processing technologies mainly target easily solubilizable NSP. To improve digestive utilization of fiber fractions from products as DDGS and RSM, however, focus should be on the more recalcitrant cell wall structures. Enzyme technologies, targeting specific structures, seem to provide more perspective. Availability of enzyme preparations with the required activities, however, remains an issue. Although theoretically, technological pretreatments may reinforce effectiveness of enzymes by enhancing accessibility, processing technologies did not enhance enzyme effects in broilers. It seems that digestive processes in the animal itself already facilitated enzyme accessibility and that technological treatments studied could not surpass this effect.

Interactions. In current feed formulation systems interactions between feed ingredients are assumed to be absent. The clear interactions between specific feed components, such as various types of fiber, and the digestive utilization of the diet, however, challenge this assumption. Instead, effects of individual feed ingredients on the physicochemical properties of the chyme, such as viscosity and WBC, and retention times in various segments of the gastro-intestinal tract may help to more accurately predict the nutritive value of diets.

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LIST OF ABBREVIATIONS

AA	Amino Acids
ADF	Acid detergent fiber
ADFI	Average daily feed intake
ADG	Average daily gain
ADL	Acid detergent lignin
AID	Apparent ileal digestibility
Ala	Alanine
Ara	Arabinosyl
Ara:Xyl	Arabinosyl:xylosyl ratio
Arg	Arginine
Asp	Aspartic acid
ASS	Alkali soluble solids
ATTD	Apparent total tract digestibility
BW	Body weight
CAID	Coefficient of apparent ileal tract digestibility
CATTD	Coefficient of apparent total tract digestibility
CF	Crude fiber
CM	Canola meal
Co	Cobalt
CP	Crude protein
Cr	Chromium
Cr:Co	Chromium:cobalt ratio
CSF	Combined severity factor
D	One full revolution around the element
DE	Digestible energy
DF	Total dietary fiber
DDGS	Maize dried distillers grain with solubles
DM	Dry matter
DMCV	Dry matter cumulative volume
DP	Degree of polymerization
EDTA	Ethylenediamine tetraacetic
FCR	Feed conversion ratio
GAX	Glucuronoarabinoxylan
G:F	Feed: gain ratio
GMD	Geometric mean diameter
Fuc	Fucosyl
Gal	Galactosyl
Glc	Glucosyl
Glu	Glutamic acid
Gly	Glycine
His	Histidine
Ile	Isoleucine
HPAEC	High performance anion exchange chromatography
HPLC	High performance liquid chromatography
HPSEC	High performance size exclusion chromatography

Leu	Leucine
Lys	Lysine
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
Man	Mannosyl
ME	Metabolizable energy
MS	Mass spectrometry
N	Nitrogen
Na	Not analyzed
NDF	Neutral detergent fiber
NGP	Non-glucosyl polysaccharides
NGP:Co	Non-glucosyl polysaccharides to cobalt ratio
NSP	Non-starch polysaccharides
Phe	Phenylalanine
RES	Residue after sequential extraction
Rha	Rhamnosyl
R_{max}	Maximum rate of gas production
RS	Resistant starch
RSM	Rapeseed meal
Pro	Proline
SEM	Standard error of the mean
Ser	Serine
T × E	Technology × Enzyme interaction
t-	Terminal linked glycoside
T/B	Terminally linked residues: branching points ratio
Thr	Threonine
T_{max}	Time of R_{max}
Tr	Trace amounts
Tyr	Tyrosine
UA	Uronyl
UA:XYl	Uronyl:xylosyl ratio
Val	Valine
WBC	Water binding capacity
WHC	Water holding capacity
WSS	Water soluble solids
WUSS	Water unextractable solids
Xyl	Xylosyl

SUMMARY (EN)

The increased use of fiber-rich feedstuffs in pig and poultry diets requires an optimal utilization of these feed ingredients. Hence, the animal feed industry explores opportunities to improve degradability of these feedstuffs and maximize their inclusion levels in pig and poultry diets. Processing and enzyme technologies can modify the physicochemical characteristics of fiber fractions from feed ingredients, thereby affecting their degradability. In this way, fermentability of non-starch polysaccharides (NSP) and thus their potential energetic utilization might be enhanced. In addition, technologies can be aimed at alleviation of adverse effects on digestion and absorption of other nutrients, which might be particularly of interest for young pigs and poultry. However, to understand modifications that occur during processing detailed information on the composition of fiber structures is required.

This thesis aimed at identifying limiting factors in the degradability of fiber fractions in pigs and poultry and at development of technologies to improve their degradation. Focus was on recalcitrant fiber structures as found in in maize dried distillers grain with solubles (DDGS) and rapeseed meal (RSM). Fiber degradation in growing pigs and broilers was studied in detail and limiting structures in the degradation of NSP were identified (Chapter 5 to 7). Effects of processing and enzyme technologies on fiber-rich feedstuffs were evaluated based on literature and *in vitro* and *in vivo* studies in growing pigs and broilers (Chapter 2 to 7). In addition, marker methods to study digestibility of fiber-rich diets in broilers were discussed (Chapter 8). Furthermore, interactive effects between specific fermentable fiber sources and the digestive utilization of the diet were investigated (Chapter 9). In the final chapter, results of the thesis were summarized and synthesized. Methods to analyze fiber components and evaluate fiber degradation *in vitro* and *in vivo* were discussed, suggestions for future research were given, and implications of the results for feed formulation were addressed (Chapter 10).

Recalcitrant fiber fractions in DDGS and RSM

The fiber fraction of maize DDGS was found to consist of complex, highly substituted glucuronoarabinoxylans (GAX) that are cross-linked to or associated with cellulose and lignin within the cell wall matrix. In pigs, total tract degradation of non-glucosyl polysaccharides (NGP) from DDGS was between 51 and 62 %. Coumaric acid and ferulic acid associated (ester)-linkages were found to contribute to the recalcitrance of DDGS fiber to fermentation in the pig. The fiber fraction of RSM consists of pectic polysaccharides, xyloglucan, and cellulose that are linked via ester-linkages or H-bonds, forming a rigid cell wall matrix. This rigid matrix was found to hinder the complete degradation of NSP from RSM. In pigs, total tract degradation of NSP from RSM was ~70 %. Nearly 50 % of the unfermented carbohydrate structures in feces were tightly bound pectins (e.g. rhamnogalacturonan and arabinan), xyloglucan, and cellulose. The other half consisted of smaller uronyl-rich carbohydrates, presumably ester-linked or H-bound. In broilers, total tract degradation of NSP from RSM was ~24 %.

Processing and enzyme technologies

Common feed processing technologies may improve degradability of easily solubilizable NSP, but are not sufficient to affect rather recalcitrant fiber fractions, such as those found in DDGS and RSM. Particle size reduction, hydrothermal treatment with or without shear, acid hydrolysis, and cell wall degrading enzymes improved *in vitro* degradability barley (13-43 % units, $P < 0.01$), whereas only severe hydrothermal acid treatment increased *in vitro* degradability of fiber fractions from DDGS (30-60 % units, $P < 0.01$). In pigs, however, hydrothermal acid treatment did not improve degradability of NSP from DDGS, despite the increased solubility of the fiber fraction. Acid treatment shifted fermentation to more proximal gastrointestinal segments, but total extent of NSP degradation was not affected. Apparently, acid-extrusion accelerated degradation of NSP structures that are not resistant to degradation by microbial enzymes in the pigs' gastrointestinal tract, whereas the most recalcitrant NSP structures were still not affected. Furthermore, acid treatment reduced feed intake, digestibility of crude protein (CP; 3 % units, $P = 0.06$) and starch (1 % unit, $P = 0.10$), and tended to reduce digestibility of crude fat (0.4 % units, $P < 0.10$). Degradability of NGP from rapeseed meal was found to be successfully improved by addition of pectolytic enzymes (9-20 % units, $P < 0.01$), due to increased degradation of branched water-soluble arabinans. This coincided with an increased NGP concentration in the ceca (4-7 g/g cobalt, $P < 0.01$), indicating that more NGP were solubilized such that they could enter the ceca and become available for fermentation. Particle size reduction, through wet milling and extrusion, facilitated solubilization of NSP, but solubilized structures could still not be degraded by the cecal microbiota. No interaction between processing technologies and enzyme addition was found. Apparently, the processing technologies studied were not facilitating accessibility of NSP to pectolytic enzymes added to the feed in vivo.

In conclusion, both processing and enzyme technologies can be effective in solubilizing NSP from DDGS and RSM, but *in vivo* research demonstrated the limited potential to improve the degradation, and thus feeding value, of recalcitrant fiber fractions. Future research should aim at targeted degradation of recalcitrant NSP structures only, while minimizing the effects on relatively easy degradable NSP and other nutrients. Enzyme technologies, targeting specific structures, seem to provide more perspective than more rigorous processing technologies. In DDGS and RSM, ester-linkages or H-bonds seem to be involved in the recalcitrance of the fiber fraction to degradation in the animal, presumably due to anchorage of NSP in the rigid cellulose-lignin matrix. Hence, technologies that degrade such linkages, as alkali treatments and especially esterases could be of interest for future research.

Marker methods

Digestibility measurements are a crucial tool in the evaluation of the nutritive value of feedstuffs. The marker method, where digestibility is estimated from the ratio between an indigestible marker and the nutrient of interest in feed and digesta or excreta, is commonly used as alternative for the laborious total collection method. In broilers, separation of marker and specific digesta fractions occurs, and especially when degradation of fiber fractions is the matter of interest, the marker method has limitations. When estimating apparent ileal digestibility (AID), separation of marker and digesta resulted in unrealistic high estimates for the digestibility of non-glucosyl

polysaccharides (54-66 %), exceeding ATTD values by 16-42 % units. Moreover, the effect of pectolytic enzyme addition on the AID of non-glucosyl polysaccharides was in opposite direction when compared with total collection.These data illustrate that fractionation of digesta, particularly in high-fiber diets, complicates accurate ileal digestibility measurements in broilers, regardless the choice of markers used. It is recommended to add a soluble marker when fiber degradation is of interest, even though it does not allow quantifying fermentative degradation of nutrients.

Interactions between fiber and digestive utilization of the diet

In current feed formulation systems interactions between feed ingredients are assumed to be absent. This assumption can be challenged as interactions between specific feed components, such as various types of fiber, and the digestive utilization of the diet exist. Although the effects of fiber inclusion on the digestive utilization of the diet are complex, specific properties can be ascribed to certain fiber types. β-Glucan, a rapidly fermentable, viscous, fiber source, enhanced the degradation of xyloglucan from RSM (ATTD of NGP increased by 6 % units, $P < 0.001$) but did not seem to affect the recalcitrant fiber fraction of DDGS. Furthermore, β-glucan decreased enzymatic digestion of CP and starch in the small intestine. In contrast, resistant starch (RS), a more slowly, but-well fermentable fiber, decreased degradation of fiber-fractions from DDGS as well as RSM (> 10 % units, $P < 0.01$). These results clearly show the interactive effects between specific fiber fractions in the diet and the degradation of NSP and other nutrients. It is suggested to include effects of individual feed ingredients on the physicochemical properties of the chyme, such as viscosity and water binding capacity, and retention times in various segments of the gastro-intestinal tract in feed formulation, to more accurately predict the nutritive value of diets.

SAMENVATTING (NL)

Door de toenemende beschikbaarheid van vezelrijke grondstoffen voor varkens- en pluimveevoeders zoekt de diervoederindustrie naar mogelijkheden om de benutting van deze grondstoffen door het dier the maximaliseren. Door technologische behandeling of het toevoegen van enzymen kunnen de fysisch-chemische eigenschappen van vezels uit grondstoffen en voeders bewerkt worden. Op deze manier kunnen deze vezels potentieel beter afbreekbaar worden gemaakt. Hierdoor kan de energetische benutting van vezelrijke grondstoffen worden verbeterd. Daarnaast kunnen negatieve effecten van vezelfracties op de vertering van andere nutriënten verminderden. Dit is voornamelijk interessant voor jonge biggen en kuikens. Om technologieën te ontwikkelen die de afbraak van vezels faciliteren is het van belang dat de vezelfractie goed gekarakteriseerd is.

Dit proefschrift had tot doel om de limiterende structuren in de vezelafbraak in varkens en pluimvee in kaart te brengen en om technologieën te ontwikkelen om de vezelfracties uit grondstoffen beter afbreekbaar te maken. De studies richtten zich in het bijzonder op de vezelfracties uit DDGS, een bijproduct van de bio-ethanol productie uit mais, en raapzaadschroot (RSM), een bijproduct van de olieproductie. Verteringsstudies met vleesvarkens en vleeskuikens werden uitgevoerd om de afbraak van deze vezelfracties te bestuderen en om de structuren die limiterend zijn voor de afbraak van de verschillende polysachariden te identificeren (Hoofdstuk 5 tot en met 7). Daarnaast werden verschillende proces- en enzymtechnologieën om de vezelafbraak te beïnvloeden bestudeerd (Hoofdstuk 2 tot en met 7). Ook werd ingegaan op het gebruik van de marker-methode bij gebruik van vezelrijke voeders in verteringsstudies met vleeskuikens (Hoofstuk 8). Tevens werd de interactie tussen specifieke fermenteerbare vezelbronnen en de vertering van DDGS- en RSM-rijke voeders bestudeerd (Hoofdstuk 9). In het laatste hoofdstuk werden de resultaten van het proefschrift samengevat en geëvalueerd. Methoden om vezelcomponenten te analyseren en om vezelafbraak *in vitro* en *in vivo* te bestuderen werden bediscussieerd en er werden aanbevelingen voor vervolgonderzoek gedaan. Daarnaast werden implicaties van de resultaten voor voederwaardering besproken.

Recalcitrante vezelstructuren in DDGS en raapzaadschroot

De vezelfractie van mais DDGS bestaat grotendeels uit complexe, sterk vertakte glucuronoarabinoxylanen, die doormiddel van cross-links met cellulose en lignine verankerd zijn in de celwand. In vleesvarkens werd de vezelfractie van DDGS voor 51 tot 62% afgebroken. De verhoogde concentraties aan coumaar- en ferulazuren in de onafgebroken DDGS fracties wijzen er op dat deze fenolzuren bijdragen aan de recalcitrantie van vezelstructuren uit DDGS. De vezelfractie uit raapzaadschroot bestaat uit pectinen, xyloglucanen, en cellulose. Deze polysacchariden zijn door middel van esterbindgen en waterstofbruggen met elkaar verbonden en vormen zo een rigide celwandmatrix. Deze rigide matrix bleek limiterend voor de afbraak van vezels uit RSM. In vleesvarkens werd de vezelfractie van RSM voor ca. 70% afgebroken. De helft van de onafgebroken structuren bestond uit dicht opeengepakte pectinen (zoals rhamnogalacturonanen en arabinanen), xyloglucanen en cellulose. De andere helft bestond uit kleine uronyl-rijke koolhydraten welke waarschijnlijk door middel van

esterverbindingen of waterstofbruggen in de celwand verankerd waren. In vleeskuikens was de afbraak van vezels uit RSM ca. 24%.

Proces- en enzymtechnologieën

Gangbare procestechnologieën kunnen gebruikt worden om de afbraak van makkelijk oplosbare NSP (niet-zetmeel koolhydraten, onderdeel van de vezelfractie) te verbeteren. Deze technologieën zijn echter niet rigoureus genoeg om meer recalcitrante vezelfracties, zoals aanwezig in DDGS en RSM, af te breken. Deeltjesgrootteverkleining, hittebehandelingen – met en zonder shear –, zuurhydrolyse en celwand afbrekende enzymen verhoogden de *in vitro* afbreekbaarheid van gerst (13-43 % punt, $P < 0.01$). De *in vitro* afbreekbaarheid van vezels uit DDGS kon echter alleen vergroot worden met behulp van hittebehandeling gecombineerd met zuur (30-60 % punt, $P < 0.01$). Ondanks de verhoogde oplosbaarheid van NSP, had zuurbehandeling van DDGS geen effect op de afbreekbaarheid van de vezelfractie in varkens. Zuurextrusie versnelde de afbraak van makkelijk afbreekbare NSP-structuren maar de totale NSP-afbraak bleef gelijk. Blijkbaar had zuurextrusie voornamelijk effect op NSP-structuren die in het dier makkelijk worden afgebroken en bleven de recalcitrante NSP-structuren onaangeroerd. Daarnaast waren voeropname en ileale eiwit- (3 % punt, $P = 0.06$), zetmeel-(1 % punt, $P = 0.10$), en vetvertering (0.4 % punt, $P < 0.10$) lager voor het zuurbehandelde voer.

De afbraak van de vezelfractie uit RSM in vleeskuikens kon wel worden verbeterd met behulp van pectolytische enzymen (9-20 % punt, $P < 0.01$). Dit was voornamelijk toe te schrijven aan een verhoogde afbraak van water-oplosbare arabinanen. Gelijktijdig werd een verhoogde concentratie aan niet-glucose polysachariden (NGP) in de ceca gevonden (4-7 g/g cobalt, $P < 0.01$). Dit wijst erop dat meer NGP oplosbaar gemaakt zijn en zodoende de ceca konden bereiken waardoor ze beschikbaar kwamen voor fermentatie. Deeltjesgrootteverkleining – bewerkstelligd met nat-malen en extruderen – droeg bij aan verbeterde oplosbaarheid van de NSP-fractie maar oplosbare structuren konden nog steeds niet worden afgebroken door de microbiota in de ceca. Er werd geen interactie gevonden tussen technologische behandeling en enzymtoevoeging. Het lijkt erop dat de technologische behandelingen gebruikt in deze studie, *in vivo* niet de toegankelijkheid voor toegevoegde pectolytische enzymen verhoogde.

Geconcludeerd kan worden dat zowel technologische behandelingen als enzymen de oplosbaarheid van NSP uit DDGS en RSM kunnen verhogen. Echter, *in vivo* studies laten zien dat de effecten op afbreekbaarheid van vezels in het dier beperkt zijn. De mogelijkheden om met behulp van proces- en enzymtechnologieën de nutritionele waarde van vezelrijke bijproducten te verbeteren, lijken daarom beperkt. Vervolgonderzoek moet gericht zijn op afbraak van de recalcitrante NSP-fractie zonder daarbij de makkelijk oplosbare NSP en andere nutriënten te veel te beïnvloeden. Enzymen zijn hiervoor doorgaans geschikter dan technologische behandelingen door de doelgerichte werking. De afbraak van vezelfracties uit DDGS en RSM lijkt gedeeltelijk gehinderd te worden door esterbindingen of waterstofbruggen, waarschijnlijk doordat NSP doormiddel van deze bindingen in de rigide cellulose-lignine matrix verankerd zijn. Technologieën die specifiek deze bindingen verbreken, zoals alkalibehandelingen en in het bijzonder esterases bieden potentie.

Markers voor verteringsstudies

Verteringsstudies zijn cruciaal voor het inschatten van de nutritionele waarde van voedermiddelen. De markermethode, waarbij vertering wordt berekend met behulp van de ratio tussen een onverteerbare marker en het nutriënt, is een veel gebruikt alternatief voor de arbeidsintensieve kwantitatieve verzamelmethode, waarbij alle mest of chymus verzameld dient te worden. Echter, in kuikens scheiden de oplosbare en onoplosbare chymusfracties van elkaar in het maagdarmkanaal, waardoor de passage van de marker niet gelijk is aan de passage van verschillende nutriënten. Hierdoor is deze methode minder geschikt voor verteringsstudies in kuikens, in het bijzonder bij vezelrijke voeders. Deze scheiding van marker en nutriënten blijkt uit de onrealistisch hoge ileale verteringcijfers die gevonden werden voor NGP (54-66 %). Ileal vertering was daarmee 16-42 % punten hoger dan de totale vertering. Bovendien was het effect van enzymtoevoeging tegengesteld aan het effect dat gevonden werd voor totale vertering met behulp van de kwantitatieve verzamelmethode. Deze data illustreren dat scheiding van chymusfracties de meting van ileale vertering in vleeskuikens beïnvloeden, voornamelijk in vezelrijke voeders en onafhankelijk van de marker die gebruikt wordt. Indien de studie gericht is op vezelafbraak, dient het de aanbeveling om, naast de standard verteringsmarker, een oplosbare marker te gebruiken.

Relatie tussen vezels en de nutritionele benutting van voeders

In de huidige voederwaarderingssystemen wordt aangenomen dat er geen interactie is tussen de verschillende voedermiddelen. Dit is echter niet geheel juist, daar er wel degelijk interactie tussen verschillende voedingscomponenten en de benutting van het voer is, zoals bijvoorbeeld het geval is voor verschillende soorten vezels. De effecten van vezels op de nutritionele benutting van voeders zijn complex maar er kunnen toch specifieke eigenschappen aan verschillende vezelsoorten worden toegeschreven. Om de interactie tussen specifieke vezelbronnen en de vertering van voeders te illustreren werden β-glucanen, een goed fermenteerbare, vezelbron met visceuze eigenschappen of resistent zetmeel, een langzamer fermenteerbare, doch goed afbreekbare vezelbron, aan DDGS- en RSM-rijke voeders toegevoegd. β-Glucanen verhoogden de afbraak van xyloglucanen uit RSM (6% punt, $P < 0.001$) maar hadden geen effect of de afbraak van vezels uit DDGS. Daarnaast hadden β-glucanen een negatief effect op de enzymatische vertering van eiwit en zetmeel in de dunnen darm. Resistent zetmeel daarentegen had een negatief effect op de afbraak van vezels uit zowel DDGS als RSM (> 10 % units, $P < 0.01$). Dit betekent dat de voedingswaarde van voeders beter voorspeld kan worden als deze interacties tussen individuele voedermiddelen meegewogen worden. Dit kan mogelijk door het opnemen van effecten van specifieke componenten op fysisch-chemische eigenschappen van de chymus, zoals viscositeit en waterbindend vermogen, evenals verblijftijd in verschillende delen van het maagdarmkanaal.

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‘If the opposite of recalcitrant is ‘degradable’, as a human, you’d better be recalcitrant’

About the author

Curriculum Vitae

List of publications

Training and supervision

CURRICULUM VITAE

Sonja de Vries was born on February 18, 1984 in Leidschendam, The Netherlands. In 2003 she graduated from high school 'Stedelijk College Zoetermeer', after which she worked full-time for one year as sales assistant at Hema B.V. (The Hague, The Netherlands) and as horse trainer at Pureform Farm (Abbotsford, BC, Canada). In 2004, she started her study 'Animal Sciences' at Wageningen University. She was among the first to complete the Research Master 'Animal Sciences' with the major specializations 'Animal Health and Welfare' and 'Animal Breeding and Genetics' and the minor specialization 'Animal Nutrition'. For the specialization Animal Health and Welfare she investigated the effects of incubation temperature and a hole in the air cell on perinatal development and physiology of layer hatchlings, commissioned by HatchTech Incubation Technology B.V. (Veenendaal, The Netherlands). For the specialization Animal Breeding and Genetics she investigated the effects of selecting for social breeding values for growth on behaviour and dominance relationships in pigs at the Institute for Pig Genetics B.V. (Beilen, The Netherlands). For the minor specialization Animal Nutrition she focussed on calcium and phosphorous metabolism in laying hens for which part of the research was carried out at the Poultry and Rabbit Research Centre of Nutreco B.V. (Casarrubios del Monte, Spain). In 2009, Sonja started her PhD research focussing on fiber fermentation in pig and poultry feed (Animal Nutrition Group, Wageningen University, The Netherlands), within the framework of the Carbohydrate Competence Centre. The research described in chapter 9 of this thesis was performed at the Department of Agricultural, Food, and Nutritional Science of University of Alberta (Edmonton, AB, Canada) under supervision of Prof. Dr R.T. Zijlstra. From February 2014, Sonja is employed as researcher feed evaluation at the Ingredient Research Centre of Nutreco R&D (Boxmeer, The Netherlands).

LIST OF PUBLICATIONS

Peer reviewed scientific publications

Pustjens, AM, de Vries, S, Bakuwel, M, Gruppen, H, Gerrits, WJJ, and Kabel, MA. *Unfermented recalcitrant polysaccharide structures from rapeseed (Brassica napus) meal in pigs.* Indust Crop Prod. **2014**:58, p. 271-279.

Pustjens, AM, de Vries, S, Schols, HA, Gruppen, H, Gerrits, WJJ, and Kabel, MA. *Understanding carbohydrate structures fermented or resistant to fermentation in broilers fed rapeseed (Brassica napus) meal to evaluate the effect of acid treatment and enzyme addition.* Poult Sci. **2014**:93, p. 926-934.

de Vries, S, Kwakkel, RP, Pustjens AM, Kabel, MA, Hendriks, WH, and Gerrits WJJ. *Separation of digesta fractions complicates estimation of ileal digestibility using marker methods with* Cr_2O_3 *and Co-EDTA in broiler chickens.* Poult Sci. **2014**: *accepted for publication*.

de Vries, S, Pustjens, AM, Kabel, MA, Kwakkel, RP, and Gerrits, WJJ. *Effects of processing technologies and pectolytic enzymes on degradability of nonstarch polysaccharides from rapeseed meal in broilers.* Poult Sci. **2014**:93, p. 589-598.

de Vries, S, Pustjens, AM, Kabel, MA, Salazar-Villanea, S, Hendriks, WH, and Gerrits, WJJ. *Processing technologies and cell wall degrading enzymes to improve nutritional value of dried distillers grain with solubles for animal feed – an in vitro digestion study.* J Agric Food Chem. **2013**:61, p. 8821-8828.

Pustjens, AM, de Vries, S, Gerrits, WJJ, Kabel, MA, Schols, HA, and Gruppen, H. *Residual carbohydrates from in vitro digested processed rapeseed (Brassica napus) Meal.* J Agric Food Chem. **2012**:60, p. 8257-8263.

de Vries, S, Pustjens, AM, Schols, HA, Hendriks, WH, and Gerrits, W.J.J. *Effects of processing technologies combined with cell wall degrading enzymes on in vitro degradability of barley*. J Anim Sci. **2012**:90 (suppl 4), p. 331-333.

de Vries, S, Pustjens, AM, Schols, HA, Hendriks, WH, and Gerrits, WJJ. *Improving digestive utilization of fiber-rich feedstuffs in pigs and poultry by processing and enzyme technologies: A review*. Anim Feed Sci Technol. **2012**:178, p. 123-138.

Molenaar, R, de Vries, S, van den Anker, I, Meijerhof, R, Kemp, B, and van den Brand, H. *Effect of eggshell temperature and a hole in the air cell on the perinatal development and physiology of layer hatchlings.* Poult Sci. **2010**:89, p.1716-1723.

Rodenburg, TB, Bijma, P, Ellen, ED, Bergsma, R, de Vries, S, Bolhuis, JE, Kemp, B, and van Arendonk, JAM. *Breeding amiable animals? Improving farm animal welfare by including social effects in breeding programmes.* Anim Welfare. **2010**:19 (suppl. 1), p. 77-82.

de Vries, S, Kwakkel, RP, and Dijkstra, J. *Dynamics of calcium and phosphorus metabolism in laying hens.* In: Vitti, DMSS, and Kebreab, E (eds), *Phosphorus and calcium utilization and requirements in farm animals* (p. 133-150). **2010**, CAB International, Wallingford, United Kingdom.

Conference and symposia proceedings

Pustjens, AM, de Vries[§], S, Kabel, MA, and Gerrits, WJJ. *Rigid pectin-cellulose-lignin matrix limits fermentation of canola (Brassica napus) meal polysaccharides in pigs.* In: Advances in pork production (vol 25), proceedings of the 2014 Banff Pork Seminar, Banff, AB, Canada, January 21-23 **2014**, p. abstract # 19.

de Vries[§], S, Pustjens, AM, Kabel, MA, and Gerrits, WJJ. *Hydrothermaal acid treatment of DDGS improves rate but not extent of fiber fermentation in growing pigs.* In: Advances in pork production (vol 25), proceedings of the 2014 Banff Pork Seminar, Banff, AB, Canada, January 21-23 **2014**, p. abstract # 20.

de Vries[§], S, Pustjens, AM, Schols, HA, Hendriks, WH, and Gerrits, W.J.J. *Effects of processing technologies combined with cell wall degrading enzymes on in vitro degradability of DDGS*. In: Book of abstracts of the XII International symposium on digestive physiology of pigs, Keystone, CO, USA, May 29 – June 1 **2012**, p. 141.

de Vries[§], S, Pustjens, AM, Schols, HA, Hendriks, WH, and Gerrits, W.J.J. *Effects of processing technologies and cell wall degrading enzymes on in vitro degradability of barley*. In: Book of abstracts of the XII International symposium on digestive physiology of pigs, Keystone, CO, USA, May 29 – June 1 **2012**, p. 136.

de Vries[§], S, Pustjens, AM, Schols, HA, Hendriks, WH, and Gerrits, W.J.J. *Effects of processing technologies combined with cell wall degrading enzymes on in vitro degradability of barley*. In: Proceedings of the 37th Animal nutrition research forum, Wageningen, The Netherlands, April 18 **2012**, p. 31.

Molenaar[§], R, de Vries, S, van den Anker, I, Meijerhof, R, Kemp, B, and van den Brand, H. *Effect of eggshell temperature and drilling a hole in the air cell on survival and development in layer hatchlings.* In: World Poult Sci J. 66, book of abstracts of the XIIIth European poultry conference, Tours, France, August 23-27 **2010**, p. 496.

Rodenburg[§], TB, Bijma, P, Ellen, ED, Bergsma, R, de Vries, S, Bolhuis, JE, Kemp, B, and van Arendonk, JAM. *Breeding amiable animals? Improving farm animal welfare by including social effects into the genetic model.* In: UFAW International symposium 2009 on Darwinian selection, selective breeding and the welfare of animals, Bristol, United Kingdom, June 22-23 **2009**, p. 77-82.

[§] Presenting author

TRAINING AND SUPERVISION ACTIVITIES[**]

Basic package (3 ECTS[††])

WIAS Introduction course 2009
Graduate School WIAS; Wageningen, The Netherlands
Ethics and philosophy in life sciences course 2010
Graduate School WIAS; Soest, The Netherlands

Scientific exposure (13 ECTS)

International conferences

14th Gut day Symposium 2012
Leuven, Belgium; November 9
12th International symposium on the digestive physiology of pigs 2012
Colorado, USA; May 29 - June 2
Banff Pork Seminar: Advances in Pork production 2014
Banff, AB, Canada, January 21-23
MAFIC Feed Evaluation Symposium 2014
Beijing, China; March 4-5

Seminars and workshops

35th Animal Nutrition Research Forum (ANR) 2010
Lelystad, The Netherlands; April 16
WIAS Science day (2x) 2010, 2012
Wageningen, The Netherlands
CCC Symposium (3x) 2010, 2012, 2013
Carbohydrate Competence Centre; Groningen, The Netherlands
Veetelers Symposium: Voedselproductie 'How to keep the planet alive' 2010
Ede, The Netherlands; March 11
Seminar: Dietary lysine and the importance of processing food-and feedstuffs 2010
Wageningen, The Netherlands; September 28
Seminar: Fibers for monogastrics 2010
Boxmeer, The Netherlands; February 17
Workshop Waarden van het land: 'Commissie van Doorn: en wat nu?' 2011
Moerdijk, The Netherlands, October 12
37th Animal Nutrition Research Forum (ANR) 2012
Wageningen, The Netherlands; April 18
Seminar: 100 Doctors Philosophy in animal nutrition 2012
Wageningen, The Netherlands; September 12
Symposium: Fibers in food and feed 2013
Wageningen, The Netherlands; October 31
Symposium: Healthy Food & Living Environment 2013
Wageningen, The Netherlands; December 10

[**] Completed in fulfilment of the requirements for the education certificate of the Graduate School WIAS (Wageningen Institute of Animal Sciences)
[††] One ECTS equals a study load of 28h

TRAINING AND SUPERVISION ACTIVITIES (*cont.*)

Presentations

Summer course glycosciences — 2010
Wageningen, the Netherlands; May 17; Poster presentation

12th International Symposium on the digestive physiology of pigs — 2012
Colorado, USA; June 1; Poster presentation (2x)

Animal Nutrition Research Forum (ANR) — 2012
Wageningen, The Netherlands; April 18; Oral presentation

Banff Pork Seminar
Banff, AB, Canada; January 21-23; Poster presentation (2x) — 2014

In-depth studies (6 ECTS)

Analytical work and possibilities within animal Nutrition Sciences — 2009
Animal Nutrition Group; Wageningen, The Netherlands

Summer course glycosciences — 2010
Graduate School VLAG; Wageningen, The Netherlands

Statistics of experimental design — 2011
Graduate School WIAS; Wageningen, The Netherlands

Advanced statistics for the life sciences — 2011
Graduate School WIAS; Wageningen, The Netherlands

Statutory courses (3 ECTS)

Laboratory animal science course — 2011
Radboud University; Nijmegen, The Netherlands

Professional skills support courses (4 ECTS)

PhD competence assessment — 2010
Wageningen Graduate Schools; Wageningen, The Netherlands

Supervising MSc thesis work — 2010
Docentenondersteuning; Wageningen, The Netherlands

Techniques for writing and presenting a scientific paper — 2010
Graduate School WIAS; Wageningen, The Netherlands

NWO Talent day — 2011
NWO; Utrecht, The Netherlands

Research career event — 2011
Nobiles and PNN; Utrecht, The Netherlands

High impact writing in science — 2012
Graduate School WIAS; Wageningen, The Netherlands

Research skills training (8 ECTS)

Preparing own PhD proposal — 2010
Improving utilization of fiber-rich products in pigs and poultry

External training period — 2012-2013
Department of Agricultural, Food and Nutritional Science
University of Alberta, Edmonton, AB, Canada; December 2012 – April 2013

TRAINING AND SUPERVISION ACTIVITIES (*cont.*)	
Didactic skills training (16 ECTS)	
Supervising practicals; Principles of Animal Nutrition	2009-2012
Supervising farmers project; Inleiding Dierwetenschappen	2010
Reviewing research proposals; Research Master Cluster	2010
Supervising research project, Feed Technology	2011
Guest lecture; Feed Technology	2013
Supervising and examine MSc major thesis (7x)	2010-2012
Examine MSc major/minor thesis (3x)	2010,2011
Management skills training (16 ECTS)	
Board member; PhD Candidate Network of The Netherlands (PNN)	2010,2011
Council member; WIAS Associated PhD Student (WAPS)	2011,2012
Chairman, general member, and Wageningen PhD Council representative	

Total 71 ECTS

COLOPHON

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