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Section 1

The substrate story

It is important to understand how plant fibres or non-starch polysaccharides (NSPs) are synthesised and structured when the target is to achieve effective and targeted breakdown using exogenous enzymes.

There is clear evidence of variation in the structure of the arabinoxylans which dominate cereal cell walls, as highlighted by both chemical analysis and microscopy using fluorescently-labelled inactivated xylanase.

Definitions of and analytical methods for NSPs in feedstuffs and feeds are available and clearly understood. Unfortunately, methodologies currently used to analyse soluble plant fibres do not use the extraction conditions found in the animal gut. As the plant cell wall is complex and

divergent, our knowledge of the structure and function of individual fibres in monogastrics is still limited. Nevertheless, the characteristics of fibre important to animal nutrition are influenced by feed processing, cereal type, variety and agronomic conditions.

The degree of polymerisation and branching of the xylose backbone with arabinose dictates the extent of viscosity and where xylanases can act to break down long-chain molecules. Similarly, the insoluble fibre structures vary in branching and identity of the side-chain sugars and/or phenolic compounds. Review of fibre structure alone is clearly only the first step in identifying the characteristics of feed enzymes necessary for a successful intervention.

Cellulose synthesis in higher plants

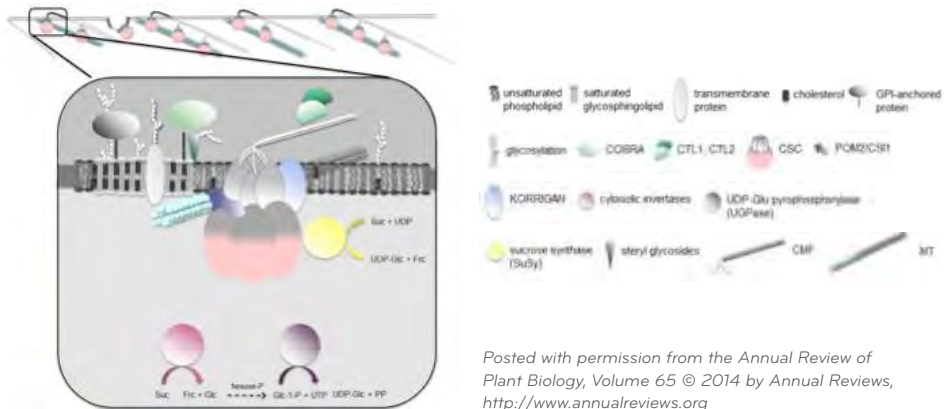
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Plant cells are encased by cellulose-containing walls that are essential for plant morphogenesis and for protection against environmental factors (Somerville *et al.*, 2004). Cellulose consists of β -1,4-linked glucan chains assembled into paracrystalline microfibrils that function as the major load-bearing elements in the cell wall. This means that the organization of the microfibrils also directs the growth of the plant cell, e.g. if cellulose microfibrils are transversely oriented the cells will expand along the longitudinal axis. Considerable evidences suggest that the cellulose chains

are synthesized by plasma membrane-located cellulose synthase (CesA) complexes (McFarlane *et al.*, in press). As the cellulose microfibrils become secured in the cell wall the synthesis of further cellulose cause the CesAs to move with constant speed in the plasma membrane. This movement is directed by an underlying cytoskeletal framework, i.e. cortical microtubules (Figure 1). The seminar at the International Non-Starch Polysaccharide forum (2014) aims at describing the current view of how cellulose is synthesized and how the CesA complex is guided by the cortical microtubules.

Figure 1. The intracellular, membrane, and extracellular environment of the cellulose synthase at the plasma membrane



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CesA speed and direction are influenced by the intracellular environment, including cortical microtubules and the supply of UDP-glucose (via Sucrose Synthase, invertase or pyrophosphorylase activity). Cellulose synthase interacting proteins, such as POM2/CS1, affect the motility and directionality of the CesAs. Extracellular proteins, such as GPI-anchored proteins, including COB, and CTL1, can also influence CesA velocity, as can nascent cellulose microfibrils and other cell wall polysaccharides.

Two types of cell walls are typically distinguished in maturing plant cells, namely primary and secondary cell walls. The primary cell walls are continuously synthesized during cell growth, whereas secondary walls typically are synthesized in certain cells that need additional support after the cells have ceased to expand. Cellulose is a major contributor to both these wall types, but the CesA complexes active during primary and secondary wall production are different. Both complexes contain CesA subunits; however, while the primary wall CesA complex contain the subunits CesA1, 3 and 6-like (Persson *et al.*, 2007; Desprez *et al.*, 2007), the secondary wall complex hold CesA4, 7 and 8 (Taylor *et al.*, 2000). Recent structural elucidations of bacterial cellulose synthase (Morgan *et al.*, 2013) and structural homology modeling of the plant CesAs (Sethaphong *et al.*, 2013) support the view that the addition of glucan moieties occur on the cytosolic side of the complex and that the incorporation of the new glucans drive the complex forward. Furthermore, Morgan *et al.*, (2013) nicely showed how certain conserved residues in the CesA protein can coordinate the substrate (UDP-glucose) and the terminal disaccharide of the glucan chain to add new glucan residues.

The CesA complexes are assembled in the Golgi, or potentially in the Endoplasmic Reticulum (McFarlane *et al.*, in press). Once assembled, the complexes are secreted to the plasma membrane where they begin synthesizing cellulose. While the secretion

system and regulatory aspects of this part of the cellulose synthesis is unresolved, it is clear that the delivery of the CesAs to the plasma membrane preferentially occur next to cortical microtubules (Gutierrez *et al.*, 2009). After an initial phase of non-motile behavior the CesAs begin to move in a steady and bidirectional fashion along the underlying microtubules. The speed with which the movement occurs has been estimated to be around 200 to 300 nm/min, with corresponds to an addition of 300 to 1000 glucose units per minutes (Paredes *et al.*, 2006). The observation that cellulose microfibrils and microtubules are arranged in similar patterns was already made in the 1960s by Green (1962) and by Ledbetter and Porter (1963). These observations led to the alignment hypothesis that stated that in many plant species and tissues the microtubules guide cellulose deposition (Baskin, 2001). However, the underlying mechanism for this principle has until recently remained obscure.

Several factors that contribute to cellulose synthesis have been obtained through forward genetic screens (Hauser *et al.*, 1995; Arioli *et al.*, 1998; Turner and Somerville, 1997). Apart from the CesAs, these studies have revealed several additional components, including POM1/CTL1, KORRIGAN, KOBITO1, COBRA and POM2/CSI1 (reviewed in McFarlane *et al.*, in press). Most of these components currently lack a clear biological function. However, recent studies have clarified the role for at least the POM1/CTL1 and the POM2/CSI1. POM2/CSI1 was

found to directly interact with the CesA proteins and therefore obtained the name Cellulose Synthase Interacting1 (CSI1; Gu *et al.*, 2010). This protein may therefore be viewed as a member of the CesA complex. Mutation in the CSI1 protein also impair cellulose synthesis and lead to slight dwarfism and cell file twisting (Gu *et al.*, 2010; Landrein *et al.*, 2013). Subsequent studies also found that the POM2/CSI1 protein can bind to microtubules (Mei *et al.*, 2012; Li *et al.*, 2012) making this protein an ideal candidate for microtubule-based guidance of the CesA complex. Indeed, when studying the coordinated action of CesAs and microtubules (dual-labelled seedlings of CesAs and microtubules using compatible fluorescent markers) in *pom2/csi1* mutant seedlings it became clear that in the absence of POM2/CSI1 the CesAs do not track along the microtubules anymore (Bringmann *et al.*, 2012a; Li *et al.*, 2012; Bringmann *et al.*, 2012b). Hence, the POM2/CSI1 protein acts as a guiding factor for the CesA complexes during synthesis.

Associations between the cellulose microfibrils and other polysaccharides, such as hemicelluloses, are important for microfibril spacing, and for maintaining cell wall tensile strength (Cosgrove, 2005). These interactions are largely affected by the action of expansins and xyloglucan endotransglucosylases that can alter the interactions between the cellulose and the hemicellulose xyloglucan and regulate the xyloglucan framework, respectively. However, recent data also suggest that the POM1/CTL1 may affect

the integration of xyloglucan chains into the cellulose microfibrils. The microfibrils typically contain regions of well-organized glucan chains, called crystalline regions, and regions with less ordered chains that are referred to as amorphous. In the latter regions it is proposed that certain other poly-saccharides can be incorporated, perhaps to integrate the microfibrils into the cell wall matrix. The POM1/CTL1 is a member of glycosyl hydrolase family 19 family and has sequence homology to chitinases, which also provided for its name; Chitinase Like 1 (CTL1). However, no catalytic activity has been found for the protein and plants do not naturally contain chitin, suggesting another function of the protein. Recent studies suggest that the POM1/CTL1 is secreted together with the CesA complex to the plasma membrane, and that the protein is deposited into the apoplast (Sanchez-Rodriguez *et al.*, 2012). Here, the protein may bind to cellulose strands and this interaction appears to affect the ratio of crystalline to amorphous ratio of the microfibrils. This, in turn, affects the xyloglucan extractability from the cell wall, suggesting that the POM1/CTL1 may control the incorporation of xyloglucans into the growing cellulose microfibrils.

Apart from the CesA proteins, these two components represent some of the best characterized proteins associated with cellulose synthesis in plants. However, tentative functions of other components are emerging. For example, KORRIGAN is an endo-glucanase possibly involved in regulating the length of the cellulose

microfibril or perhaps in substrate supply to the CesA complex (Endler and Persson, 2011), COBRA (named based on a mutant screen) is a glycosyl-phosphatidyl inositol (GPI)-anchored protein that affect cellulose crystallinity (Roudier *et al.*, 2005), and KOBITO1 encodes a protein of unknown function that is important for cellulose synthesis as mutant plants contain less than 40% cellulose as compared to wild-type (Pagant *et al.*, 2002).

It is estimated that several thousands of proteins partake in the synthesis and modification of plant cell walls (McCann and Carpita, 2008). However, only a fraction of proteins have been associated with distinct biological functions related to cell wall metabolism. For cellulose synthesis, the main bulk of components have been identified via forward genetic screening or through the identification of

genes that are co-regulated with the CesA genes (also referred to as co-expression). One important step will be to identify more gene products associated with cellulose synthesis and to put these components in context to the CesA complex. It is perhaps unlikely that this will come about via more forward genetic screens as many screens have focused on the typical mutant phenotypes (e.g. cell swelling and dwarfism) associated with cellulose defects. While the co-expression approach still offer a viable route forward, it is perhaps anticipated that protein interaction screening will provide valuable information in the near future. In this seminar I will discuss known concepts in cellulose synthesis as also summarized here, new components that affect cellulose production and give a view of what might be expected from future efforts in this field.

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Cereal cell wall structure from a botanical perspective – Microscopy

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Cereals, all belonging to the family of the grasses or Poaceae, are a major staple food worldwide and form an important source of dietary fiber. Indeed, cereal grains, and cereal cell walls in particular, are rich in arabinoxylans (AX) and mixed-linkage β -glucan. AX consist of a linear backbone of β -(1,4)-linked xylose units to which no, one or two arabinose units are attached (Perlin, 1951). Mixed-linkage β -glucan, further referred to as β -glucan, consist of a linear chain of glucose units that are mostly linked by β -(1,4)-glycosidic bonds, interrupted by single β -(1,3)-glycosidic bonds (Lazaridou and Biliaderis, 2007). Apart from AX and β -glucan, cereal cell walls may contain cellulose, glucomannan and lignin, but generally in lower concentrations (Stone and Morell, 2009). Both the concentrations and structural features of AX and β -glucan can differ strongly between cereal species and between different tissues of the same species. Similarly, their spatial distribution and content changes clearly during grain development. Microscopy forms an excellent tool to visualize these differences in a convenient way.

Several microscopic techniques are available for studying cell wall compounds and they can be grouped based on the type of interaction with the sample. Light is used for optical microscopes, whereas a probe

and electrons interact with the sample surface in scanning probe microscopy and electron microscopy, respectively. Despite the relative modest magnification levels used for optical microscopy, it covers a useful range for studying grain cell walls. Chemical stains, such as calcofluor or congo red for β -glucan, are the most straight forward stains for visualizing cell wall compounds (Autio and Salmenkallio, 2001). More specific staining of AX and β -glucan can be obtained when antibody probes are used for specific recognition (Gullion *et al.*, 2004) (Ordaz-Ortiz *et al.*, 2011). Finally, also labeled enzymes that bind solely to the cell wall compound of interest are used as a selective probe. Hereunder, we describe the use of a fluorescently labeled xylanase to visualize AX. Furthermore, AX and β -glucan distribution were studied in cell walls of wheat kernels at different stages of grain development. Finally, AX staining with fluorescently labeled xylanase was compared with immunolabeling of AX in cell walls of different types of mature cereals (wheat, barley, oat and rye). Similarly, calcofluor staining of β -glucan was compared with β -glucan immunolabeling for the four different cereal types.

AX labelling with an inactive fluorescently labeled xylanase

Bacillus subtilis xylanase (XBS) was labeled with an Alexa Fluor488 C₅ dye and its use for AX staining was optimized. Its practical use was validated by examining stained wheat sections with epifluorescence microscopy (Dornez *et al.*, 2011). Since the wild-type XBS contains no cysteine residue, introduction of a sole cysteine residue by site-directed mutagenesis enabled a 1:1 labeling of the enzyme. The most intense signal was obtained when the xylanase was inactivated by creating a mutation in the active site. The signal with the wild-type xylanase was lower, probably because the active enzyme degraded AX during staining. The xylanase probe stained AX in wheat seed coat, nucellar epidermis, aleurone layer and starchy endosperm, but not the highly substituted AX of the pericarp layer. The latter was likely caused by the high arabinose to xylose ratio in the pericarp AX which may hamper xylanase binding to the xylan chain.

Study of grain cell wall structures by microscopic analysis in developing wheat kernels

The evolution of wheat cell wall polysaccharides was investigated during kernel development, both through quantification of the levels of the different compounds and through visualization of their spatial distribution using fluorescence microscopy (Verspreet *et al.*, 2013). The results were compared to these of other important kernel components such as storage carbohydrates and proteins. During

the first phase of grain development, the phase of cell division and expansion, grains were characterized by a rapid accumulation of water and high concentrations of the water soluble carbohydrates fructan, sucrose, glucose and fructose.

AX was not present in the kernels of the first days after anthesis (DAA) in contrast to β -glucan (Figure 1 A to 1C). At 5 DAA, calcofluor clearly stained the cell walls of the maternal pericarp and the highest β -glucan concentrations (1.58 ± 0.12 %) were measured at 9 DAA. AX accumulation took place during the second phase of grain development when grain filling occurred and large amounts of starch and protein were deposited (Figure 2A to 2F). During this phase, the most intense AX staining was observed for the nucellar epidermis and AX were not seen in the maternal outer tissue of the pericarp. β -Glucan could be labeled during grain filling in the endosperm cell walls, especially those close to the crease region and a clear calcofluor staining of the nucellar epidermis was observed (Figure 1D to 1F). However, in the mature kernels (Figure 1G to 1I), only a weak signal was observed in the nucellar epidermis and likewise the signal in the outer pericarp had almost disappeared. In the endosperm, the thick aleurone cell walls were the most prominently stained. The final three weeks of development were characterized by moisture loss and an almost constant grain dry matter composition. Based on these results and literature data, different functions may be attributed to β -glucans and AX in developing wheat kernels. β -glucans appear to be the structural elements of the

early cell walls of growing cells whereas AX might strengthen the walls of cells that have reached or almost reached their final size.

Figure 1. Fluorescence micrographs of immature grain sections stained with calcofluor
Fluorescence micrographs of grain sections stained with calcofluor, 5 DAA (A, B and C), 16 DAA (D, E and F) and 57 DAA (G, H and I). Bars measure 1000 μm (B, E and H), 200 μm (A, D and G) or 50 μm (C, F and I). Nucellar epidermis (NE) and aleurone cells (AC) are indicated. The large and small rectangles in image B, E and H indicate the zones in which image A, D and G and C, F and I, respectively, were taken.

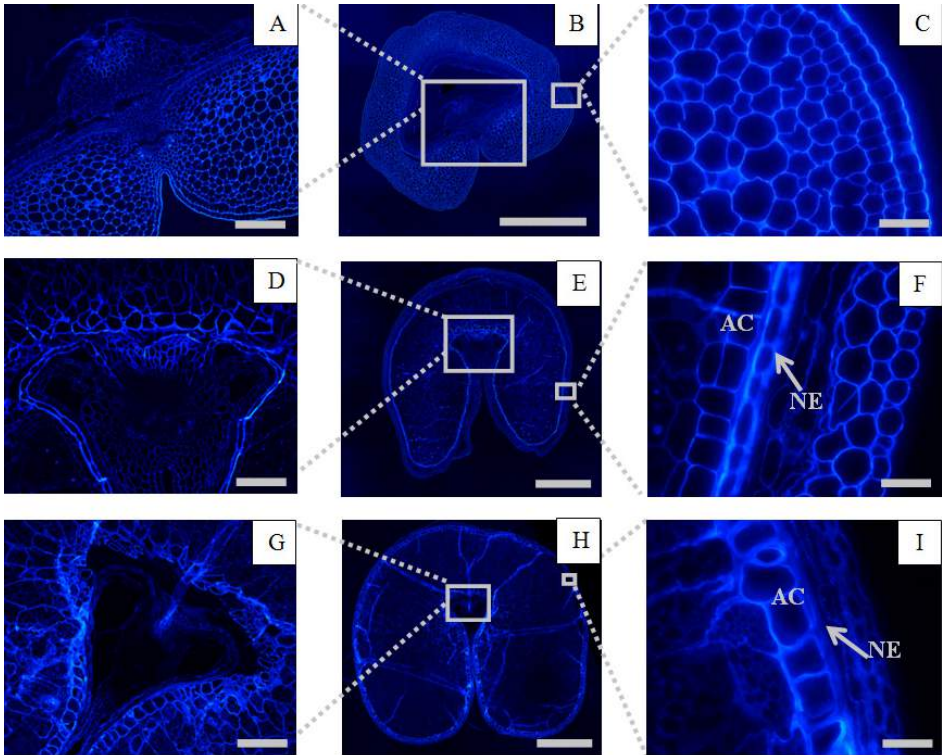
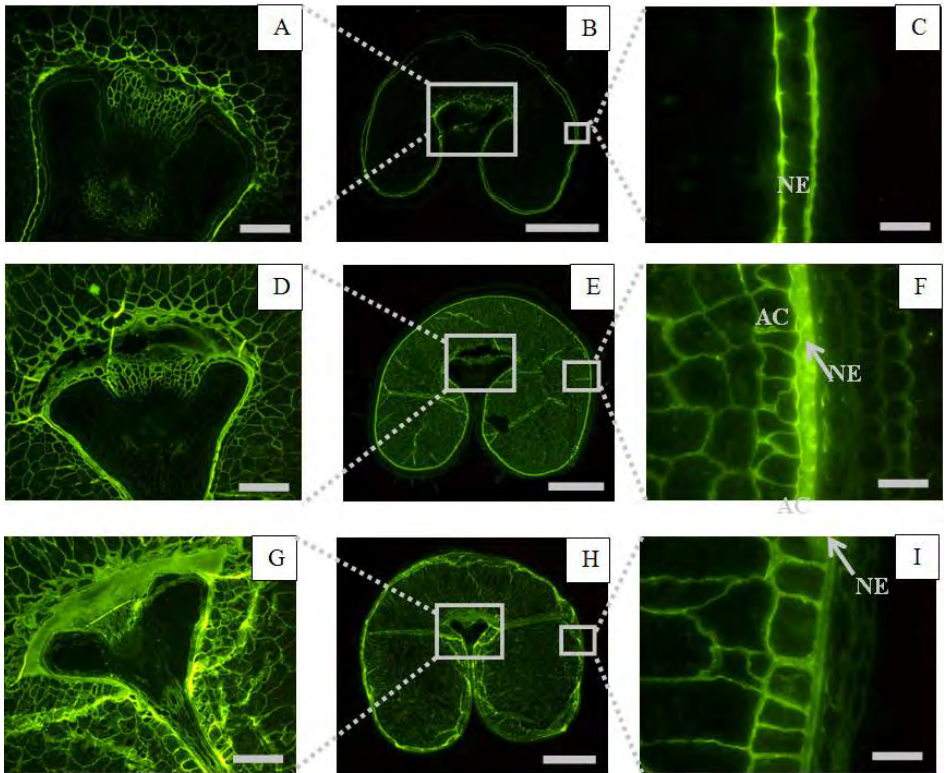


Figure 2. Fluorescence micrographs of immature grain sections stained with inactive fluorescent labeled xylanase, 12 DAA (A, B and C), 28 DAA (D, E and F) and 57 DAA (G, H and I). Bars measure 1000 μm (B, E and H), 200 μm (A, D and G) or 50 μm (C, F and I). Nucellar epidermis (NE) and aleurone cells (AC) are indicated. The large and small rectangles in image B, E and H indicate the zones in which image A, D and G and C, F and I, respectively, were taken.



Study of grain cell wall structures by microscopic analysis with four different staining techniques

The newly developed XBS based staining technique described above was compared with immunolabeling of AX with monoclonal antibodies. Besides, β -glucan staining with calcofluor was compared with immunolabeling using monoclonal antibodies (Dornez *et al.*, 2011). The staining of cell wall polymers by these four

techniques in wheat, barley, rye and oat grains is summarized in Table 1. None of the AX labeling techniques stained pericarp, but the xylanase staining gave generally a stronger signal. Another difference between the two techniques was that AX antibody labelling was more species dependent than the xylanase labelling. It is however difficult to generalize these results, obtained with only one specific antibody, to immunolabeling in general as each antibody has its own

specificity. Indeed, each antibody has its own capacity to recognize AX with distinct degrees of arabinose substitution and the same can be expected for different xylanases. Differences were also observed between calcofluor and antibody based β -glucan staining (Table 1). Both techniques allowed to stain the cell walls in (sub) aleurone cells but the starchy endosperm cell walls and pericarp of wheat could only

be visualized with the β -glucan antibody. Hence, once again it can be inferred that some differences occur because of the different probe specificities. This should be taken into account when a staining technique is selected together with the amount of workload and time needed for producing the stain and the staining itself. Indeed, the antibody based staining is generally much more time consuming.

Table 1. Staining of the cell walls of the grain tissues by the different staining techniques

	XYLANASE PROBE	LM11 AX ANTIBODY	ACID FUCHSIN – CALCOFLUOR	β -GLUCAN ANTIBODY
Pericarp	-	-	-	+/-
Seed coat and nucellar epidermis	+++ wheat/oat/rye ++ barley	++ wheat/oat/rye + barley	-	+/-
Aleurone layer	+++ wheat/oat/rye ++ barley	+++ barley +/- other grains	++	+++ wheat/rye + barley/oat
Subaleurone layer	++ wheat/barley/ rye + oat	+ wheat - other grains	+++ oat ++ other grains	+++ oat ++ other grains
Starchy endosperm	++ wheat + other cereals	+ wheat - other grains	+++ barley/rye ++ oat +/- wheat	+++ barley/rye ++ wheat/oat
Transfer cells	-	-	-	-
Nucellar projection	+++	+	+	-

- no staining
- + limited staining
- ++ moderate staining
- +++ strong staining

When staining was compared between different cereal types, an intense AX staining was noted in the seed coat and nucellar epidermis of all grain types (Figure 3). This can be explained by the high AX concentrations in these tissues and their low arabinose:xylose ratios. Yet, the nucellar epidermis was markedly thicker in wheat and rye than in barley and oat. In contrast to AX, β -glucan was not stained in the

nucellar epidermis whereas the subaleurone cell walls were intensively stained in all grain types (Figure 4). The aleurone layer of barley consisted of two to four cell rows and so clearly differed from the aleurone of the other cereals, having only one aleurone cell row. In the case of barley, oat and rye, the starchy endosperm cell walls gave a more pronounced signal after β -glucan staining than after AX staining.

Figure 3. Outer kernel layers, central starchy endosperm and crease region of kernel cross sections of wheat, barley, oat and rye stained with the inactive fluorescently labeled xylanase probe

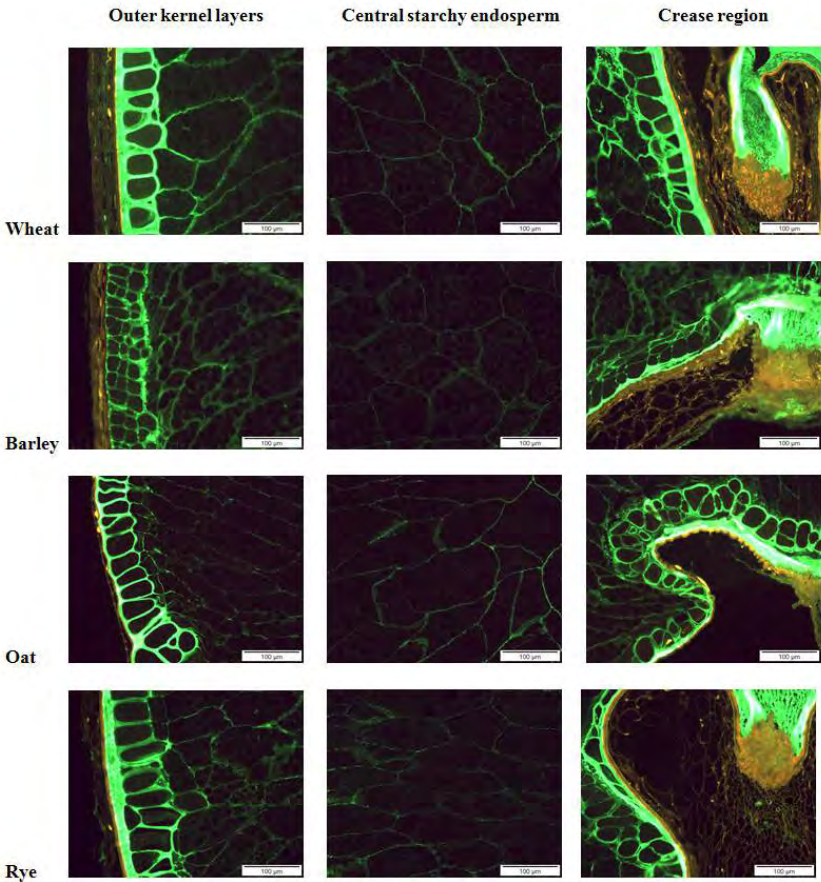
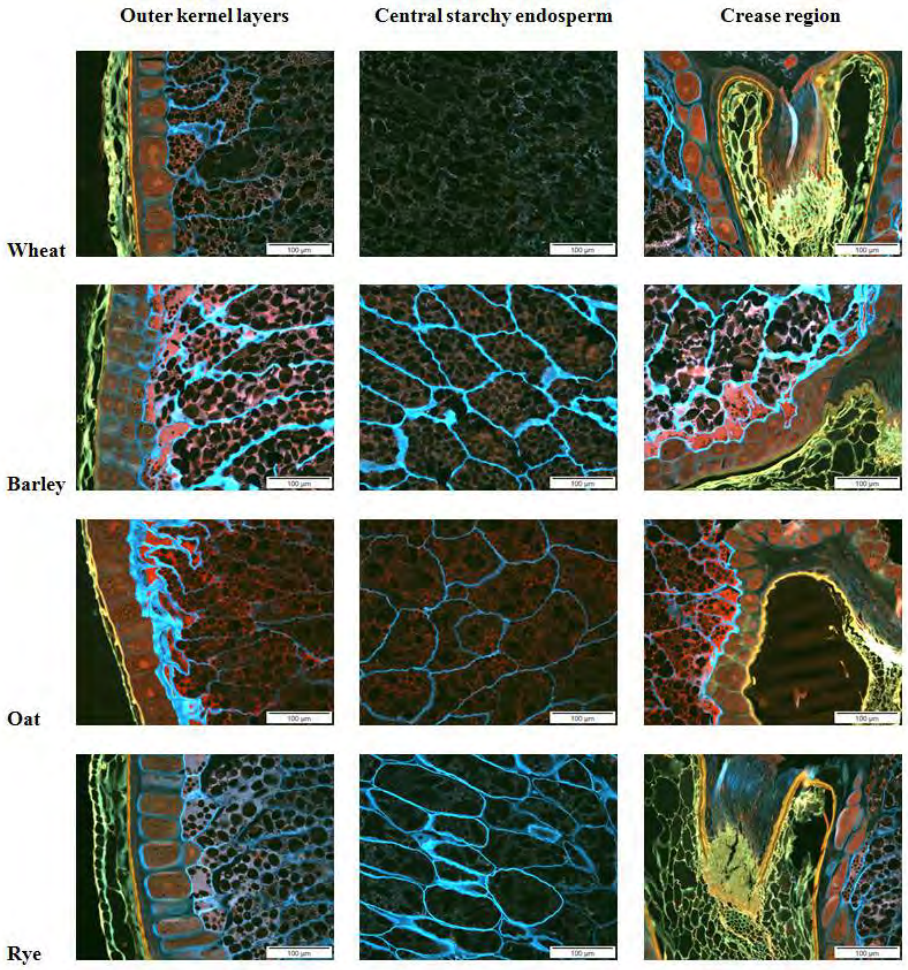


Figure 4. Outer kernel layers, central starchy endosperm and crease region of kernel cross sections of wheat, barley, oat and rye stained with Acid Fuchsin and Calcofluor



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Fibre and xylanases in baking

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Introduction

In the baking industry the main interest in the application of hemicellulase enzymes is not to increase the nutritional functionality of the products of cereal grain milling, rather it is to use less biologically obvious reactions which deliver significant technological functionality in the manufacture of bread. It may be that there are some direct and beneficial nutritional changes for human beings, but these are perhaps of less consequence than is the case with animal nutrition. To understand the technological importance of hemicellulases in the bakery we have to first understand a little of how bread is made.

The cereal chemist describes the baking process as being one of creating a foam and by employing various processing steps converting that foam to a sponge (Cauvain, 2012). The foam is created when flour, water, yeast, salt and a few other functional ingredients are mixed together. The main proteins of wheat flour, the glutenins and the gliadins, swell when they come into contact with water and with progressive mixing of the ingredients a smooth homogenous mass is formed with a mixture of viscous and elastic properties (Stauffer, 2007).

The transition at this stage is referred to as the development of a gluten network and key

amongst the various properties of gluten is its ability to trap and retain small air bubbles in the dough. It is the presence of these tiny air bubbles, commonly less than 150µm in diameter which is crucial to establishing the initial foam. Not least because there are no significant opportunities to introduce more air bubbles once the dough has formed because the rheological properties of the gluten network largely prevent this occurring.

Thus once mixing is completed the key properties of the foam are already in place and in some ways the final bread quality has already been decided even though there is much more processing to undertake before baking is complete. To put this into context dough mixing takes about 10 min while processing continues for around another two hours. From the bulk dough formed at mixing, smaller individual pieces are cut out and moulded into various shapes. At this stage we are still dealing with a foam in the dough and little happens to the air bubbles. However, bakers actively avoid damaging the gluten network otherwise they compromise final bread quality. The individual pieces are placed in a warm, moist environment to encourage fermentation by the yeast in the dough.

As carbon dioxide is released into the dough it migrates into the small, trapped air bubbles, they grow in size and the dough

rises. Eventually the bubbles become large enough to touch one another and coalescence occurs. This is not a simple process, but essentially bakers seek to limit coalescence of gas bubbles before the end of the process they call proof (the fermentation period). Too many factors control bubble expansion and coalescence to be covered in this brief overview, but there is a powerful role for fibre-rich materials as discussed below.

As the dough moves into the oven the foam to sponge conversion occurs as a warm front moves from the crust with the transfer of heat to the dough piece centre by conduction. Expansion and coalescence of the gas bubbles proceeds rapidly and as the gluten network loses its ability to limit bubble coalescence expansion of the dough stops and the embryonic loaf structure is formed. Now the gas cells are open and connected, they are much larger in size, typically for 0.5 to 3 mm or larger and they are far fewer in number. In a typical slice of bread we see about 10,000 cells, some estimates suggest that this number represents only 10-20% of the air bubbles that were present in the starting dough.

Flour type and bread quality

White flour is relatively low in fibre as the flour milling process has been geared to removing as much of the wheat bran as possible (Catterall and Cauvain, 2007). We can debate the virtues or otherwise of using white flour to make bread but it remains a fact that the vast majority of bread produced and eaten around the world is based on white flour and that is the choice

that consumers make. Raising the fibre content of bread presents no problem, we simply use wholemeal flour which is 100% of the grain milled to flour. However, there is a penalty in that in many cases and for many years, wholemeal bread was far denser than white bread and lacked appeal to many sectors of the population. As we developed a better understanding of the role of fibre in the human diet and the demand for fibre-rich breads increased, innovation by bakers was to make a wider range of non-white breads available and sales of such products dramatically increased as the products became less dense, had a softer crumb and kept softer longer.

Now we have to be careful as we unravel the role of fibre in breadmaking and recognise that all bread production in the UK is governed by a short, positive list of permitted ingredients and that ingredient additions are not solely responsible for the improvement in bread qualities discussed below. In bread making the key contributors to quality come from a series of complicated interactions between ingredients, recipe and process. We must also be aware that the manufacture of bread from wholemeal flour is based on an even shorter list of permitted ingredients than we can use for white bread.

Apart from improving the function of our bowels what is it that fibre does in bread? To answer this question we have to descend to the microscopic level in the dough to see what its structure looks like. In the dough we would see the air bubbles trapped in the gluten network showing as roughly spherical dots of different sizes. The continuity of



the gluten network is critical for retaining the air bubbles and accommodating their expansion during fermentation. It appears that the starch granules in the flour are mostly stuck onto the surface of the gluten strands and do not greatly interfere with extensibility of the gluten network and its expansion during fermentation.

Particles of fibre on the other hand do reduce the overall ability of the dough to expand, especially when the dough enters the oven and the expansion potential is at its greatest. It has been postulated that the bran particles which are main source of fibre from wheat, puncture the gas bubbles and release the trapped gas. It is difficult to imagine that a hydrated and therefore soft particle could achieve this effect and it is more likely that the main effect comes from the bran particles constituting points of weakness in the gluten network which permit the coalescence of gas bubbles in the dough at an earlier stage in the oven baking process.

Non-starch polysaccharides and water in dough

The wheat flour components of greatest concern in this paper are often grouped under the general heading of non-starch polysaccharides (NSPs) and it is they which will provide the substrate for the class of enzymes known as hemicellulases. The NSPs includes the pentosans which constitute around 1-3% of white flour (Stauffer, 2007). There are roughly equal quantities of water-soluble and insoluble pentosans in the flour. The general view is that the pentosans, more specifically the

arabinoxylans, form a network with the gluten-forming proteins and have an overall negative effect on the development of a network with optimum rheological properties for dough processing. In part this is because the pentosans are able to absorb much larger quantities of water than all of the other major flour components when considered on a weight for weight basis.

For bakers there is little that they can do in the flour specification to limit the level of pentosans which are present since the level of NSPs are a consequence of wheat choices made by the miller and the specific set-up of the mill. In the past millers themselves have not been greatly concerned with pentosan levels in white flours but with more recent changes in milling practices more attention is being paid to their presence. In practice there is not a lot that current milling practices can do to limit or enhance flour pentosan levels because the complex shape of wheat grains and its troublesome crease make it difficult to deliver in a commercial context the purity of component separation that might be ideal. The complications arising from the milling of wheat into white flour predicate that it is the baker and specialist ingredient supplier who are best placed to exploit the naturally occurring pentosans in wheat flour and this is where xylanases play their part.

The critical role that water plays in determining the final quality of bread products has long be recognised (Cauvain and young, 2008). Most commonly it is related to the rheological nature of the dough and what bakers loosely term its

consistency. In general terms bakers seek a dough which is easily changed in shape during processing but which retains a cohesive and extensible gluten network so that control of the bubble coalescence mechanism is optimised. One of the consequences of not having the appropriate dough rheology during dough processing is that damage to the relatively delicate gluten network can make a direct contribution to the occurrence of large and unwanted holes in the crumb of the product (Cauvain and Young, 2008). In the later stages of the process the mobility of water between dough components in the oven is important in controlling final bread volume and cell structure.

In this context it has long been known that the role of enzymes is very important. One of the first identifications in the 1980s of the importance of such actions was for fungal *alpha*-amylase (Cauvain and Chamberlain, 1988) when it was observed that as a result of the breakdown of the starch from the amylase action, the water released from the starch was taken up by the gluten which allowed it to stretch further and 'set' a little later in the process. The net effect was to increase loaf volume and contribute to improved cell structure and crumb softness.

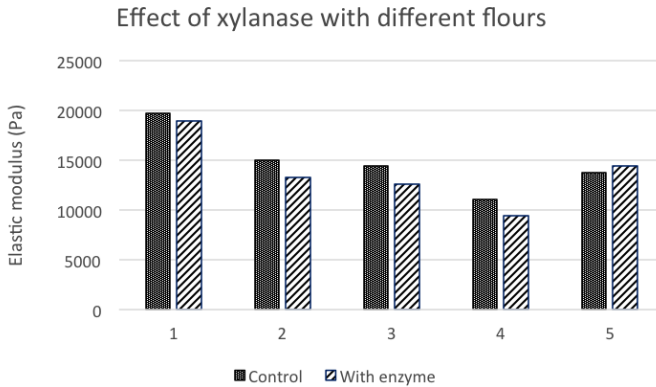
Xylanases in bread baking

In baking probably the most commonly enzymes emerging from the hemicellulase group are the xylanases and in particular the endo-xylanases which cleave the xylose backbone from within (Kornbrust *et al.*, 2012). Claims are made for modification of dough handling properties with additions of

xylanase, in part as a result of the xylanase degrading the insoluble fraction of the pentosans rendering them soluble. As a result of such actions there is an increase in the quantity of smaller sized molecules and a loss of water-binding capacity. Far from being a negative effect it is likely that the loss of water binding capacity in the pentosans works in favour of the gluten network and contributes to its greater extensibility, especially in the oven, and so the net effect is comparable to that of *alpha*-amylase. One of the practical problems with using xylanases is that their particular effectiveness varies depending on the microbial source from which the enzyme is derived along with variations in flour characteristics, recipe and dough making procedure and so it is often hard to be definitive about the quality contributions that xylanase make to bread quality.

The effect of a standardised xylanase addition on the rheology properties of different flours is illustrated in Figure 1 which shows the impact on the elastic modulus of the dough; in most cases the elastic modulus falls slightly though in the case of flour 5 there is no change. While dough is commonly described as being visco-elastic, too much elasticity can be a problem as bakers have to increase the force required for the final shaping of the dough to compensate for any elastic regain in the dough. An unfortunate consequence of subjecting dough to excess pressure during shaping is that the gluten network may become damaged and this may lead to the formation of unwanted large holes in the crumb (Cauvain and Young, 2008).

Figure 1: Effect of xylanase addition on dough rheology (elastic modulus)

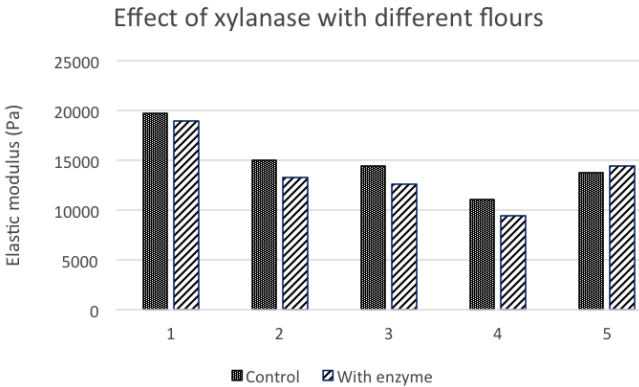


Source: Sahi, 2002

As shown in Figure 2 the addition of xylanase has brought about an immediate increase in loaf volume but that further increases in the level of addition actually resulted in bread volume falling. The results show just how sensitive the response to enzyme addition can be in breadmaking. While a response of only 80ml in volume (around 2.6% increase) would be considered small, if we look at commercial practice we can begin appreciate why bakers would be concerned. Many loaves in this country have a rectangular shape because they are baked in a pan with a lid to retain that shape. You will be familiar with the type of bread if you ever buy a sandwich on your travels

round the UK. The sandwich assemblers give the baker a tight specification with regard to the dimensions of the slice cross-section because they want the triangle of the sandwich to fit neatly into the pack. Since the dough is constrained by the pan during baking the only way that it can really expand is upwards so that variations in measured volume are really related to variations in loaf height. So if a specification says that the loaves should be 145mm high, then a 2.6% variation in height would be around 4mm, that is from 143-147mm and in practice sandwich assemblers restrict variation to a range of 5mm or so, so very close to the example given.

Figure 2: Effect of level of xylanase addition on bread volume

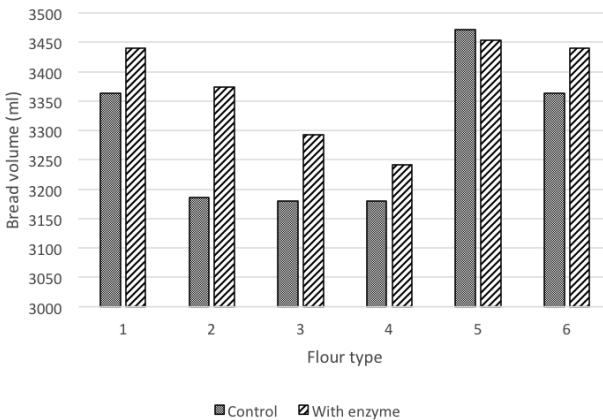


Source: Sahi, 2002

The relationships between flour properties and ingredient additions in baking are complex. In Figure 3 the results show the effect of adding a constant level of xylanase with different flours on loaf volume is shown. When we consider the percentage change resulting from the change from one flour

to another we can see that this is around 6% and would most certainly this change in flours supply would take me outside of the specified height range for my sandwich making customer unless I compensated with a change in xylanase level for example.

Figure 3: Effect of level of xylanase addition on bread volume with different flours



Source: Sahi, 2002



The relationship between effects of xylanase and flour in baking is further complicated by the presence of endogenous xylanase inhibitors in wheat and this inhibitory effect will almost certainly carry through to baking and may go some way to understanding why there are variations in the reported effects of xylanase additions. Some of the other variability in results which have been reported may be attributed to the source of the xylanase, particularly the microorganism used in the initial manufacturing process. The practicality of using xylanase addition in baking is that it is seldom if ever, used as the sole enzyme addition. Probably all of the so-called bread improvers which are used in the baking industry will contain a mixture of different types of enzymes and in some cases, different types (sources) of the same enzyme.

Conclusions

Current EU legislation classifies enzymes as processing aids since they do not survive the baking process unaltered. This means that they do not need to be declared on the product label as an ingredient and this has

given rise to the concept of so-called clean label improvers. I should emphasise that this concept has no legal standing but has become a driving force for change in bread improver composition. There is a negative in this approach for the baker; namely that they often have no idea as to what enzyme they are adding and consequently they do not always know what the effect will be in their product. We all know that the activity of enzymes depends on the availability of a substrate and the conditions under which it is operating, such as pH, temperature and how long it has to act. Commercial baking is not a precise science and so problems occur when all the facts are not known. Xylanases and many other enzymes have important role in delivering bread of the qualities required by modern consumers but as with many other materials used in baking, the use of xylanase is not straightforward and requires not just an understanding of the chemistry involved but also the contribution of those chemical changes to the interaction of dough with the processing technologies employed in baking.

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Analysis of dietary fibre – Relevance to animal nutrition

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Introduction

Crude fibre, acid detergent fibre (ADF), neutral detergent fibre (NDF) and more recently dietary fibre have been important analytes for feed characterization. In the traditional analysis system the old crude fibre method is used (Henneberg and Stohmann, 1859). The sample is sequentially extracted with acid and alkali, and the dry matter of the residue is reported as crude fibre (AOAC 962.09). Many of the components included in modern dietary fibre definitions are lost during the extractions with acid and alkali and therefore the method results in very incomplete accountings for total dietary fibre. However, the method is still used for feed characterization due to long experience about its relation to the feeding value. Another early approach was the development of detergent methods. The ability of detergent solutions to solubilize fat, nitrogen-containing compounds, simple sugars and some starch, under relatively mild conditions, is the basis for these methods. Two analytes, ADF, containing mainly cellulose, lignin, acid insoluble ash and silica, and NDF, including cellulose, hemicellulose and lignin as major components, are commonly used for feed characterization (Van Soest *et al.*, 1991). Lignin in ADF can be determined after acid hydrolysis or by difference after oxidative degradation. The two detergent methods are widely used in

ruminant nutrition; however, they exclude important components included in the modern definitions of dietary fibre and therefore have limited value for dietary fibre estimations of human foods and monogastric feeds.

Dietary fibre is a diverse group of molecules with varying degrees of solubility, molecular size and structure, which will influence rheological properties of the gastrointestinal content, flow of digesta and the digestion and absorption process (Bach Knudsen, 2001). In the stomach and small intestine the effect is essentially a physical one, where fibre acts as a barrier to the release of nutrients or increase viscosity of the liquid phase and form gel and thereby restricting nutrient absorption. In the large intestine, dietary fibre is degraded by the microflora to a variable degree, depending on fibre solubility, structure and cross-linking. Today effects of fibre structure on microbiota composition, gut hormones, gut-brain signalling, metabolism, satiation and satiety are important research areas.

In this overview we will mainly deal with more modern approved method for dietary fibre analysis and characterization.

Definition of dietary fibre

The term dietary fibre was introduced by Hispley in 1953 (Hispley, 1953). In Europe

(EU 2008/100/EG), dietary fibre was defined in 2008 as carbohydrate polymers with at least 3 sugar units which are not digested nor absorbed in the small intestine of humans. This is a broad definition that includes many types of carbohydrates of different origin, such as resistant oligosaccharides with 3-9 sugar residues and resistant polysaccharides with more than 9 sugar residues. Resistant polysaccharides contain plant cell wall polysaccharides and certain polysaccharides that are not present in the wall, for example resistant starch and fructan. Compounds of non-carbohydrate nature associated to the carbohydrate polymers are included in the definition if included in the analyses. Lignin is one of these components and it can significantly influence both dietary fibre content and properties.

Internationally, *Codex Alimentarius* has launched a similar definition for dietary fibre (CAC/GL 2-1985, revised 2010), following protracted discussions spanned over 16 years. In this definition it is, however, up to the national authorities to decide if resistant oligosaccharides with 3-9 sugar residues should be included in the definition or not.

An exact definition of fibre is very important since it will dictate method development and the choice of analytical method, and it is therefore unfortunate that the European and International definitions differ.

Methods for determination of soluble, insoluble and total dietary fibre

The European Commission has recently (December 2012) launched an evolving

guidance document with regard to methods of analysis for determination of the fibre content declared on a food label. Methods are divided into:

1. General methods that measure both higher (more than 9 sugar residues) and lower (3-9 sugar residues) molecular weight fractions
2. General methods that do not measure the lower molecular weight fraction
3. Methods that measure individual specific components

In the first group, the most promising method is AOAC 2009.01 which was developed in accordance with the European and *Codex* definitions (McCleary *et al.*, 2010). The method quantifies both high- and low-molecular weight dietary fibre components, and includes extended enzymatic digestion at more physiological conditions to simulate human intestinal digestion of starch followed by liquid chromatography with RI detection for the resistant oligosaccharides. Ideally, the method thus quantifies the complete range of dietary fibre components included in the new definitions and therefore in many cases should give higher values compared to the classical methods. This method has also been updated to include separate quantifications of soluble, insoluble and total dietary fibre. (McCleary *et al.*, 2012).

The second group includes two of the most commonly used methods. AOAC 985.29 (the Prosky method) for the high-molecular weight fractions of the dietary fibre. Samples are firstly incubated at ~ 95°C in a phosphate buffer containing

thermo-stable alpha-amylase. pH is then adjusted, protease added and samples incubated at 60°C and then adjusted again and incubated with amyloglycosidase at 60°C. Available carbohydrates have now been solubilized and degraded and the total dietary fibre content can be obtained after ethanol precipitation of soluble fibre components, filtration and drying. Duplicate samples are always processed, allowing the subtraction of protein and ash for calculation of the total dietary fibre content. This method does not include the low molecular weight fraction of the dietary fibre and only part of the resistant starch, and should not be used when these fibre components are present in significant amounts. Some years later the method was modified for separate determination of soluble and insoluble dietary fibre (AOAC 991.43, the Lee modification). This modification has the same limitations as discussed above.

In a recent paper, empirical and rational methods for the measurement of dietary fibre, including methods AOAC 991.43 and AOAC 2009.01, were compared using real and model foods (Englyst *et al.*, 2013). The main conclusion was that for most samples the two methods gave similar results, which may be due to a low content of resistant oligosaccharides in the samples and that retrograded amylose was the only major resistant starch present in most samples analysed. They also concluded that the rational method (AOAC 2009.01) is useful in identifying specific carbohydrate constituents.

Total dietary fibre contents in wheat grain-

based products have been determined with both AOAC 985.29 and AOAC 2009.01 (Brunt and Sanders, 2013). They found a good agreement between the two methods for the high molecular weight dietary fibre fraction. With AOAC 2009.01, however, 1-3 % of low molecular weight dietary fibre was also determined, but some of this was found to be un-hydrolysed starch or malto-oligosaccharides. By introducing an extra amyloglucosidase hydrolysis steps in the method, these oligosaccharides were fully hydrolysed and this resulted in lower contents of this analyte. In another study, the two methods for total dietary fibre were compared on fifteen bread and bakery products (Hollman *et al.*, 2013). They also found significantly higher values with AOAC 2009.01 due to high levels of the low molecular weight fraction. These studies thus indicate that caution should be taken when analysing this type of samples.

The AOAC 2009.01 method is relatively new and food and feed tables have therefore generally not been updated with results from this method. It has been suggested that these tables need to be developed to include both "classic" and new data since re-analysing all feeds and foods on a short notice is impossible due to financial restrictions (Westenbrink *et al.*, 2013).

The second group also contain AOAC 994.13, the Uppsala method). This method includes preparation of a residue after treatment with thermostable alpha-amylase and amyloglucosidase and ethanol precipitation of solubilized dietary fibre components while leaving low-molecular weight carbohydrates

in solution. By centrifugation, soluble and insoluble components can be fractionated and analysed separately (Anderson *et al.*, 1999). The residue is hydrolysed in two steps, neutral polysaccharide residues are quantified as alditol acetates by gas-liquid chromatography, uronic acid residues by colorimetry and the ash-free acid insoluble residue (Klason lignin) is determined gravimetrically (the non-carbohydrate part of dietary fibre). With this method, total dietary fibre, including starch resistant to the amylase treatments used, is calculated as the sum of analysed polysaccharides and Klason lignin. Fructo-oligosaccharides and fructan are completely destroyed during the two acid hydrolysis steps. For cereals, fructo-oligosaccharides are the major resistant oligosaccharides and therefore total dietary fibre according to the new European definition can easily be determined by adding the value from a separate determination of these components to the result from the Uppsala method. Results for total dietary fibre from the Uppsala method have been shown to correlate well with corresponding results determined with AOAC 991.43. The Uppsala method gives information about the total content of dietary fibre and also

the constituents of the dietary fibre. In many cases this is important information, for example when effects of processing and changes during fermentation are followed.

The third group includes several methods for separate analysis of specific dietary fibre components. In the Uppsala laboratory we use method AOAC 995.16 for mixed-linked β -glucan analysis and method AOAC 999.03 for analysis of fructo-oligosaccharides and fructan.

Some application examples

Winter wheat samples from different cultivars and breeding lines were analysed with the Uppsala method and AOAC 999.03 for fructo-oligosaccharides/fructan (Andersson *et al.*, 2013). Average content of total dietary fibre in whole grain wheat was 13.4% with a range of 11.5-15.5% (Table 1). The dominating dietary fibre component was arabinoxylan, with an average content 6.5 % and a variation from 5.5-7.4%. The content of glucose residues, from cellulose and β -glucan but not resistant starch since the samples were not heat-treated, was on average 2.9%, of which the majority was cellulose (average 2.1%).

Table 1: Contents of total dietary fibre and dietary fibre components in 129 winter wheat varieties from the European HEALTHGRAIN project (% DM) (Andesson *et al.*, 2013).

COMPONENT	RANGE	MEAN
Total dietary fibre ^a	11.5-15.5	13.4
Arabinose residues	2.11-2.95	2.52
Xylose residues	3.58-4.88	4.20
Mannose residues	0.31-0.56	0.39
Galactose residues	0.28-0.40	0.33
Glucose residues	2.39-3.71	2.85
Uronic acid residues	0.41-0.61	0.50
Klason lignin	0.74-2.03	1.33
Fructooligosaccharides/fructan ^a	0.84-1.85	1.28

^aTotal dietary fibre calculated as the sum of dietary fibre analysed with AOAC 994.13 and fuctan analysed with AOAC 999.03

Winter rye samples harvested in Sweden were analysed for total dietary fibre and dietary fibre components as for wheat above (Andersson *et al.*, 2009). Average

content of total dietary fibre was 19.9% in whole grain rye with a range from 18.7-22.2% (Table 2).

Table 2: Contents of total dietary fibre and dietary fibre components in 18 winter rye varieties grown in Sweden (% DM) (Andesson *et al.*, 2009).

COMPONENT	RANGE	MEAN
Total dietary fibrea	18.7-22.2	19.9
Arabinose residues	2.8-3.9	3.3
Xylose residues	4.7-6.4	5.6
Mannose residues	0.6-0.7	0.6
Galactose residues	0.3-0.5	0.4
Glucose residues	3.9-5.0	4.4
Uronic acid residues	0.2-0.3	0.2
Klason lignin	0.9-1.4	1.1
Fructooligosaccharides/fructan ^a	3.6-4.6	4.1

^aTotal dietary fibre calculated as the sum of dietary fibre analysed with AOAC 994.13 and fuctan analysed with AOAC 999.03

This is significantly higher than for wheat and as much as 37% of the dietary fibre components are soluble. Arabinoxylan was the dominating dietary fibre component also in rye with an average content of 8.9%. The average content of glucose residue was 4.4% and that of fructo oligosaccharides/fructan 4.1%.

In the next example, whole grain triticale harvested in Sweden were analysed for dietary fibre components with the Uppsala method as well as for fructo-oligosaccharides/fructan and β -glucan

(Rakha *et al.*, 2011). Average content of total dietary fibre was 14.5% and arabinoxylan was also in this case the dominating polysaccharide (average content 6.7 %), followed by fructo-oligosaccharides/fructan, cellulose, β -glucan and arabinogalactan. Klason lignin was also present in significant amounts (Table 3). The average content of dietary fibre in triticale was between that of wheat and rye, although more similar to wheat.

Table 3: Contents of total dietary fibre and dietary fibre polysaccharides and Klason lignin in 8 winter triticale varieties grown in Svalöv, Sweden (% DM) (Rakha *et al.*, 2011).

COMPONENT	RANGE	MEAN
Total dietary fibre ^a	13.4-15.6	14.5
Arabinoxylan	5.9-7.4	6.7
β -glucan ^b	0.6-1.0	0.7
Fructooligosaccharides/fructan ^a	1.6-2.9	2.2
Cellulose	1.8-2.5	2.1
Arabinogalactan	0.16-0.23	0.20
Klason lignin	1.4-1.7	1.5

^aTotal dietary fibre calculated as the sum of dietary fibre analysed with AOAC 994.13 and fructan analysed with AOAC 999.03

^b β -glucan was analysed with AOAC 995.16

Relevance of dietary fibre analysis in feed analysis and animal nutrition

These recent developments in dietary fibre analysis methods, for example to include fructo-oligosaccharides, fructan and resistant starch, are a benefit to animal nutrition as these fractions are likely to be degraded by the gastro-intestinal microflora in the same way as other fibre components.

From a nutritional point of view, methods which differentiate between soluble and insoluble fibre are important. However, the analysed content of soluble fibre is highly influenced by extraction method (Graham *et al.*, 1988), and fibre components can be solubilized during transit through the animal digestive tract (Graham *et al.*, 1986). Further, while the content of dietary fibre may be of some interest, from a nutritional point of

view it would be more critical to know how this would influence parameters such as the viscosity in the target animal small intestine. Similarly, while knowing the content of total dietary fibre is important in animal nutrition, the fermentability of this fibre as well as its influence on the digestion of other dietary components, such as starch, protein and fat, would be of great interest.

Gravimetric methods such as ADF and NDF are still widely used in ruminant nutrition, where they are well correlated with feed quality and animal performance. However, these methods do not account for the important soluble fibres and give little information on the types of polysaccharides present, and thus are of limited use in monogastric nutrition. Quantification of the polysaccharide components, for example by the Uppsala (Andersson *et al.*, 1999) and β -glucan methods (AOAC 995.16), are also critical to developing a better understanding of the role of fibre in nutrition. However, while an experienced analyst will know that arabinose residues in a wheat sample originate primarily from arabinoxylans, in a complete feed this arabinose could come from arabinoxylans or pectins such as arabinans or arabinogalactans. From the point of view of someone targeting this fibre with a specific enzyme, this type of information would be important!

Concluding remarks

During recent years, dietary fibre in relation to human nutrition has been defined internationally and in Europe. Unfortunately, in the Codex definition it is up to the individual countries to decide if resistant

oligosaccharides should be included in the definition or not, which may create confusions when results are compared between countries. New analytical methods or combinations of methods for both total and soluble/insoluble dietary fibres in accordance with the new definitions have been developed. These methods give in many cases higher values due to inclusion of all types of resistant starch as well as resistant oligosaccharides (if included) and therefore databases need to be developed to include both more classical and newer results since re-analysing all feed- and foodstuffs at short notice will be impossible. In the future, methods giving a more detailed picture of dietary fibre composition and properties will most probably be developed. The link between these analytical methods and nutritional value and animal production results needs to be better understood.

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Section 2

The fibre debate

Dietary NSPs have both negative and positive effects on nutrient utilisation and health in pigs and poultry.

The issue of how dietary fibre acts within the gut is complex, with numerous interactions throughout the digestive process, and affected by the fibre itself, animal factors and the make-up of the diet.

Whilst insoluble and soluble fibre has the ability to retain water in both swine and poultry, the effects differ between species. For example, the digesta is much drier in poultry, which concentrates the soluble fibre and results in much higher gut content viscosity. Interestingly, such conditions vary with age in poultry, with viscosity peaking at 21d of age, a critical stage in the bird's development. However, fibre can also be beneficial. Up to one third of the energy requirements of mature sows can come from fibre, compared to maybe 3-10% for poultry.

The fermentation of fibre in the large intestine not only supplies energy in the form of volatile fatty acids, but also suppresses the toxin-producing putrefaction of protein, which can dominate if fibre supply is limited. The ideal is to supply sufficient 'slow burn' fibre to maintain fermentation throughout the length of the large intestine. Such fermentable fibre tends to be oligomeric

fragments generated from the soluble and insoluble fibre by enzymatic action (either exogenous or endogenous microbial). The benefits of such fragments are lost if they are reduced to monomeric sugars. Many fibre-digesting bacteria have been found to directly produce butyrate, a metabolite with multiple regulatory functions and also involved in the maintenance of epithelial integrity.

There is a clear link between increased gut viscosity, created by high levels of soluble long-chain NSPs, and an increased risk of necrotic enteritis. The bacteria responsible, *Clostridium perfringens*, thrives in a low oxygen environment and needs amino acids to grow rapidly, and that is just what high gut viscosity facilitates. Using NSP-degrading enzymes to reduce viscosity and optimise digestion can therefore help reduce risk of a necrotic enteritis outbreak.

The NSP-degrading enzyme market is today dominated by xylanase products, but the lack of a standard protocol for assaying in-feed activities means research often fails to report enzyme types or levels as-fed. Enzyme inclusion levels can vary enormously between xylanases, as can activity losses during feed processing due to differences in thermostability. As a result trial results are sometimes inconsistent, or cannot support the claims being made.

The role of soluble and insoluble fibre in poultry nutrition

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Introduction

Dietary fibre in poultry feed is the target of a variety of exogenous carbohydrate degrading enzymes. As with any enzyme reaction, a clear understanding of the chemistry and structure of the fibre substrate is required for successful enzyme application. In addition, understanding how dietary fibre affects nutritional value and poultry feeding is important in defining the need for enzyme use and the nature of the enzymes required. This review will attempt to summarize fibre effects in broilers and also identify where enzyme use has found or could find application. Although, the use of fibre degrading enzymes in poultry feeding is not the primary focus of the review, it is often difficult to define a fibre effect without discussion of dietary enzymes. Because of the large volume of research in this area, this review should not be considered to be comprehensive. However, research in this area has been reviewed extensively (e.g. Campbell and Bedford, 1992; Bedford, 2000; Choct, 2006; Slominski, 2011; Masey O'Neill *et al.*, 2014).

Fibre

As a prelude to the discussion, it is relevant to mention that dietary fibre effects are the result of the interaction between the consuming animal, components of the

diet and the gastrointestinal tract (gut) microbiota. As a result, changes in any of these interacting factors can impact dietary effects. Similarly, dietary fibre is inherently complex with a variety of composite monosaccharides, structure and linkages to other plant components. Therefore, when considering the effects of dietary fibre in poultry or other species, it is important to recognize that positive or negative effects on the animal will be defined by the specific fibre source and importantly the level found in the diet. This logic encourages caution in the extrapolation of effects from one ingredient to another.

By definition, poultry species lack the enzymatic complement to digest fibre found in the plant-based ingredients and this is verified by usually low fibre digestibility in poultry research (e.g. 4.8% – wheat, soybean meal, fish meal (SBM) diet, 15.0% – wheat, wheat screenings, barley, SBM, canola meal (CM); Boros *et al.*, 2004). However, exogenous enzymes can play a role in increasing fibre digestibility. Although the degree of increase is variable and generally small, reports of increased “apparent” digestibility of fibre as a result of exogenous enzyme use are common (Boros *et al.*, 2004; Choct *et al.*, 2004). For digestibility of fibre to occur, fibre must either be initially soluble or have been solubilised

by gut microorganisms or exogenous enzymes. The ability of dietary enzymes to solubilise insoluble fibre fractions has been clearly demonstrated with a common finding that digestibility of insoluble fibre increases and concurrently the digestibility of soluble fibre decreases (Choct *et al.*, 2004). A possible goal of enzyme use to increase fibre digestibility is the release of monosaccharides for animal absorption and metabolism. However, arguments against this possibility are the complex nature of fibre components, the variety and amount of enzymes required for hydrolysis to monosaccharides, and the relatively rapid feed passage rate in birds. Further, the ability of birds to effectively use released monosaccharides would be dependent on the nature of the monosaccharide. Release of glucose is an example of a positive effect while the pentoses, arabinose and xylose, are examples of monosaccharides that produce negative responses in birds, even at relatively low levels (Schutte, 1990). Therefore, evidence to date does not support an enzymatic strategy of hydrolysis of fibre to monosaccharide constituents. More probable is that the disappearance of fibre relates to the depolymerisation of fibre to oligosaccharides not measured by fibre analysis and/or smaller polysaccharides that along with oligosaccharides are substrates for microbial fermentation. It is recognized that the energy derived from animals via fermentation is much less than when monosaccharides are directly absorbed and utilized (e.g. glucose), particularly in poultry species with limited fermentation capacity.

Fibre solubility is an important characteristic and it is generally recognized that the soluble fraction is more biologically relevant than the insoluble fraction. It is therefore appropriate to discuss soluble and insoluble fractions separately. However, *in vitro* assessment regardless of approach, may not match the degree of solubilisation found in the digestive tract of animals and as such *in vitro* measures are only estimates of solubility (Bedford and Classen, 1992). *In vivo* solubilisation is affected by the combined effects of the host animal (chemical and mechanical actions), gut microbiota, and additives and other factors in the diet. As noted above, exogenous enzymes are a good example of an additive that can increase fibre solubility in the avian gut (Choct *et al.*, 2004).

Insoluble fibre

A traditional view is that insoluble fibre is largely inert and dilutes the nutrient content of the diet. In turn, it is considered a "bulking" agent and is thought to increase the speed of digesta passage. This interpretation has been questioned with a number of effects suggested (for review see Hetland *et al.*, 2004).

As noted above, insoluble fibre fermentation is possible and in poultry species the highest potential exists in the caeca (Svihus *et al.*, 2013). Entry into the caeca is generally considered to be restricted to soluble material, but entry of small particle size insoluble material also occurs. The extent of entry, however, is not well understood. If solubilised in this location, fermentation of insoluble fibre is possible. Feed processing

(fine grinding) or gizzard action can reduce particle size and enhance the possibility of caecal entry. Because of the questions regarding entry of insoluble fibre entry into the caeca, the energy contribution from its fermentation is likely of little consequence in commercial poultry production. Exogenous enzymes or other mechanisms such as heat processing of feed can solubilise fibre to permit caecal entry. Caecal entry of soluble material is not guaranteed with higher molecular weight viscous soluble fibre possibly not entering the caeca (Choct *et al.*, 1996). Fermentation can provide energy in the form of volatile fatty acids (VFA), as well as modify the microbial community thereby providing inherent benefits to animal health and the colonization of zoonotic organisms (Bedford and Cowieson, 2012).

Insoluble fibre has been found to increase digestibility of nutrients such as starch via mechanisms derived from gizzard action and increased gastric exposure (Svihus and Hetland, 2001; Hetland *et al.*, 2004; Svihus, 2011). Feeding insoluble fibre can increase the digesta content in the gizzard, increase gizzard size and induce gastro duodenal flux, with the effect most associated with increased particle size fibre. No obvious benefit can be seen for dietary enzymes in this scenario, unless the increased hydrolysis of insoluble fibre in this setting would not reduce the benefit of increased diet digestibility via gizzard action.

The most important consequence of insoluble fibre is reduced nutrient digestibility as a result of encapsulation of nutrients and prevention of digestive enzyme exposure.

This action has long been thought to be of importance (Pettersson and Åman, 1989), and the evidence that exogenous enzymes can release nutrients for absorption is strong. It is probable that encapsulation is more of a factor in raw than processed ingredients like soybean meal and canola meal where pre-press solvent extraction is more likely to have disrupted cell walls (Meng *et al.*, 2005). Encapsulation is an obvious issue in full-fat ground or whole canola and flax seeds (Meng *et al.*, 2006; Slominski *et al.*, 2006) and dietary enzymes have been used to increase fat digestibility.

Soluble fibre

The anti-nutritional effects associated with feeding soluble fibre are often associated with their ability to produce viscous conditions in the avian gut (Burnett, 1966; Antonio and Marquardt, 1981; White *et al.*, 1983; Bedford and Classen, 1992; Choct and Annison, 1992b; and many others). This has been demonstrated with partially purified fibre sources as well as cereal grains such as barley, oats, wheat, triticale and rye where β -glucans and arabino-xylans are partially soluble and are responsible for the increased viscosity. Viscosity associated with these grains and in particular with rye relates in a linear fashion with high molecular weight (>550 kDa) soluble fibre fraction (Bedford *et al.*, 1991; Bedford and Classen, 1992). With higher levels of viscosity, a range of mechanisms are suggested to be responsible for the negative effect. Digesta passage rate is delayed (Salih *et al.*, 1991; Van der Klis and Van Voorst, 1993) resulting in reduced feed intake, nutrient digestibility in reduced with

fat digestibility affected more than starch and protein (Edney *et al.*, 1989), diffusion of substrates and enzymes reduced (Fengler and Marquardt, 1988), changes in digestive tract size (Brenes *et al.*, 1993) and enzyme capacity, and an increase in gut microbes (Choct and Annison, 1992a). Consequences of these effects plus the water holding capacity of this fibre are wet litter and poor environmental conditions in litter floor broiler barns, which further add to the negative effect on broiler production. Of importance for the use of enzymes, depolymerisation of large molecular weight materials readily eliminates the viscosity effects. The negative effect of high viscosity grains is without question, but in less viscous conditions, other effects of soluble fibre become more evident. It is important to understand that fibre solubility does not equate to viscosity.

Fermentation of dietary fibre and in particular soluble fibre plays a role in the negative and positive effects of dietary fibre. Highly viscous materials as described in the previous paragraph produce an environment that is conducive to negative bacterial proliferation in the small intestine. Adding 6.6% soluble and viscous arabino-xylans to the diet increased volatile fatty acid (VFA) production in the ileum, but of interest, not in caeca (Choct *et al.*, 1996). This suggests that highly viscous material does not enter the caeca and may also reduce entry of other fermentable undigested material (starch, protein) associated with high viscosity mal-absorption. Appropriate dietary enzymes reduce and increase fermentation in the small intestine and caeca, respectively. It is probable that the nature of the bacterial

response to dietary fibre will vary with the degree of physicochemical changes due to viscosity in the gut and the nature of the fibre polymers available after enzyme treatment.

Of note, gut bacterial populations evolve with age and in response to the nature of fibre found in digesta. This is shown in a comparison of ileal viscosity determined at weekly intervals from 7 to 42 days of age in broilers fed corn, wheat, or wheat plus enzyme diets (Fischer, 2003). As expected, digesta viscosity was lower for birds fed corn or wheat plus enzyme diets. Of interest was the finding that viscosity of digesta from birds fed wheat diets increased to 21 days of age and then decreased. Fischer (2003) speculated that intestinal bacterial populations adapt with age and are able to solubilise fibre, with initially fibre release exceeding depolymerisation to produce the increased viscosity at 21 days of age. With increasing broiler age, release and depolymerisation rates equalize and viscosity is reduced. Of relevance in her work, is that the degree of depolymerisation (arabinose and xylose in digesta fraction <100 kDa) by the exogenous enzyme exceeded that achieved by gut microorganisms. This work demonstrates several factors that complicate the interpretation of fibre effects, gut microbiota and bird age. It is possible that the benefits of enzyme addition change with age based on the "negative or positive" effects that occur at specific ages.

The ability of the gut microbiota to ferment soluble dietary fibre has already been mentioned primarily from a negative

perspective. However, the potential for positive effects of fibre hydrolysis products have also been shown. Adding arabinoxylooligosaccharides derived from wheat bran to broiler diets improves feed efficiency increases caecal *Bifidobacteria* numbers and provides dose dependent protection against oral infection by *Salmonella* Enteritidis (Courtin *et al.*, 2008a, b; Eeckhaut *et al.*, 2008). The amount of fibre solubilisation and the degree of fibre depolymerisation, which are both exogenous enzyme dependent, are likely to affect the benefits gained.

The changes in gut microbiota in response to fibre (and enzymes), has potential to impact digestive tract disease (Bedford and Cowieson, 2012; Kiarie *et al.*, 2013). Use of dietary enzymes has been shown to reduce digestive tract *Clostridium perfringens* numbers and the severity of necrotic enteritis (endoxylanase – Sinlae and Choct, 2000 and Jia *et al.*, 2009a,b; β -mannanase – Jackson *et al.*, 2003), but the mechanism(s) of these effects are not established and results are not

always consistent (Riddell and Kong, 1992). The removal of substrate suitable for fermentation (increased digestibility), an increase in beneficial substrate (promote proliferation of beneficial bacteria) or an impact on bird immune function have been suggested as mechanisms. Additional research on the effects of dietary fibre and enzyme depolymerisation of fibre on gut microbiota and health are likely fruitful areas of research. Regardless, it is likely safe to say the factors like dietary enzymes that stabilize the microbial ecology of the gut are beneficial to bird health.

Conclusions

Dietary fibre can affect the digestive process via viscosity, encapsulation and other mechanisms that in turn adversely affect poultry performance and health. Enzymes capable of solubilising and depolymerising fiber can negate these effects and also provide probiotic lower molecular weight polymers and oligosaccharides that impact gut microbiota and health, and impact colonization of bacteria that influence food safety.

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What does the fibre fraction mean for swine?

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Introduction

Until recently, the topic of fibre in the nutrition of the pig captured very little interest in the US and other regions feeding diets based primarily on corn and soybean meal. This certainly was not the case in Europe and other parts of the world, where more complex diets employing a wide variety of feed ingredients, many high in fibre, were being fed. The situation in the U.S. changed in 2008 when the price of corn doubled,

then tripled and almost quadrupled (Figure 1), and left the pork industry seeking ways to reduce the cost of feeding pigs. Concurrently, co-products of the biofuels sector as well as other crop and food processing industries were adopted with surprising speed. Indeed, by 2011/12, many practical diets in the U.S. had been successfully switched from 75% corn to less than 40%, and in some cases, less than 20%.

Figure 1. 10 year history of the futures contract price of corn



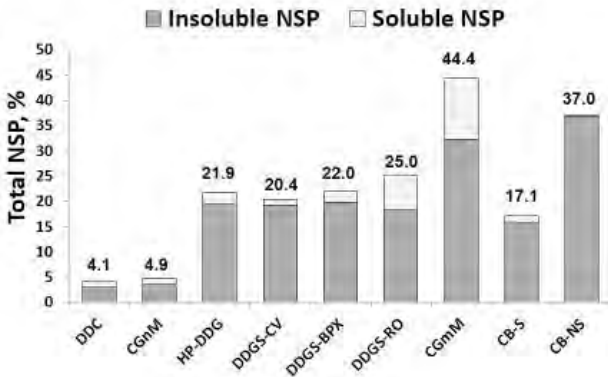
Source: NASDAQ, 2014

The speed with which new ingredients were adopted, and concurrent changes in feed manufacturing and diet formulation strategies, inevitably led to many questions about fibre use by the pig. Questions arose about the impact of fibre in the diet on the utilization of other dietary constituents such as fat, starch and amino acids. It also resulted in inquiries about the selection of optimum energy concentration in the diet, and about ways to enhance the daily intake of lower energy, higher bulk diets. Estimating the energy content of diets higher than usual in fibre content proved to be problematic (Acosta Compargo *et al*, 2014), and will be addressed below.

Because corn grain and its co-products contain an unusually high portion of insoluble as opposed to soluble fibre (Figure 2), at least compared to other common crops such as wheat, barley and peas

(Bach Knudsen, 1997), the challenges in understanding fibre utilization in North American diets at the practical level is a bit unique. It differs somewhat from those experienced in other parts of the world, although truth be told more in degree if not in substance. Fortunately, researchers on fibre in Europe took a holistic approach in their studies and generated a great deal of valuable information that can now be applied to North American circumstances. However, understanding the impact of fibre on the pig is a complex subject. Fibre can at once be an energy diluent and a benefit to gastrointestinal health. It can impede enzymatic digestion in the pig, but also stimulate gastrointestinal microbiota. The same fibre that impedes digestion and absorption in the upper gut can stimulate fermentation and the release of energy in the colon.

Figure 2. Soluble and insoluble NSP content of corn co-products (as-fed basis)



Source: Gutierrez *et al*, 2014

Therefore, it has been a challenge to utilize fibrous ingredients effectively in practical pig diets while maintaining the level of growth performance previously experienced using corn-based diets, reducing the overall cost of feeding the pig herd, maximizing net income, enhancing the pig carcass and in some instances improving pig health and welfare. Understanding the impact of fibre in pig nutrition has been very challenging, but advancing the science will advance the competitiveness of pork production as a source of high quality protein in the human diet.

Definition of dietary fibre in swine nutrition

Dietary fibre can be, and most certainly has been, defined in many different ways. A commonly referenced definition in human nutrition was presented by Trowell (1976) as "all plant polysaccharides and lignin that are resistant to hydrolysis by human digestive secretions." In 2001, the Institute of Medicine suggested that total fibre can be defined as the sum of dietary fibre and functional fibre, where dietary fibre is non-digestible carbohydrates plus lignin and functional fibre is isolated, non-digestible carbohydrates having beneficial physiological effects on humans (Slavin, 2003). More definitions have evolved in the past decade, but from the perspective of the pig, we can avoid some of the controversy that exists in human nutrition. For example, in human nutrition, there is a desire to evaluate fibre on its ability to enhance satiety and encourage reduced caloric intake, something that has little interest in pork production other than perhaps

the gestating sow, where satiety could be beneficial.

In swine nutrition, most definitions of fibre fall into two broad categories: chemical and physiological (Bach Knudsen, 2001). From a physiological perspective, fibre is often referred to as that portion of the diet which is resistant to digestion by endogenous enzymes, similar to Trowell's definition above. Chemically, fibre refers to the sum of non-starch polysaccharides (NSP) and oligosaccharides plus lignin. NSPs typically comprise about 90% of the cell wall of plants. The main NSP present in plant cell walls is cellulose. The main matrix polysaccharides are β -glucan in barley and arabinoxylans in wheat, rye, sorghum, corn and triticale (Choct, 1997; de Lange, 2000). The NSP content of common cereal grains and co-products is presented in Table. 1. While more than 100 monosaccharides are known to exist, only about 9 are of importance in NSP structure: the pentoses (arabinose and xylose), the hexoses (glucose, galactose and mannose), the 6-deoxyhexoses (rhamnose and fucose, and hexauronic acid), galacturonic acid and gluconic acid. Unlike cereal grains, legumes tend to be rich sources of oligosaccharides, which are also poorly digested by the pig. Finally, so-called resistant starch that avoids enzymatic degradation in the upper gut can be considered fibre. As one can appreciate, starch that is fermented in the lower gut and absorbed as short chain fatty acids will provide less available energy to the pig for metabolic purposes than that which is enzymatically digested in the upper gut and absorbed as glucose (Bindelle *et al.*, 2008).

Table 1. Types and levels of non-starch polysaccharides in cereal grains and cereal by-products

Cereal	ARABINO-XYLAN	SS-GLUCAN	CELLULOSE	MANNANS	GALACTANS	URONIC ACID	TOTAL NSP
Wheat							
- Soluble	1.8	0.4			0.2		2.4
- Insoluble	6.3	0.4	2.0		0.1	0.2	9.0
Barley							
- Soluble	0.8	3.6			0.1		4.5
- Insoluble	7.1	0.7	3.9	0.2	0.1	0.2	12.2
Corn							
- Soluble	0.1	0.1					0.2
- Insoluble	5.1		2.0	0.2	0.6		8.0
Rye							
- Soluble	3.4	0.9		0.1	0.1	0.1	4.6
- Insoluble	5.5	1.1	1.5	0.2	0.2	0.1	8.6
Sorghum							
- Soluble	0.1	0.1					0.2
- Insoluble	2.0	0.1	2.2	0.1	0.2		4.6
Rice bran¹							
- Soluble	0.2				0.1	0.1	1.7
- Insoluble	8.3		11.2	0.4	1.0	0.4	21.3

¹ Defatted. Source: Choct, 1997

Soluble fibre includes pectic substances, gums, mucilage and some hemicelluloses. Insoluble fibre includes cellulose, other hemicelluloses and lignin. The solubility of fibre helps to define its physiological effects. For example, soluble fibres will increase viscosity of the intestinal contents and will be better fermented in the lower gut. Insoluble fibre, on the other hand, will increase fecal volume, and decrease intestinal transit time.

The challenge of defining fibre is a significant one in pig nutrition. Physiological definitions often lead to a component of

the diet that is difficult to define chemically. And chemical definitions may not quite align with physiological function. While the differences may be small in some situations, they do pose significant challenges in others, particularly where fibre is viewed as both a negative and a positive dietary constituent. It can be viewed as negative, because it reduces diet digestibility and positive, because it may provide benefits to the health of the gastrointestinal tract, and thus the pig.

Assays used for quantify fibre in swine diets

I will not enter into a detailed discussion on the various assays of fibre that have been or are being used in pig nutrition. That is well beyond the scope of this paper, and there are far more qualified speakers on the program able to address this topic in detail. However, a brief historical review and summary is relevant to the current discussion, and helps to set the foundation for later discussion on the biological implications of dietary fibre in swine.

Crude fibre is an assay with strong historical roots in animal nutrition; it dates back to at least 1820, if not before. It represents the residue of plant material remaining after sequential extraction by diethyl ether, dilute acid and dilute alkali. It represents, imprecisely and incompletely, the hemicellulose, cellulose and lignin content of feedstuffs. The degree of recovery varies widely among ingredients and thus is no longer considered an acceptable fibre assay (Asp *et al.*, 1983). Regrettably, it is still in broad use in North American private analytical laboratories.

The detergent system developed by van Soest at the U.S.D.A. in Beltsville, MD (van Soest, 1963) and referred to as acid detergent fibre (ADF) and neutral detergent fibre (NDF) was intended for use in ruminant species, but earned increasing acceptance in monogastric nutrition. ADF quantifies the cellulose and lignin content of a feedstuff, and NDF includes cellulose, hemicellulose and lignin content. The detergent system has limitations, in particular its exclusion

of the soluble fibres; for example, pectins, mucilages, gums and β -glucans will not be included in ADF or NDF determinations (Grieshop *et al.*, 2001). Since many cereal grains contain significant quantities of soluble fibre, this can be a serious limitation in certain situations. Also, ADF and NDF do not identify the constituents of fibre like the newer assays are able to do. While ADF and NDF serve useful purposes in practical application in the swine industry today, their limitations need to be understood. They may have their greatest use in diets consisting primarily of corn and corn co-products since most of the fibre is present in insoluble form. Ultimately, there is a need to move away from the detergent system to more precise and definitive analytical methodologies, but van Soest can be considered an early pioneer in the field of fibre and animal nutrition.

"I began my work on fibre in 1957 while employed in the Agriculture Research Services, USDA, at the instigation of the late Dr. Lane Moore, who believed that fibre was one of the Cinderellas of nutrition. Other administrators and biochemists thought, and I was told by several, that money and time was being wasted on a fruitless topic that would lead to no future."

Dr. Peter van Soest, 1978

There are three main methodological categories of assays employed in the determination of dietary fibre in human nutrition, and this approach is increasingly being adopted in swine nutrition as well: non-enzymatic-gravimetric, enzymatic-gravimetric and enzymatic-chemical

(Elleuch *et al.*, 2011). The determination of crude fibre and the detergent fibre system, both described previously, are examples of the non-enzymatic-gravimetric method. The most commonly used methods in human nutrition, and increasingly in animal nutrition, are the enzymatic-gravimetric procedure of the AOAC (Prosky *et al.*, 1988) and the enzymatic-chemical method (Englyst *et al.*, 1994).

The Prosky method uses a combination of enzymatic and gravimetric procedures to determine total dietary fibre (TDF). It evolved from the Van Soest detergent method, adding an enzymatic step to minimize interference from starch. Briefly, the procedure includes enzymatic treatment to remove starch and protein, precipitation of soluble fibre with ethanol, filtration and weighing of the dietary fibre residue. The result is corrected for protein and ash in the residue (Prosky *et al.*, 1988). This method will include polysaccharides, lignin, and some, but not all resistant starch and other compounds such as waxes, phenolics and Maillard reaction products. It excludes oligosaccharides and some resistant starch. The method has been enhanced to include indigestible oligosaccharides and therefore allows for determination of soluble and insoluble fibre.

Enzymatic-chemical methods are also known as component analysis; this procedure uses a very different approach, by removing starch enzymatically. Precipitation with ethanol or by dialysis separates the soluble NSPs from lower molecular weight sugars and starch hydrolysis products. The

sugars are then quantified by gas-liquid chromatography or high-performance liquid chromatography. A single value for the sum of all sugars can be determined by spectrophotometry (Englyst *et al.*, 1994). Klason lignin is determined on the residue and then added to the sum of total NSPs to generate a value for total dietary fibre. This method measures only NSPs and lignin. Oligosaccharides and resistant starch will not be included.

The Uppsala method (Theander *et al.*, 1995) is another example of an enzymatic-chemical method and quantifies neutral sugars, uronic acid and Klason lignin, using enzymatic removal of starch and recovery of fibre by precipitation with 80% ethanol. The fibre is hydrolyzed in acid and the neutral sugars are measured using GLC, uronic acids by colorimetry and Klason lignin by gravimetry.

No assay is perfect, but the more sophisticated methods developed over the past 25 years have greatly aided in our understanding of fibre metabolism, and the role of fibre in swine nutrition. Europe has been a clear leader in this field. We encourage continued work in this regard, to not only refine the most sophisticated methodologies, but also to create methods that can be widely used by the pig industry to evaluate and characterize feed ingredients on a higher volume, lower cost basis. While the detergent system is less precise than more modern methods, it is attractive in commercial practice, as opposed to research laboratories, due to its cost and capacity. Perhaps Near Infrared



Reflectance will offer opportunities "on the ground" to enhance the characterization of large quantities of feed ingredients and thus contribute to the more effective use of high fibre ingredients in practical pig diets.

Fibre digestion

As described above, fibre is not digested by endogenous enzymes in the pig, but rather must undergo bacterial fermentation (Just *et al.*, 1983). Fermentation in the large intestine, and to a smaller extent in the ileum, produces short chain fatty acids (SCFA), mainly acetate, propionate and butyrate. It also produces some gases and water, and of course, bacterial biomass. The SCFA are rapidly absorbed from the gut in most situations. Acetate is carried to the liver, where it acts as a substrate for energy metabolism in muscle. Propionate is converted to glucose in the liver, while butyrate is used locally by the colonocytes to help support their energy needs. Butyrate is believed to be associated with gut health, because it stimulates epithelial cell proliferation in both the small and large intestine, and also encourages water and sodium resorption (Pluske and Kim, 2014). The hindgut plays a major role in digestion of highly fibrous diets; Shi and Noblet (1993) reported that across a wide range of diet composition, the large intestine contributed 16% of the total DE of the diet in growing pigs. The digestibility coefficients of energy, fibre and crude protein in the lower gut were 35%, 39% and 15%, respectively. Keys and deBarthe (1974) reported that approximately 100% of the digestion of cellulose and 80% of the digestion of hemicellulose took place in the large intestine.

The extent of digestion of fibre, and its impact on the digestibility of other nutrients, is broadly explained on the basis of viscosity, water holding capacity and solubility (Souffrant, 2001; Hooda *et al.*, 2011). Solubility depends on the type of polymers in the fibre and their structural associations with other cell wall components (deVries *et al.*, 2012). The hull is generally heavily lignified and thus resists digestion or even fermentation. Noblet and Le Goff (2001) reported that total tract digestion of dietary fibre ranges from virtually 0 for lignin to 80% to 90% in fibre that is rich in pectin or is highly soluble.

Physiological factors also influence the degree of digestion of fibre, including level in the diet, intestinal transit time, the age and weight of the pig and the innate bacteria present in the gut (Le Goff *et al.*, 2003; Montagne *et al.*, 2003). With so many factors involved, it is no wonder that modifying diets with different fibre sources has often led to uncertain results.

Ngoc *et al.* (2011) reported that particle size reduction, from 810 to 341 microns, increased apparent total tract digestibility of crude fibre, NDF, organic matter and gross energy in the weanling pig, but not in the growing pig. De Vries *et al.* (2012) reached the same conclusion; reducing particle size increases the surface area of the material, making it more accessible to enzymes and to bacteria. However, grinding also alters the physicochemical nature of feedstuffs. Adding heat, either dry or wet, to the processing equation further enhances digestibility by breaking the seed coat and endosperm.

Viscosity is also impacted by various physical processing procedures. The exact impact of the process on viscosity depends on the nature of the NSP and the severity of the treatment. Simple grinding will tend to reduce viscosity by reducing molecular weight of the NSP fragments. If heat treatment is added, however, this will tend to burst cell wall structures and release the matrix contents without particle size reduction; this will result in an increase in viscosity (De Vries *et al.*, 2012).

Finally, the addition of enzymes is another process that can be applied to the topic of fibre digestion. Other speakers will be covering this topic in detail.

Hindgut fermentation can represent a substantial portion of the total energy supplied to the pig by the diet. For example, Anguita *et al.* (2006) reported that on a high fibre diet (24% NSP), fermentation represented 18% of the total daily supply of energy, and even on a low fibre diet (7.7% NSP), it contributed 7% of the total.

Impact of fibre: Animal performance

Changes in the content of fibre in the diet may have substantial impacts on growth performance. Increasing the NDF content of growing and finishing diets may result in a reduction in rate and efficiency of gain, as explained below, unless steps are taken

to maintain equivalency of dietary energy, such as by adding the appropriate amount of fat to the diet. Beaulieu *et al.*, (2009) reported that replacing wheat and oil in a diet with increasing proportions of barley and canola meal increased NDF. Since the dietary energy content was allowed to float, it caused the pigs to eat more feed and experience reduced feed efficiency (Table 2). Presumably, the pigs ate more feed as the NDF increased in order to maintain constant daily energy intake; however, when the NDF content became too great, and the energy content of the diet declined too much, the pigs were not able to further increase feed intake and daily energy intake declined as feed intake plateaued. Presumably, the limit to increased feed intake was diet bulk; the physical capacity of the gut was simply exceeded when the NDF content was too high. The extent to which pigs can adjust to higher fibre, lower energy diets will depend on a large number of factors, but suffice it to say that in commercial practice, when lower energy, higher fibre diets are fed, attention to maximizing feed intake in the pig takes on added importance. Indeed, in commercial practice, where a variety of factors conspire to restrict feed intake, any reduction in dietary energy will often result in slower growth rates.

Table 2. Impact of decreasing dietary energy concentration – and increasing dietary NDF content, on growth performance and daily energy intake

DIET DE, MCAL/KG	3.05	3.19	3.33	3.47	3.61
Initial wt., kg	31.2	31.1	31.5	31.2	31.1
Final wt., kg	115.1	115.5	115.3	115.0	115.6
Daily gain, kg	1.00	1.02	1.04	1.02	1.03
Daily feed, kg ¹	2.66	2.62	2.62	2.52	2.44
Feed conversion ¹	0.39	0.40	0.41	0.42	0.44
DE intake, Mcal/d	8.22	8.49	8.76	8.61	8.71

¹Effect of diet ME content significant, $P < 0.05$. Source: Beaulieu *et al.*, 2009
Low DE diet contained 16.4% NDF; high DE diet contained 9.6% NDF

Impact of fibre: Nutrient requirements

Increasing the content of fibre in the diet may alter nutrient requirements. The new NRC (2012) quantifies the increase in threonine required in the diet as fermentable fibre levels increase.

Impact of fibre: Energy digestibility and content

There is no question that increased dietary fibre will result in a reduction in the

apparent total tract digestibility of energy and nutrients (Table 3 & 4). Pilcher *et al.*, (2013) reported an average 3.5% reduction in the standardized ileal digestibility of indispensable amino acids. It must be noted that the increase in fibre could result in an underestimation of standardized ileal digestibility of amino acids, since SID accounts only for basal endogenous losses and does not account for specific endogenous losses, which are expected to increase with higher fibre ingredients.

Table 3. The impact of increased neutral detergent fibre on the standardized ileal digestibility of amino acids

AMINO ACID	NDF = 7.0% ¹	NDF = 11.4% ²	SEM	P-VALUE
Lysine	84.1	79.6	1.02	<0.0001
Methionine	86.0	83.9	0.91	0.0003
TSAA	81.5	77.9	1.26	<0.0001
Threonine	79.5	74.9	0.97	0.0403
Tryptophan	85.6	84.0	1.52	<0.0001
Isoleucine	84.2	81.1	0.90	<0.0001
All Indispensable amino acids	84.55	81.62		

¹ 7.0% NDF diet contained no corn DDGS; ² 11.4% NDF diet contained 25% corn DDGS. Source: Pilcher *et al.*, 2013

Table 4. Impact of increasing total dietary fibre on apparent digestibility of energy and TDF in the upper and lower gut and over the total length of the digestive tract

ITEM	0 ²	10	20	30	40	SEM	L ³	Q ³
AID, %								
GE	78.8	75.1	74.6	72.5	72.2	1.3	<0.01	0.19
TDF	14.9	17.5	16.5	15.9	19.9	2.8	0.15	0.62
Hindgut fermentation, %¹								
GE	6.5	8.1	5.9	5.7	4.8	1.0	0.07	0.43
TDF	21.9	16.4	16.8	10.8	9.7	3.0	<0.01	0.81
ATTD, %								
GE	85.4	83.3	80.6	78.2	76.9	0.9	<0.01	0.36
TDF	36.6	34.4	33.5	26.4	29.1	2.2	<0.01	0.66

¹Hindgut disappearance, calculated from the difference of ATTD and AID

²Percent corn bran with solubles added to the diet

³P-values for linear and quadratic effect of diet

Source: Gutierrez et al., 2013

In the same way, fibre can also reduce the digestibility of energy. Gutierrez *et al.* (2013) reported that increasing corn bran with solubles from 0 to 40% of the diet reduced the apparent ileal digestibility of energy by 8.4% and the apparent total tract digestibility by 10.0%. Hindgut fermentation was also affected, but since corn fibre is poorly fermented, the contribution to the overall digestibility of energy was very small.

Recently, Newman and Patience (unpublished data) compared corn of differing quality and measured digestibility

in the upper gut, lower gut and across the total gastrointestinal tract. They compared two corn samples that were previously determined to be higher in digestible energy content versus two corn samples that were lower in DE content (Table 5). They reported that there was no difference in apparent digestibility at the terminal ileum, but that hindgut fermentation was 40% higher in the higher energy corn samples. These samples were evaluated with and without enzymes and apparent total tract digestibility was improved in the presence of a xylanase (Econase XT®).

Table 5. Comparison of apparent ileal and total tract digestibility, and hindgut fermentation in 2 higher quality (HQ) and 2 lower quality (LQ) corn samples

Corn Samples						
Item	HQ-1	HQ-2	LQ-1	LQ-2	SEM	P-Value
AID, %						
GE	79.12	80.18	80.20	79.67	1.04	0.744
DM	77.46	78.22	78.86	78.52	1.11	0.686
Fermentation, %						
GE	5.59	5.48	2.76	3.79	1.49	0.092
DM	6.30	6.50	3.67	4.52	1.69	0.113
ATTD, %						
GE	84.39	85.68	83.15	83.60	1.05	0.008
DM	83.47	84.82	82.82	83.25	1.11	0.007

Source: Newman and Patience, Unpublished data

However, a note of caution is appropriate. Measurement of apparent total tract digestibility of energy is generally interpreted to mean that any energy not appearing in the feces is available to the pig for metabolic purposes. There are two flaws with this assumption. First, some energy not appearing in the feces will be energy that was consumed during fermentation and released as gas or heat – and is therefore not available to the pig. Second, energy absorbed by the pig as short chained fatty acids is less efficiently utilized by the pig, something which is not addressed in a simple digestibility experiment (Patience, 2012). Bach Knudsen estimated that for every 1% increase in energy digested in the large intestine, the utilization of metabolisable energy will be reduced by 0.27%.

Although not well defined quantitatively, it

is known that fibre will alter the transit time of digesta. For example, Wilfart *et al.* (2007) reported that increasing the concentration of wheat bran in the diet of the growing pig, a source of highly insoluble fibre, had no effect on mean retention time in the stomach, reduced mean retention time of the solid phase of the digesta in the small intestine and decreased mean retention time of both the solid and liquid phases in the large intestine. Earlier, Potkins *et al.* (1991) evaluated both soluble and insoluble fibre sources on rate of passage. They concluded that the effects of wheat and oat bran on rate of passage was more substantive in the large intestine than the small intestine. This suggests that rate of passage would not be a factor in any effects these fibre sources might have on nutrient digestibility in the upper gut, but that differences may occur in the lower gut.

Impact of fibre: Carcass yield

It has long been known that feeding diets higher in fibre will result in growth of visceral tissue and also in increased mass of intestinal contents. The consequence of this is potentially misleading growth performance information when comparing diets with varying fibre content. Weber (2012) reported that increasing corn distillers dried grains with solubles from 30 to 60% of the diet reduced dressing percentage from 76.1 to 75.2. As shown in Table 5, when performance data were expressed on a whole body basis, there appeared to be no change in growth rate and a tendency for improved feed efficiency. However, when performance was expressed on a carcass basis, growth rate was impaired and there was no difference in feed conversion. These data, along with numerous other examples in the literature, suggest caution be exercised when comparing feeding programs of varying fibre content on a live weight basis; erroneous conclusions may be occur.

Impact of fibre: Environmental impact

Dietary fibre shifts nitrogen excretion from urea in the urine to microbial mass in the feces. The inclusion of soluble fibre in the diet of the growing pig increased fecal nitrogen output and lowered urinary nitrogen output, although overall nitrogen balance was unaffected (Zervas and Zijlstra, 2002).

Impact of fibre: Gut health

An important balance exists between the mucosa of the gut, the commensal bacteria in the gut and the diet (Montagne *et al.*, 2003). Disturbance of this balance can

lead to gastrointestinal disease, or at least greater susceptibility to disease; on the other hand, a proper balance can actually be protective. The microbial population in the gut is generally considered to exist in three sub-populations: those in the lumen, in the mucous layer and in the mucosal surface (Salanitro *et al.*, 1977).

Recently, Pluske and Kim (2014) reported that the addition of soluble and/or insoluble fibre in a factorial experimental design with weanling pigs experimentally infected with enterotoxigenic *E. coli* resulted in improved growth performance as well as feed conversion. There was, however, no effect of dietary fibre on diarrhea index or number of therapeutic treatments given to the pigs.

De Lange *et al.* (2010) reported that insoluble as opposed to soluble fibre sources in the diet provided protection against certain gastrointestinal diseases such as diarrhea due to haemolytic *E. coli*. However, the optimal concentration of insoluble fibre has not yet been elucidated. Bikker *et al.* (2006) proposed that fermentable carbohydrates would counter the negative effects of protein fermentation in the gut of the newly weaned pig. While they were able to reduce the quantity of ammonia produced in the gut, they were not able to enhance pig performance.

It appears that while no consensus exists, insoluble fibre tends to be viewed as protective against diarrhea, especially in the young pig, and that soluble fibre may actually exacerbate the problem. Answers are by no means definitive on this topic, and

much more work is required to develop firm guidelines and achieve consistent outcomes. One of the problems with increasing the quantity of insoluble fibre in the diet is that while it may protect against diarrhea, it may also slow growth in an unacceptable manner.

Impact of fibre: Feed handling

Often overlooked – initially at least – is the impact of higher fibre ingredients on feed storage, mixing and handling capacity, due to changes in bulk density (Table 6). Ingredients such as wheat

middlings, though commonly used in pig diets, substantially reduce the capacity of feedmills as well as feeders in the barn, all of which has important implications for feed processing and handling. For example, if a feed mill is operating at maximum capacity, and an opportunity comes along to save money by using a new ingredient, if its bulk density is lower than current ingredients, it may not be a feasible option. This not only demonstrates yet another way in which fibre affects swine nutrition, but also the close integration of all aspects of the pork production chain.

Table 6. Impact of increasing corn DDGS content of the diet from 30 to 60 percent on growth performance, when expressed on both whole body and carcass basis

	DIET		SEM	P-VALUE
	D30	D60		
BW, kg				
d 57	30.3 ^a	29.3 ^b	0.25	0.005
Market	122.4	121.9	0.45	0.41
ADG, kg	0.91	0.92	0.008	0.59
ADG carcass, kg	0.71	0.69	0.005	0.07
ADFI, kg	2.07	2.03	0.021	0.21
G:F, live	0.44	0.45	0.005	0.11
G:F, carcass	0.34	0.34	0.004	0.91

Source: Weber, 2012

Conclusion

Fibre is an increasingly important part of swine diets. As corn and other cereal grains are used for other food and non-food purposes, the pork industry will be forced to utilize higher fibre ingredients in the future. This presents a strong case for continued research on the chemistry of fibre and its

physiological role in the pig. It also speaks to growing interest in the use of enzymes to help the pig extract the maximum quantity of nutrient from fibrous ingredients.

At the present time, there is a wide gap between the edge of scientific knowledge on fibre and its application in practical swine nutrition. Nowhere is this more apparent

than in analytical techniques, where research laboratories measure soluble versus insoluble fibre, individual sugars and various other fibre fractions, while commercial laboratories are typically determining crude fibre or ADF and NDF. This gap in adoption represents a significant opportunity to advance the field of swine nutrition.

Finally, the successful adoption of exogenous

enzyme use in swine production represents a substantial opportunity to assist the industry in utilizing diets that increasing contain significant quantities of fibre. Other options for enhanced use include feed processing technologies – some of which can be employed in concert with enzymes – and genetic selection of animals with enhanced capabilities to handle lower energy, higher fibre diets.

Table 7. Differences in the bulk density of common feed ingredients

Ingredient	NDF, % ¹	Density (kg/m ³)	Index ²
Barley, ground	18.29	400.5	68
Corn, ground	9.11	592.7	100
Corn DDGS	30.46	560.3	95
Oats	25.30	320.4	54
Peas	12.84	800.9	135
Soybean meal	8.21	656.8	111
Soybean hulls	59.39	368.4	62
Wheat, ground	10.60	608.7	103
Wheat, middlings	34.97	320.4	46

¹NRC, 2012

²Relative to corn = 100



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What are the unique benefits of fibre cross species?

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Introduction

The gastrointestinal tract consists of different compartments – mouth, stomach, small intestine and large intestine – and supplying organs – liver, pancreas – involved in the digestion and absorption processes. The whole assembly is integrated with the peripheral organs through a large set of receptors (distension, tactile, chemo) that monitor the digestive and absorptive processes through neural and hormonal feedback signals. In this way, variations in blood nutrient content are minimised and the provision of nutrients to the different organs are regulated and optimised. Fibre is an important component of plant based feedstuffs used in the feeding of non-ruminant species and is the foremost important dietary factor influencing the digestion and absorption processes. Fibre also plays an important role for the regulation of nutrients to organs and tissues not in direct proximity to the gastrointestinal tract.

The main purpose of this paper is to discuss, in a generic way, the role of fibre in digestion, absorption and metabolism. The paper has a generic approach but most of the results will be based on results obtained on pigs.

Definition and terminology

Fibre is not a well-defined chemical entity,

but a term that in the nutrition literature has been defined by the methods applied for its analysis – crude fibre, neutral detergent fibre and dietary fibre (DF). In human nutrition, fibre is now defined as: “Carbohydrate polymers with three or more monomeric units which are neither digested nor absorbed in the human small intestine” (de Menezes *et al.*, 2013). In animal nutrition, however, it appears more relevant to stick to the chemical definition of DF; the sum of non-starch polysaccharides (NSP) and lignin (Theander *et al.*, 1989). This term is better related to the classic methods used for the characterisation of fibre in feeds, i.e. neutral detergent fibre and crude fibre and to the components of the feed with low overall digestibility (Bach Knudsen *et al.*, 2013). The neutral detergent method measures most of the insoluble components and crude fibre, primarily cellulose and lignin (Table 1).

The main polysaccharides of NSP are cellulose, and a wide variety of non-cellulosic polysaccharides (NCP): β -glucan, arabinoxylan, xylans, xyloglucans and pectic substances to mention the major ones (Bach Knudsen *et al.*, 2013; Theander *et al.*, 1989). The composition of the DF fraction varies in the different feedstuffs; cereals, legumes, protein crops and fibre rich materials as shown in Table 1. From the monomeric

composition of the NCP it can be seen that the main polysaccharides of the NCP fraction in cereals are arabinoxylan and beta-glucan

whereas in legumes, protein crops and fibre rich materials pectic substances and xyloglucans are the main polysaccharides.

Table 1. Fibre composition (% of dry matter) of common feedstuffs

	CORN	WHEAT	BARLEY	WHEAT BRAN	SOYA BEAN MEAL	PEAS	SUGAR BEET PULP
Cellulose	2.1	1.8	4.0	7.0	5.9	5.3	19.5
Non-cellulosic polysaccharides	7.0	9.5	14.6	29.4	15.1	12.1	58.4
Rhamnose	-	-	-	-	0.2	0.1	1.0
Arabinose	2.0	2.8	2.7	8.5	2.6	3.6	18.9
Xylose	2.7	4.5	5.7	14.7	1.8	1.3	0.9
Mannose	0.2	0.2	0.4	0.5	1.3	0.2	0.8
Galactose	0.5	0.4	0.3	0.8	4.2	0.6	4.7
Glucose	0.8	1.2	5.0	3.4	0.6	3.1	1.2
Uronic acids	0.7	0.4	0.5	1.5	4.5	3.0	30.4
Total NSP	9.0	11.3	18.6	36.4	21.0	17.4	77.9
Klason lignin	1.1	1.8	3.3	7.0	1.8	1.0	3.5
Dietary fibre	10.1	13.1	21.9	43.4	22.8	18.4	81.4
Neutral detergent fibre	10.1	11.2	16.5	37.1	15.3	14.6	42.0
Crude fibre	2.5	3.0	5.3	10.2	7.7	6.4	20.7

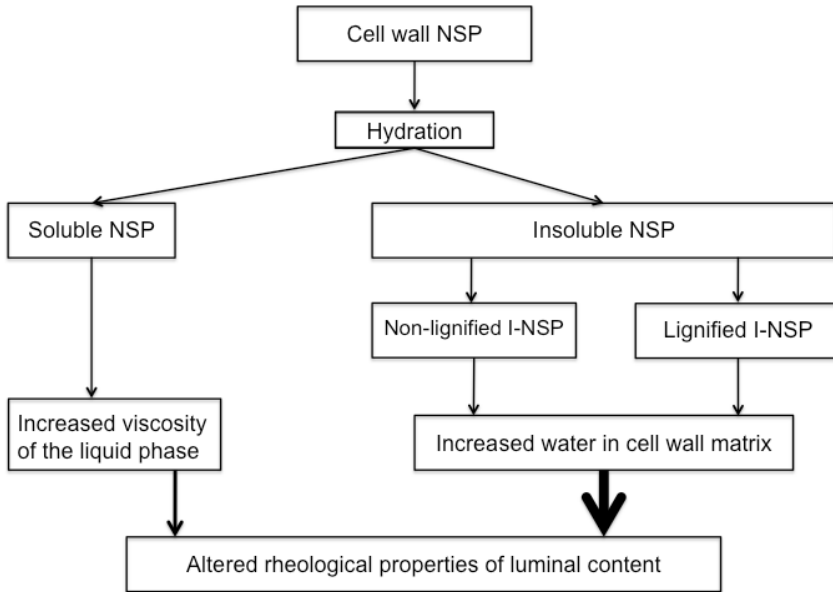
NSP, non-starch polysaccharides
Data from Bach Knudsen (1997)

Physicochemical properties

A common feature of all DF sources is the ability to swell, hold water in the cell wall matrix and to increase viscosity when exposed fluids (Figure 1) (Thibault *et al.*, 1992). However, while all DF sources swell and hold water, the viscous properties depend on the type and chemical nature of the polysaccharides making up the DF fraction. For instance, although sugar beet pulp has a high content

of soluble DF, it will primarily increase the water binding capacity of digesta whereas the viscosity elevating properties of sugar beet pulp is relatively low. Contrary to that, beta-glucan and arabinoxylan will solubilise from the cell wall matrix to a larger extent and raise luminal viscosity. Both the water binding capacity of digesta as well as the viscosity will both have a profound influence on the rheological properties of digesta.

Figure 1. Impact of soluble and insoluble non-starch polysaccharides on the rheological properties of digesta



Influence on the digestion and fermentation processes

DF influences the digestion and absorption processes at all sites of the gastrointestinal tract as well as the secretion of fluids from pancreas and liver (Figure 2, Table 2). Raised DF increases the weight and content of the gastrointestinal tract and leads to a higher flow of nutrients at all sites (Table 2). Concentration and composition of ileal NSP are primarily determined by dietary concentration and composition although the proportion of NSP that reaches the terminal ileum depends on

source and composition of the DF fraction (Bach Knudsen *et al.*, 2013). For instance, beta-glucan from barley and oat is heavily degraded and modified during passage of the small intestine in contrast to arabinoxylan that is almost quantitatively recovered in ileum and with a much lower modification (Bach Knudsen and Lærke 2010; Fadel *et al.*, 1988; Johansen *et al.*, 1997). Studies also show that the degradation of beta-glucan in the small intestine may be influenced by the presence of slowly digestible carbohydrates, i.e. lactose (Bach Knudsen 2012).

Figure 2. Influence of soluble and insoluble DF on digestion and absorption processes in the various segments of the gastrointestinal tract

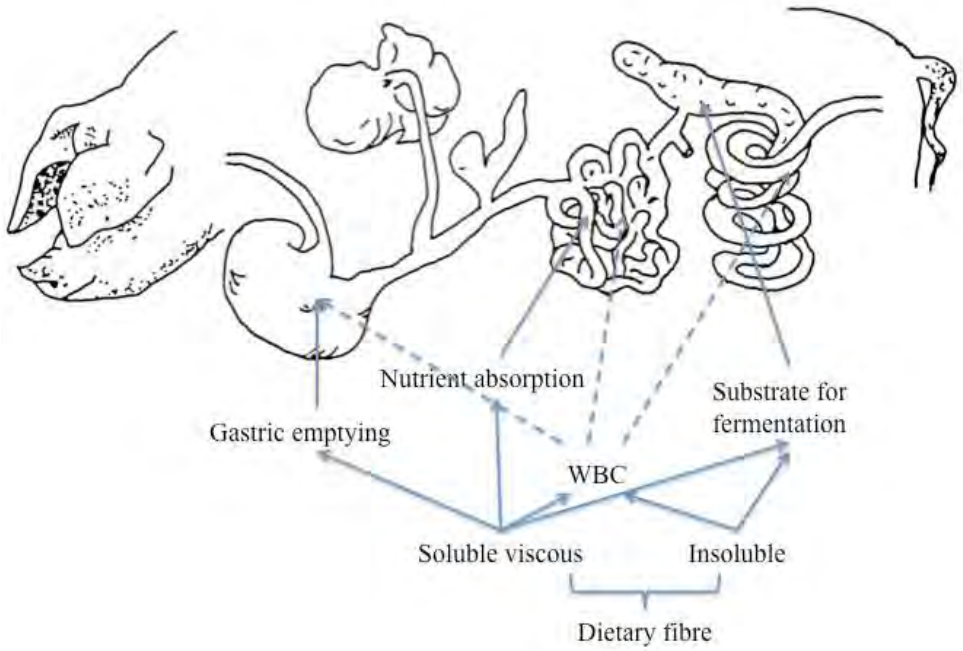


Table 2. Digesta flow, marker index, and concentration (g/kg DM) of carbohydrates in diet and ileal digesta

		DIGESTA FLOW	MARKER	DIG CHO		NDC	
		G/D	INDEX	SUGARS	STARCH	FRUCTANS	NSP
GROWING PIGS	Low dietary fibre						
	Diet		100	6	517		56
	Ileum	2,126	652	7	17		366
	Medium dietary fibre						
	Diet		100	7	454		97
	Ileum	2,584	472	8	12		372
	High dietary fibre						
	Diet		100	29	492	14	211
	Ileum	3,785	345	8	28	20	514
ADULT SOWS	Low dietary fibre						
	Diet		100	21	501	9	140
	Ileum	5,560	347	10	59	3	267
	High dietary fibre						
	Diet		100	23	210	6	363
	Ileum	9,816	187	3	33	1	507

Dig., digestible; CHO, carbohydrates; NDC, non-digestible carbohydrates; NSP, non-starch polysaccharides
 Data compiled by Bach Knudsen *et al.*, (2013)

Although all compartments of the gastrointestinal tract of pigs are colonised, it is the large intestine that is the main site for microbial degradation of DF and DF is the main substrate for the microflora (Table 2) (Jensen and Jørgensen 1994). Soluble DF is mainly degraded in caecum and proximal colon, whereas insoluble DF is degraded at more distal locations at a slower rate (Bach Knudsen *et al.*, 2013). DF stimulates the activity of the entire microbial ecosystem, but some specific oligomers and polymers may additionally have the capability of stimulating beneficial groups of microorganisms (*Lactobacillus* spp. together with *Bifidobacterium* spp.)

which potentially may protect the animals from gut infection (Bach Knudsen *et al.*, 2012; Flint *et al.*, 2012). The microbial fermentation of DF results in the production of short-chain fatty acids (SCFA; acetate, propionate and butyrate) and a reduced luminal pH (Bach Knudsen *et al.*, 2012). The proportion of produced SCFA's is influenced by the composition of fermented NSP but also the luminal pH appears to play a role, particularly for the balance between the production of acetate and butyrate (Duncan *et al.*, 2009). The produced SCFA is rapidly absorbed from the lumen, and less than 5% remains in the gastrointestinal content. From an analysis of the faecal excretion

we know that the DF components resisting digestion are lignin and polysaccharides in lignified tissue (Bach Knudsen *et al.*, 2013). Undigested DF will increase the bulk in the large intestine and faeces and is the main cause for the reduced total tract transit time seen when feeding high DF diets.

In general, DF has a negative impact on the apparent digestibility of protein at ileum and over the total tract (Bach Knudsen *et al.*, 2013). This is primarily caused by the raised endogenous secretion (pancreatic enzymes, slaughter epithelial cells) of nitrogen and by the encapsulation of nutrients within cell structures, (i.e. aleurone cells) which hinders access to potentially available nutrients (Johansen *et al.*, 1997; Saunders *et al.*, 1969; Tervila-Wilo *et al.*, 1996). In this way, nutrients that potentially are available for absorption in the small intestine is lost for the animal, e.g. amino acids released by microbial fermentation in the large intestine cannot be utilised by the animal.

Influence on the absorption processes

DF typically substitutes starch and sugars in diets for non-ruminant species, whereas protein and fat are reasonably constant. A consequence of more DF and lower starch and sugar is a changed provision of carbohydrate derived assimilation products, reduced average absorption rate of glucose, and increased absorption rate of short-chain fatty acids as illustrated by the experiment with sows in Table 3. Soluble DF that has the potential of elevating viscosity and the water binding capacity may further have the ability of prolonging gastric emptying and reducing the rate of uptake by hindering the contact between the substrate and digestive enzymes and by slowing down the movements of hydrolytic products of the digestion processes. However, relatively high levels of soluble DF are needed when feedstuffs high in soluble DF are used, whereas concentrates high in soluble DF are more efficient.

Table 3. The influence of fibres on the weight of gut content, concentration of insulin in plasma and absorption of carbohydrate derived nutrients

	LOW FIBRE	HIGH FIBRE INSOLUBLE	HIGH FIBRE SOLUBLE
Dietary composition, g/kg DM			
Starch	518	239	217
Dietary fibre	175	453	430
Gastrointestinal content, kg			
Stomach	4.3 ^b	5.6 ^a	6.1 ^a
Small intestine	1.5 ^b	2.1 ^a	2.4 ^a
Large intestine	5.1 ^b	8.6 ^a	7.5 ^a
Absorption, mmol/h			
Glucose ¹	419	189	124
Short-chain fatty acids	133 ^c	218 ^b	321 ^a
Concentration, pmol/L			
Insulin	138 ^a	98 ^b	87 ^b

^{a-c} Within a row, means without a common superscript are different ($P < 0.05$)

¹Diet (D), time (T) and D \times T are all statistically significant

Data from Serena *et al.* (2008a); Serena *et al.* (2009)

Influence on energy

DF is the dietary component with the largest negative effect on digestible, metabolisable and net energy. This is caused by the fact that not all energy present in DF can be degraded, that the cost of digesting DF rich diets is higher and that the utilization of absorbed energy deriving from SCFA is lower than of glucose (Bach Knudsen *et al.*, 2013).

Age effect

Adult animals can tolerate and handle higher levels of DF than is the case with young fast growing animals. For instance, the level of DF in diets for piglets and growing-finishing pigs is typically restricted to 150–250 g/kg DM because the physical capacity of the

gastrointestinal tract makes it impossible for the pigs to compensate for the lower provision of nutrients (Bach Knudsen *et al.*, 2013). Adult sows, however, particularly after their first litter, can tolerate substantially much higher levels of DF. In an experiment with sows fed either a low DF diet (170 g/kg DM) or diets high in insoluble DF or soluble DF (430–450 g/kg DM) it was found that the high DF diets increased the amount of undigested residues in the gastrointestinal tract and influenced the absorption profile of nutrients (Table 3) (Serena *et al.*, 2008b; 2009). Thus, a substantial reduction in absorption of glucose and a concomitant increase in the absorption of SCFA were seen when feeding the high fibre diets. Reduce diurnal variation in the uptake of energy and fluctuation in insulin was also

seen (Serena *et al.*, 2009). These conditions will keep the sows satiated for a longer period of time and can potentially reduce the incidence of aggressiveness, stress and/or stereotype behavior in sows incurred by hunger.

Conclusion

DF has a direct effect at all sites of the gastrointestinal tract but is also a dietary component that can be used to modulate the digestion and absorption of carbohydrate derived nutrients and the hormonal response. In the wider perspective, this has consequences for gut health and animal welfare.

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Impact of fibre on intestinal microbiota

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The concept of fibre in regard to different animal species

An average consumer would say that fibre in the daily diet is good for health. The reasons for the beneficial effects of fibre are not so well known. One of the reasons is undoubtedly that fibre adds bulk in the diet, thus enhancing satiety and reducing the overall energy intake. This is certainly a significant aspect for the growing number of individuals with obesity problems. The definition of fibre requires several aspects to be considered. The American Association of Cereal Chemists takes a physiological (rather than chemical) approach in their definition (<http://www.aacnet.org>):

"Dietary fiber is the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine, with complete or partial fermentation in the large intestine. Dietary fiber includes polysaccharides, oligosaccharides, lignin, and associated plant substances. Dietary fibers promote beneficial physiologic effects including laxation, and/or blood cholesterol attenuation, and/or blood glucose attenuation."

According to the AACC definition, carbohydrates susceptible to digestion by digestive enzymes of the host or small-intestinal microbiota should not be called

fibre. However, structures resistant to small-intestinal digestion are included in dietary fibre pool regardless of being degraded or not by the microbiota of the lower digestive tract. At first sight, dietary fibre does not stand out as being a nutritionally desired component in the diets of monogastric animals, when aiming at maximum feed conversion efficiency. Indeed, the physiological health benefits referred to by above definition of fibre are highly important for humans, but not necessarily relevant for production animals.

Potential benefits of fibre

Fibre may potentially provide benefits also for production animals through multiple mechanisms. The role and importance of fibre are dependent on the animal species and the health status of the animal.

Energy source. Carbohydrates resistant to digestive enzymes of the host may be susceptible to digestion by the anaerobic microbial community of the gastrointestinal tract, producing metabolites possessing energy value for the host (Bergman, 1990). This concept is highly pronounced in ruminants, in which 80% of their energy is produced through this mechanism. Also in swine (and human), hindgut fermentation may have as high as a 10 to 30% contribution to the total dietary

energy capture. The percentage is naturally dependent on the diet composition, especially its fibre content (Bergman, 1990). Birds have physiological limitations to the fermentation of fibre in their distal intestine. The fermentative capacity of the avian lower intestine is dominated by the paired caeca, a sieve-like entry which excludes insoluble fibre and most likely also large soluble polysaccharides. The large intestine of chickens is short and therefore its role as a compartment with intense bacterial fermentation is likely to be insignificant.

Promoter of intestinal health. Some fibre components have been shown to affect the composition of intestinal microbiota. In monogastric animals, a product category referred to as prebiotics has gained reputation as a group of oligosaccharides serving as specific growth substrates for beneficial bacteria (Gibson & Roberfroid, 1995; Roberfroid, 2007). Some short-chain prebiotics (e.g. fructo-oligosaccharides with low degree of polymerisation; DP) can be also metabolised by small intestinal bacteria (Alles *et al.*, 1997; Apajalahti *et al.*, unpublished data). According to the fibre definition such products should not be classified as fibres since they are not resistant to digestion in the small intestine. Indeed, strictly speaking true fibres can only support the growth of bacteria inhabiting compartments distal to the ileum. When the term prebiotic was originally launched, mainly lactic acid producing bacteria, such as bifidobacteria and lactobacilli, were referred to as beneficial. The complexity of microbiota has since then been revealed, making it increasingly difficult

to exactly define the bacteria that should be considered beneficial. For humans, lactobacilli and bifidobacteria in the colon may indicate good health, whereas for broiler chickens their abundance has instead been found to correlate with poor feed conversion efficiency (Apajalahti *et al.*, unpublished data). It is important to note these potential discrepancies, and, it might be again useful to redefine what is meant by beneficial bacteria and the term "prebiotic".

Fibre has been proposed to enhance intestinal health and digestion in many different ways. The physicochemical structure of fibre has been reported to influence intestinal motility, morphology of the gut, the villus height, crypt depth as well as mucus production and structure, to mention some proposed effects on intestinal epithelium (Montagne *et al.*, 2003). Through such mechanisms fibre can be assumed to affect intestinal health in many ways and also interact with the intestinal microbiota. Certain fibres have been found to exhibit positive effects on the nutrient-absorbing epithelium (e.g. increased villus/crypt ratio), while for some fibres opposite effects have been reported (Langhout *et al.*, 1999). Since different carbohydrate structures appear to affect these mechanisms in a different manner, we cannot univocally state that fibre has a certain uniform health effect on intestinal morphology.

Fermentation of fibre

A vast variety of intestinal microbes participate in the digestion of one or more carbohydrate structures referred to as fibre.

While some bacteria use polysaccharidases to attack insoluble fibre by rendering it soluble and accessible to other micro-organisms, others adhere on the fibre and digest it by cell wall-bound enzymes. Soluble polysaccharides are hydrolysed to oligosaccharides and monomers and eventually converted to metabolites of sugar fermentation, i.e. organic acids and gases most often in intestinal systems. In anaerobic habitats primary metabolites can be further utilised by a second array of bacteria, until the metabolites have no more energy to be captured under the prevailing conditions. Under anaerobic systems such as rumen, colon or caecum this means that the metabolites are reduced to an extent which is possible in the habitat; in other words, the compounds can no longer accept electrons. It is worth noting that compounds (e.g. methane) that have no energy value in the anaerobic low-redox habitat may have a lot of in-built energy at the high-redox environment in the presence of molecular oxygen. In practice this means that upon crossing the cell membrane of intestinal enterocytes, the true energy value of metabolites such as volatile fatty acids is instantly increased.

Fibre can be soluble or insoluble. For monogastric animals soluble fibre can be harmful and anti-nutritional (Choct *et al.*, 1996). This is largely due to the fact that soluble fibre increases small-intestinal viscosity, reduces digesta passage, nutrient diffusion and digestibility of many important nutrients. The changed environment in the small intestine may also lead to bacterial overgrowth and, as a consequence, reduced

nutrient and energy capture by the host. Soluble fibre is at least partly fermented by bacteria in the lower digestive tract, thus affecting the ecology of the habitat and providing high-energy metabolites for the host. The degree of fermentation of insoluble fibre is low especially in the intestine of young monogastric animals. Bacteria in the rumen are instead efficient degraders of all types of soluble and insoluble fibre. Intact plant fibre may appear as tightly packed polysaccharide bundles, the rate of digestion of which is limited by the exposed surface area for bacteria hydrolysing fibre (Varga & Kolver, 1997).

Many fibre-associated problems can be alleviated by exogenously provided feed enzymes. In monogastric animals the anti-nutritional effects caused by viscous polysaccharides can be reversed by enzymes that hydrolyse non-starch polysaccharides (Choct *et al.* 1999). Such enzymes have been reported to stimulate bacterial fermentation in the caecum of broiler chickens, most likely by increasing the concentration of soluble, low DP polysaccharides that are able to access the caeca.

In ruminants, the surface area of fibre for the fibrolytic bacteria can be increased by endo- β -glucanases and hemicellulases, which can open up the insoluble fibre bundles and partially solubilise the polysaccharides. In a recent study performed in our laboratory we found that β -glucanase treatment increased the density of the ends of sugar chains in insoluble fibre. After being exposed to rumen bacteria, such fibre was



digested at a higher rate than the untreated fibre (Apajalahti *et al.*, unpublished data). Enzyme treatment can be thus expected to accelerate the digestion of roughage and increase the feed intake in ruminants.

Fermentation of intact dietary fibre in a complex ecosystem involves numerous bacterial species directly or indirectly and consequently maintains the diversity of the microbial community (Varga & Kolver, 1997). When introducing isolated functional fibres, such as prebiotics, it is to be expected that selected bacterial groups are favoured with a corresponding reduction in microbial diversity. The scientists that launched the term prebiotic 20 years ago described prebiotic as a carbohydrate that selectively favours bifidobacteria and lactobacilli (Gibson & Roberfroid, 1995). Perhaps the most extensively studied prebiotic, inulin, has indeed been shown to favour bifidobacteria and increase the residual concentration of lactic acid in the lower gastrointestinal tract of test animals (Apajalahti *et al.*, 2002). It should be noted, however, that lactic acid should be a transient metabolite in balanced lower intestinal fermentation; all lactic acid produced should be utilised concomitantly. Therefore, prebiotics that stimulate lactic acid-producing bacteria may be detrimental for animals with a compromised epithelium in the small intestine and, consequently, leakage of excessive monomeric glucose to lower intestine.

On the other hand, the presence of increased concentrations of lactic acid may also influence the resident microbiota; it has been shown that various organic acids,

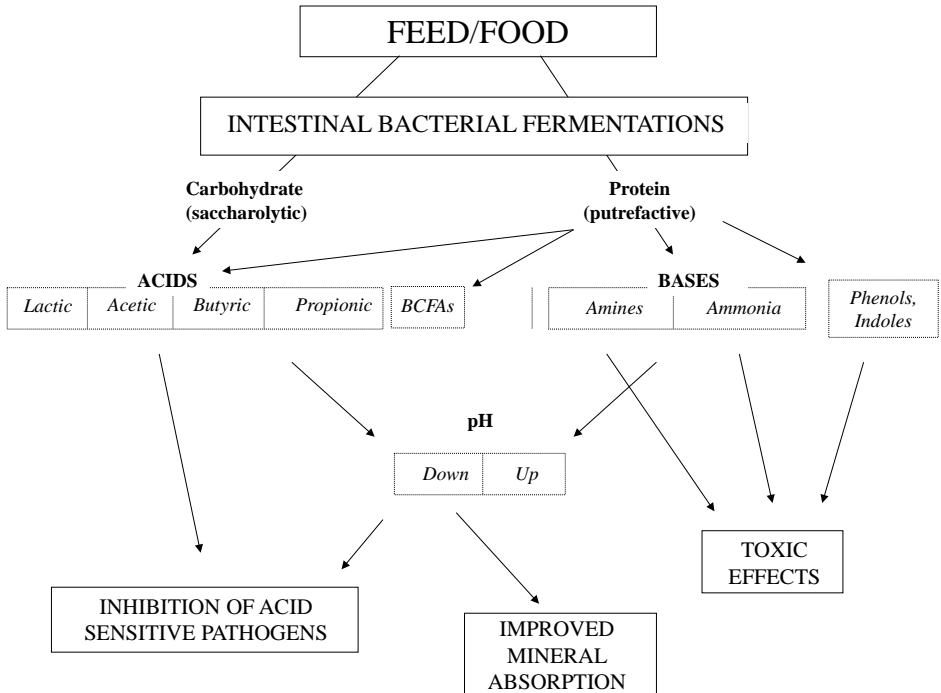
most notably lactic acid have a disrupting effect on the Gram-negative bacterial outer membrane *in vitro*, resulting in increased permeability of the cell envelope towards external substances and possibly increased susceptibility to agents that are normally excluded by the Gram-negative bacterial cell envelope (Alakomi *et al.*, 2000; Helander & Mattila-Sandholm, 2000). Indeed, through this mechanism lactic acid could potentially lead to a beneficial effect by suppressing harmful Gram-negative bacteria.

Among the most important effects of slowly digestible dietary carbohydrates is the effect on the balance between saccharolytic and putrefactive fermentation in the lower gastrointestinal tract (Figure 1). This is important because protein fermentation produces several harmful (toxic) compounds such as ammonia, amines, N-nitroso compounds, phenols, thiols and indoles. The lower intestine harbors numerous bacteria that can potentially utilise both carbohydrates and proteins for their growth. It has been found that the investigated bacteria preferentially utilised carbohydrates when both of these substrates were present. Feeding studies have revealed that oligosaccharides and resistant starch suppressed putrefaction, measured as the residual concentration of products of protein fermentation (Alles *et al.*, 1997; Le Leu *et al.* 2007). Protein fermentation also tends to increase intestinal pH, thus cancelling the pH-lowering effect of carbohydrate fermentation. A lowering effect on pH is considered beneficial, because most intestinal pathogens have a lower growth rate at low pH. In conclusion, indigestible protein

that enters the lower gastrointestinal tract should be accompanied by carbohydrates

to reduce the intensity of putrefaction and the detrimental effects that potentially follow.

Figure 1. Schematic presentation of characteristic pathways and potential health effects of saccharolytic and putrefactive fermentations by intestinal bacteria (figure from Apajalahti, 2005)



Studies involving fibre amendments

In studies investigating the effect of dietary fibre on microbiota composition, one of the most problematic issues is to construct the test diets in a manner resulting in an experimental design that fulfils the scientific criteria. Multiple components are often concomitantly altered, making it difficult to know whether the observed microbial shifts were caused by the changed fibre

structure. A typical example was a study where the wheat-rye ratio was changed in the diet of broiler chickens in order to see the effect on different intestinal lactic acid bacteria. The results clearly showed that increased inclusion of rye decreased the abundance of lactobacilli, while that of lactic acid-producing cocci increased (Figure 2). It cannot, however, be concluded whether the shift was due to the different structure of the

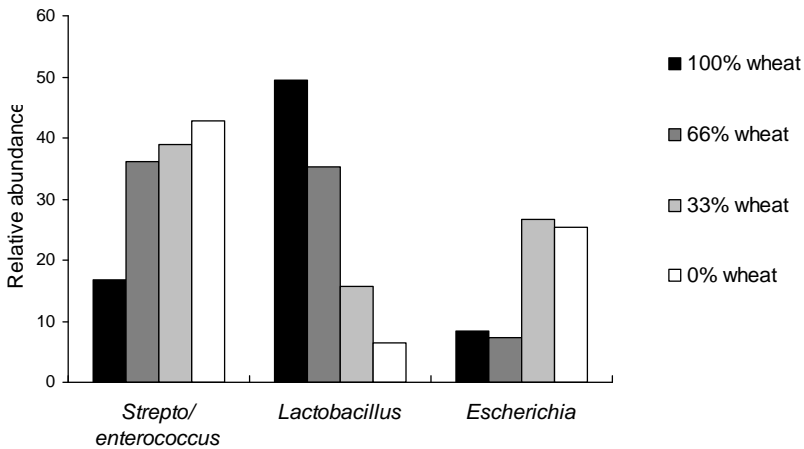


fibre component of the grains or to some other variable.

Corresponding studies have also been carried out with functional fibres or prebiotics enriched from various plant sources. Choct and co-workers have shown that dietary inclusion of non-starch polysaccharides

enriched from wheat significantly boosted bacterial fermentation, especially in the ileum of broiler chickens (Choct *et al.*, 1996). Xylanase supplementation significantly reduced the intensity of fermentation in the ileum, but boosted fermentation in the caecum (Choct *et al.*, 1996; 1999).

Figure 2. Effect of wheat-rye ratio of diet cereals on the abundance of selected bacterial gener



Inulin amendment to the Western-type high fat diet for mice caused boosted fermentation in the caecum as compared with the non-amended diet. Residual concentrations of volatile fatty acids and lactic acid increased (Apajalahti *et al.*, 2002). In the same study the microbiota composition was studied by using %G+C chromosomal DNA profiling and 16S rDNA sequencing. Both methods revealed significant changes in microbiota composition by inulin amendment. Selective stimulation of bifidobacteria has been reported to take place in numerous studies applying culturing techniques for microbiota

analysis, (Gibson *et al.*, 1995; Bounhik *et al.*, 2007). Stimulation of bifidobacteria by inulin was also noted by molecular techniques, but a numerically much greater stimulation was found for unknown bacteria that could not be assigned to any bacterial phylum. This example implies that while a prebiotic might show selective stimulation of bifidobacteria upon quantitation of only a limited number of bacteria, a culture-independent method might indicate that the stimulation was not, in fact, specific to bifidobacteria. A prebiotic might actually selectively stimulate an unknown bacterial

group, the role of which on health and disease remains to be discovered.

Conclusion

Dietary and functional fibres undoubtedly affect the composition and metabolism of microbiota of the gastrointestinal tract. Also, fibres affect intestinal health through multiple mechanisms some of which may be microbiota mediated. However, there are few or no controlled studies showing the specific effects of fibres on the entire intestinal microbiome and linking such changes to performance of monogastric production animals. Dietary fibres may well affect positively the productivity of animals with hidden intestinal health problems but impair the feed conversion efficiency of healthy animals by reducing the energy density of the diet.

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Feed, Fibre, Enzymes Modify Necrotic Enteritis

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Necrotic enteritis is a common but complex live production problem with broilers. Research conducted at understanding *Clostridium perfringens* A has generally employed models that orally gavage concentrated cultures while imposing feedstuffs that increase digesta viscosity along with coccidia to impair mucosa integrity. Such infection models (Shojadoost *et al.*, 2012; Pederson *et al.*, 2008) do well at creating necrotic enteritis in the laboratory; however, resulting observations may be inappropriate to the field. Present approach takes a cursory view of feedstuffs and commercial enzymes used at-large in terms of organism response when confronted with intestinal specific.

C. perfringens A is a gram+ rod that thrives in an anaerobic environment approximating 30-35°C when provided with proteins characteristic of flesh. Under these terms, a 20 minute turnaround ensues until sporulation. Sporulation allows accrued toxins A and/or the recently described netB to be freed upon cell lysing. Most field diagnosed necrotic enteritis involves colonization of the latter half of the small intestine which first impairs feed recovery then growth as surface loss progresses. Infection accessing the portal system provides access to the liver leading to carcass condemnation after processing.

The small intestinal lumen is normally aerobic and unfavorable for *Clostridium perfringens* A development. Extensive digesta refluxing occurs after evacuation from the gizzard until the yolk stalk (Moran, 1982). Such motility maximizes digesta convection with walls to enhance nutrient absorption while oxygen diffuses from villus arterioles into the lumen (Macagno *et al.*, 1982; Sheppard and Kiel, 1992). Villi prominence, extent of motility and oxygen transfer all decrease with distal progression along the pig's intestine to shift microbes from aerobe to favor anerobe (Hillman *et al.*, 1992). A parallel situation is assumed to occur with fowl. Given that pig motility employs peristalsis together with segmentation while fowl depend on refluxing, the loss in dissolved oxygen with distance may be more extensive.

Fiber with many grains and their by-products, particularly rye, barley and wheat, contain non-starch polysaccharides (NSP) that ultimately increase viscosity of digesta in the small intestine. Such viscosity not only impairs wall convective exchanges involving nutrients except oxygen, but, accentuates microbial favorability from aerobes to anaerobes with distance. Potential change in lumen viscosity can be further influenced by gizzard pH and activities that enhance hemicellulose "dissolution"

(Mishima *et al.*, 1998). In addition to NSP, "Fiber" contains protein in small amounts that parallels animal connective tissues in composition and structure (Rhodes and Stone, 2002; Robertson *et al.*, 1997). Pepsin has hydrolytic specificity hydrophobic end groups found extensively in connective type proteins to resemble chymotrypsin and most commercial supplemental proteases.

The large amounts of connective tissues in animal source by-products depend on pepsin for their digestion (Hegedus, *et al.*, 1989) paralleling the protein in fiber. Contributions of phytin are known to vary among plant feedstuffs. The gizzard composite at low pH is expected to solubilize phytin and create negatively charged phytic acid (Crea, *et al.*, 2004; Kaufman and Kleinberg, 1971). This prominent negative molecule is envisaged as complicating removal of an extensively positive peptide with activation of pepsinogen (Bohak, 1969). Decreasing the operational level of gizzard pepsin likely has repercussions on the cleavage of all sources of connective proteins while infringing on fiber dissolution. Effectively decreasing phytin by using supplemental phytase is viewed as relieving these complications to gastric digestion while nutritionally availing phosphorus.

Conveying gastric digesta into the duodenum via refluxing motility rapidly incorporates pancreatic juice and bile for neutralization permitting digestion to ensue. All pancreatic enzymes have an associated Ca^{+2} that stabilize their tertiary structure and lengthen the life of their activity. Delaying autolysis of this digestive enzyme complex

improves an extended duration of activity by which to recover poorly digestible feedstuffs. Duodenal phytic acid rapidly presented after gastric release and neutralization now has the potential to capture this Ca^{+2} and cause fragility. The subsequent extent of digestion with difficult nutrients suffers before reaching the large intestine (Moran, 2010). Animal meals varying in connective protein and grains heated to alter starch structure are examples of "difficult" feedstuffs in need of extended digestion. Supplemental phytase may now relieve another type of complication created by dietary phytin. If phytin had enabled continuation of microbiologically sensitive indigesta accessible concurrent with anaerobic conditions, then *C. perfringens* A would be positioned for population expansion. Animal protein feedstuffs, given their inherent difficulties at digestion, represent one situation particularly susceptible to delayed recovery at the lower small intestine and favorability to *C. perfringens* (Khattk, *et al.*, 2009; Palliyeguru *et al.*, 2010; Wilkie *et al.*, 2005). The ability of *C. perfringens* to digest cereal non starch polysaccharides is highly limited (Branto, *et al.*, 1996); thereby, extending ability to extend viscosity and an anaerobic environment.

Lumen protection by the unstirred water layer requires the cooperation of mucins from enterocytes and goblet cells. The fundamental mucin unit involves threonine and serine as major "hydroxyl-connectors" in a protein core whereby attachment of 6-10 linear sugars, amino sugars and sugar acids create a bottle brush arrangement. Membrane associated mucin connects

these units in a linear sequence such that a filament (glycocalyx) arises from CHO content and length to project from enterocyte microvilli. Goblet cell mucin has a greater CHO to water favorability while employing cystine to interconnect core proteins into a net-like structure. Negative charges associated with the sugar-acid CHO's necessitate that goblet cells incorporate Ca^{+2} to permit condensation of net into a granule (Perez-Vilar, 2007). Ca^{+2} "depletion" upon granule release into the lumen is thought to progressively "free" the net for subsequent "entanglement" in microvillus filaments and form the "unstirred" water layer.

This "molecular sieve" is thought to act by protecting enzymes finalizing digestion at the membrane surface from their destruction by pancreatic enzymes contained in the lumen while obstructing microflora. Hypothetically, "free" phytic acid accessible upon granule release enhances Ca^{+2} removal by forming insoluble calcium phytate as mucin net "opens" and filament capture. Supplemental phytase is suspected as reducing this rate of transition in mucin's physical character. In turn, phytase would seem to fulfill another means of minimizing digestive complications by indirectly conserving endogenous loss. Enhanced surface retention of mucin conserves endogenous N loss. This advantage builds on a combination of previous problems involving gastric and pancreatic enzyme difficulties while enhancing mucin layer stability. The extra-nutritional potential of phytase is well established (Cowieson *et al.*, 2006; Onyango, *et al.*, 2008; Walk *et al.*, 2013).

Actual pathogenicity attributed to *C. perfringens* relates to its "A" and "B-like" toxins which lyse enterocyte membranes enabling sub mucosal access. The unstirred water layer encapsulates the entire villus with the exception of its apex (Smedley *et al.*, 2004) where the mucin covering the extrusion zone is exceptionally thin (Holman, 1975; Potten and Allen, 1977). Binding by *C. perfringens* to the villus such that colonization and infection ensues focuses on extracellular proteins (ECMM) located as lateral enterocyte interconnections (Martin, 2010; Olkowski *et al.*, 2008). ECMM locations lie below the unstirred water layer; however, *C. perfringens* has an arsenal of glycosidases, particularly neuraminidase and fucosidase, that are adept at cleaving mucins (Chow and Lee, 2008; Macfarlane *et al.*, 1989; Wold *et al.*, 1974). Enterocyte access is least obstructed by the unstirred water layer at the villus apex. Establishing an infection would not only require a "critical" number of *C. perfringens* cells in the lumen, but anaerobic and nutritional terms to support population activity.

Necrotizing enteritis in its initial stages of development has been microscopically established at cell disruption areas of the villus extrusion zone (Kalhudaahl *et al.*, 1995; Martin, 2010). Subclinical infection progressively impairs digestive efficiency with an eventual villus failure that extends from bird to flock. Infringement of micro vessels provides an opportunity for *C. perfringens* and toxins to enter the portal system and create hepatic lesions (Jarmund and Telle, 1982; Sasaki *et al.*, 2003). Lovland and Kaldhudaahl, 1999 were able to establish



a correlation between the incidence of field necrotic enteritis and occurrence of hepatic condemnations in the processing plant.

Coccidia are known to facilitate expression of necrotic enteritis. Merazoitites can readily pass through the unstirred water layer and microvilli membrane to parasitize enterocytes. Eventual cell lysing several days later likely provides ECMM access for *C. perfringens* binding, colonization and toxin release, thereby contributing to invasion at the extrusion zone. Altered motility and gut stasis associated with infection likely extends anaerobic advantage. Eimeria species and their preferential location for invasion are meaningful for *C. perfringens* A incidence. Species favoring the lower small intestine prevail in field cases of necrotic enteritis, whereas duodenum-jejunum lesions are prevalent when laboratory terms are invoked (Baba *et al.*, 1992; Baba *et al.*, 1997; Chapman *et al.*, 2005).

Necrotic enteritis evolving in the field differs from events created by laboratory modeling. Although *C. perfringens* A is likely to be encountered at hatch and continuously consumed thereafter, an expansion of the "few" cells involved to create a debilitating infection defies terms commonly encountered. Aerobic conditions in the lumen dominate duodenum and early jejunum to discourage *C. perfringens*. Eventual decrease of lumen oxygen content while encountering animal feedstuff indigesta now favors population development. Increasing the extent of luminal viscosity by dietary NSP likely accentuates anaerobic terms to further enhance population

expansion. *C. perfringens* A activity encourages release of enzymes adept at cleaving animal connective tissues as well as mucin. Cleaving the mucin at the villus apex where poor protection exists facilitates initiation of colonization. Connective tissue like binding areas provides a basis for establishing a fixed community. Progressive loss of digestive surface as colonization descends the villus enables subclinical problems to escalate and clinical ones to eventually appear. Vascular entry of toxins and *C. Perfringens* cells provides the liver as a secondary infection site. Supplementing commercial enzymes to the feed offers an opportunity at suppressing necrotic enteritis without resorting to antimicrobials and loss of antibiotic free status. The "multifaceted" support of *C. perfringens* A to initiate and continue to thrive could suffer from dietary enzymes. Combining protease, phytase, amylase, and hemicellulase provides for action at a variety of GI locations and mitigate organism expression in a cumulative manner.

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Historical and commercial drivers of NSP-enzymes use in animal feeds

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Attendees at any animal feed exhibition will probably notice that numerous companies are offering a range of feed enzyme products, while those at any animal nutrition conference are likely to see a high proportion of papers addressed the effects of feed enzymes on animal performance. So why all this interest, and where did it start? The reason for the interest is simple; – feed enzymes are estimated to be saving the poultry and swine industries over \$3 billion per annum, with similar additional savings possible in the future through better application of enzyme technology. On the back of this, the feed enzyme market is currently estimated to be worth over \$1 billion per annum, and growing.

Where it all started is less well known. Enzymes have been used for centuries, for example in beer and cheese production. The first still functioning commercial enzyme company was started in Germany in 1907, initially producing proteases for leather treatment and now trading as AB Enzymes. As far back as the 1920's there are reports in the scientific literature of trials where supplemental enzymes improved animal performance. These enzyme products contained a wide range of activities produced by submerged fungal or bacterial fermentations, which is still the case for many feed enzyme products.

However, the first methodical research program was by the late Jim McGinnis and his colleagues in Washington State University, Puyallup, in the 1950's. Professor McGinnis was working with a range of cereal grains in poultry production, including barley and oats. Initial trials established that water-soaking of these grains could improve productive value. While the production of antibiotics by contaminating bacteria was suspected, subsequent work established that carbohydrase enzymes produced during the water treatment were primarily responsible for the improvements seen in bird performance. This paper will review the research at Uppsala and Montana State University in the 1970'2 and 1980's that helped clarify the mode of action of feed enzymes and facilitated the commercial introduction of fibre-degrading feed enzymes for monogastric animals in the mid-1980's.

The development in Uppsala of a comprehensive battery of methods for accurate carbohydrate analysis, including for dietary fibre components, mixed-linked β -glucans and starch, provided a major step forward in understanding (Åman & Hesselman, 1984 and 1985; Åman & Newman, 1986; Theander & Åman, 1979). Initial animal work focused on barley, looking at both productive value and the wet

litter problems in poultry often associated with feeding this cereal. This confirmed that digesta viscosity was a key causative factor and that supplementation with a β -glucanase-based enzyme product enzyme product could solve these problems (Table 1). It was also apparent that dietary viscosity was difficult to predict from diet composition; for example, viscosity was poorly correlated

to the content of soluble fibre. Subsequent work confirmed that supplementation with xylanase-based enzymes could have a similar positive effect on poultry and swine fed wheat, triticale and rye-based diets (Table 2). This series of trials, in both swine and poultry, established that feed enzymes improve animal performance by:

Table 1. Influence of β -glucanase supplementation and processing of a barley/wheat-based diet on diet characteristics and broiler performance (Pettersson *et al.*, 1991)

0-22 DAY PERFORMANCE	MASH DIET		COLD PELLETING		STEAM PELLETING	
	CONTROL	+ENZYME	CONTROL	+ENZYME	CONTROL	+ENZYME
Soluble β -glucans (%)	1.3	1.4	1.4	1.4	1.5	1.5
Soluble xylans (%)	0.5	0.7	0.5	0.5	0.8	0.7
Extract viscosity (cPs)	2.4	2.3	3.3	2.7	3.6	2.9
Excreta dry matter (%)	33.2 ^a	38.1 ^b	35.5 ^{ab}	34.0 ^a	34.0 ^a	33.3 ^a
Sticky droppings (%)	20.5 ^a	6.3 ^b	23.4 ^a	9.4 ^b	37.5 ^a	21.9 ^a
Weight gain (g)	400 ^a	464 ^b	508 ^c	566 ^d	517 ^c	574 ^d
Feed intake (g)	716 ^a	807 ^b	856 ^{bc}	925 ^{de}	898 ^{cd}	947 ^e
Feed conversion ratio (g/g)	1.79 ^a	1.74 ^{ab}	1.69 ^{bc}	1.63 ^c	1.74 ^{ab}	1.65 ^{bc}

^{a-e} $P < 0.05$

Table 2. Influence of β -glucanase or xylanase supplementation of rye- or barley-based diets on broiler performance (Pettersson *et al.*, 1990a)

0-24 DAYS	RYE-BASED DIET			BARLEY-BASED DIET		
	CONTROL	+GLUCANASE	+XYLANASE	CONTROL	+GLUCANASE	+XYLANASE
Liveweight (g)	401a	552b	527b	254c	716d	746d
Feed intake (g)	817a	1025b	947b	549c	1116b	1145b
FCR (g/g)	2.04a	1.86b	1.78b	2.17a	1.57c	1.54c

^{a-b} $P < 0.05$

1. Reducing digesta viscosity through the degradation of soluble fibres
2. Opening up feed cell walls through the partial degradation of insoluble fibre
3. Providing oligosaccharides for microbial growth by partial degradation of soluble and insoluble fibre

This leads to an improved ileal digestibility of nutrients such as starch, protein and fats (Hesselman & Åman, 1985 & 1986), with an increase in the degradation of some fibres prior to the ileum also apparent (Table 3). These effects are often masked in faecal samples by microbial activity in the hind-gut.

Table 3. Influence of enzyme (β -glucanase + xylanase) supplementation on ileal and faecal digestibility in pigs fed barley/wheat-based diets (Graham *et al.*, 1988)

22 KG PIGS	ILEAL APPARENT DIGESTIBILITY (%)		FAECAL APPARENT DIGESTIBILITY (%)	
	CONTROL	+ENZYME	CONTROL	+ENZYME
Crude protein	64.5	70.1*	79.4	81.2
Crude fat	60.0	65.5*	56.9	58.8
Starch	92.0	94.9*	99.2	99.6
Total NSP	12.2	13.2	52.4	55.6
Arabinoxylan	1.7	2.1	47.4	50.3
Cellulose	9.6	5.5	34.3	37.8
β -Glucan	40.1	58.6*	99.5	99.7
Xylan solubility %	15.9	22.2*	<1	<1
Soluble xylose:arabinose	1.46	1.60*	-	-

*differs from control ($P < 0.05$)

The commercial breakthrough for feed enzymes came in Finland in the Autumn of 1984, and was the result of a combination of several contributing factors. At that time locally grown Finnish barley was substantially cheaper than imported feed wheat, but was not widely used in poultry diets due to the anticipated litter and production problems. After a series of trials, lower cost broiler feeds based on barley and supplemented with a β -glucanase/xylanase-rich enzyme product were launched in Finland. This practice quickly spread to other monogastric species and countries where locally produced, relatively

cheap barley was available, and by the late 1980's similar enzymes were used in commercial barley-based animal feeds in, for example, Finland, Sweden, Canada, Spain and the UK. In 1989 wheat quality in the UK was poor, and animal production, particularly broilers and litter quality, was affected. Based on previous university trials, a xylanase-based product was tested commercially and feedback was positive, particularly regarding litter quality improvement. This application quickly spread to other countries where wheat was the main cereal in monogastric feeds. Based on the knowledge that arabinoxylans are



the main fibre-polysaccharide in corn and subsequent successful animal trials, the use of xylanase-based products in corn-based poultry diets started a few years later. Today corn-based applications are the main market for NSP-enzyme sales worldwide of close to \$500 million annually.

The initial thoughts on how to formulate with enzymes also came from this early experience in N. Europe. The original concept with barley-based diets was to formulate feeds as usual, with wheat as the primary cereal, and then replace wheat with barley + enzyme up to 40-50% of the diet, targeting the same animal performance. Thus barley + enzyme was assumed to have the same energy value as wheat. With energy values for barley and wheat of around 2760 and 3050 kcal/kg, respectively, that means the enzyme increased the energy value of the barley by approximately 10%. Practically, nutritionists then had two barleys in their formulation matrix, the first with a normal energy value and the second with 10% higher energy (i.e. 3040 kcal or 12.7 MJ/kg) and only included when the extra costs of the enzyme product was forced into the diet. A similar approach was taken with wheat, where energy was upgraded by approximately 6% with enzyme addition. By the time enzymes were introduced to the corn-based market, nutritionists were more aware that enzymes could affect the digestibility of all components in the diet, and thus an uplift of up to around 100 kcal/kg feed is applied when supplementing with enzymes.

Approximately 30 years since the first commercial in-feed use of NSP-enzymes,

a recent survey established that "single" xylanases account for approximately 36% of non-phytate feed enzyme sales, xylanase-based products blended with other activities (such as amylase and protease) for 31%, xylanase/glucanase-based "multi-enzyme" products for 18%, proteases for 8% and mannanases for 6%. Commercial products are all generally a mixture of various enzyme activities, some which are positive in the animal and others which can be negative due to the release of free sugars such as arabinose and xylose (Schutte, 1991). Modern products are optimised to contain more of the desired activity, although there are still a number of generally older "multi-enzyme products" available on the feed market. The initial problem of poor processing stability can be overcome by applying liquid products post-pelleting or by encapsulation with water-resistant materials. Both can have problems, the former of consistent application and the latter of slow release in the gut. However, in recent years suppliers have improved thermo-stability, through manipulation of the enzyme structure or by sourcing from organisms that live in high temperature environments such as hot springs, and there are now xylanases available that can be pelleted as high as 95°C/205°F (Graham, 2008). These xylanases are sufficiently thermo-tolerant to allow application into the feed mixer, prior to heat processing. This allows a better mixing of the enzyme with the feed, and, somewhat surprisingly, reduces the energy required to pellet the feed. This is presumably due to the partial degradation of the fibre during conditioning.

So almost 100 years after the first trials, the feed enzyme business has grown significantly and is again a hot research topic worldwide. Today around 70% of poultry diets and 50% of swine are supplemented with enzymes worldwide, and we are generally aware of the mode of action of feed enzymes and of the composition of diets. However, there is a general lack of understanding of the role of different enzyme activities and how these can influence, both positively and potentially negatively, animal performance. In particular, there are very few (if any) published trials where the product tested or diets fed to animals have been analysed for all a range of the enzyme activities present. Further, there is little understanding of how these different activities survive feed processing or through the acidic and proteolytic conditions of the upper gastro-intestinal tract, and there is a lack of awareness if the enzymes used in trials are endo- or exo-acting. The former,

which would rapidly reduce viscosity and produce oligosaccharides, would be expected to improve animal performance. The latter would have little influence on viscosity and produce single sugars, and thus could potentially have a negative effect on animal performance (Schutte, 1991). Until we better characterise the enzymes used in academic trials, it will prove difficult to draw concrete conclusions on the effects of specific enzymes, the benefits or otherwise of combining different activities or of dose-response relationships. As a consequence of this lack of clear understanding at both an academic and end-user levels, over 15 commercial products account for significant sales worldwide, and almost all of these products are more than 10 years old. There is a clear need to better understand feed enzymes and to clearly communicate this to end-users, to allow a more effective use of modern fibre-degrading enzyme products.

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Section 3

Examining enzyme solutions

There are important differences between NSP enzymes, even those belonging to the same class, in terms of substrate specificity, conditions for optimal activity, extent of NSP hydrolysis and resistance to in-feed inhibitors, pepsin and feed processing.

There is also variation in the environment in which NSP'ases have to act, as a result of differences in substrates, sections in the animal gut, species of animal and individual animals. All of the above can impact the efficacy of the enzyme. Given that a principal benefit of an NSP'ase is reducing variability between feeds, flocks/herd and individual animals, it is extremely important that the enzyme is consistently applied and delivered.

When NSP's are hydrolysed to fermentable oligosaccharides the effects they have depends upon their chain length and degree of branching. For example arabinoxylo-oligosaccharides (AXOS) with a chain length of 3-4 have shown to be more effective in promoting animal growth whereas longer chain AXOS are more effective in reduction salmonella contamination in the caeca of chickens. Thus the dose and specificity of the enzyme is critical if a consistent outcome is desired. Overdosing and use of multi-enzyme products may over-process these AXOS, thereby reducing efficiency. When

an optimal caecal fermentation is achieved, not only does it provide beneficial VFA's in the caeca, but it can stimulate release of peptide YY. This hormone can delay gastric emptying and may be thus responsible for increasing digestibility of the whole diet through increased gizzard retention time.

The potential to be gained from NSP-degrading enzymes is very obvious, but using more than one is clearly not additive. No advantage has yet been convincingly shown when multi-component enzyme products are used in an attempt to increase NSP hydrolysis. The AXOS/PYY mechanism described alludes to such a conclusion that more complete degradation of NSP is not necessarily desirable. For a predictable and repeatable response, the use of a specific, targeted NSP-degrading enzyme appears to offer the most consistent results.

To more effectively evaluate and compare the efficacy of NSP-degrading enzyme, research has to more closely match commercial reality. That means including a phytase, using pelleted diets and monitoring the entire growth period of the animal. A reliable, robust and consistent assay and more consistent reporting of enzyme specification and dose are imperative. The result will be better research, better products and better performance in the animal.

Variation in enzyme characteristics and efficacy in xylanases

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The substrate

Cellulose is the most abundant biopolymer on earth (est. 180 billion tons per year) and hemicellulose is the second most abundant biopolymer (est. 90-130 billion tons per year, based on typical plant cell-wall composition – 35-50% cellulose, 20-35% hemicellulose and 10-25% lignin (by dry mass)). Hemicellulose was originally defined as plant polysaccharides that could be separated from cellulose by water-alkali extraction. Hemicelluloses are a very diverse group of substances of which the most abundant is Xylan. The nomenclature is based on the major sugar in the backbone (i.e., in xylan the major backbone component is D-xylose; arabinans = L-arabinose; mannans = D-mannose; galactans = D-galactose). Hemicelluloses from different sources and from different parts of the plant vary significantly and there is also variation between different growth areas and from one season to the next. Xylan from grasses and cereals is usually referred to as arabinoxylan because it is highly substituted with arabinosyl residues at the C2 or C3 position, or both. This xylan is also acetylated and it may contain some glucuronic acid (though less than is found in hardwoods).

The xylanase enzymes

Xylanases have been shown to have a

positive effect when added to feed of monogastric animals. However, there are a number of different mechanisms how this could be achieved. One proposal is that by reducing the viscosity of the feed in the intestine, the rate of diffusion is significantly increased, allowing the nutrients to be absorbed by the stomach more readily. Soluble arabinoxylans are known to increase viscosity in an aqueous environment and the viscosity reduction by some xylanases is well documented. However, a positive effect from adding xylanases to the diets is also seen when xylanases are added to diets without high viscosity, such as maize. This suggests that xylanases may also have other effects such as releasing additional nutrients and possibly direct or indirect pre-biotic effects.

Although collectively the enzymes that hydrolyse xylan are known as xylanases, there are a number of different enzyme families and the enzymes within individual families shown significant differences, both in terms of their biochemical characteristics (pH optimum and stability, temperature optimum and stability) as well as their selectivity.

The basic enzymes for breakdown of xylan are the xylanases (β -1,4-endoxylanase (EC 3.2.1.4)). These are endo-acting and will cleave the glycosidic bonds between

the xylose subunits in the middle of the xylan backbone. In addition, there are β -xylosidases (EC 3.2.1.37), which are exo-acting and will cleave smaller oligosaccharides by attacking from the end of the xylan chain.

However, most xylan is not unsubstituted. This means that xylanases on their own will not be able to hydrolyse all or even most of the xylan, depending on the type of xylanase. The heterogeneity requires an arsenal of different enzymes, able to deal with the specific linkages found in the xylan.

In nature, most organisms that hydrolyse xylan (e.g., saprophytic fungi such as *Trichoderma* and *Aspergillus* sp.) have evolved an arsenal of enzymes that together can completely hydrolyse xylan, including multiple endo-xylanases as well as enzymes that work on the side chains and substituents. This is relevant for the fungus to be able to release monomeric sugars from its environment but may not be necessary for the animal- feed application.

The most commonly found xylanases in nature, and the most characterized biochemically are the Glucosyl Hydrolases of family 11 (GH11) and family 10 (GH10). In addition, xylan-hydrolysing enzymes in other families have also been described (GH5, GH8, GH7, GH43) of which examples of GH5 and GH8 have also been characterized.

GH11 xylanases typically display high substrate selectivity and high catalytic efficiency, a small size (~20 kD) and have a variety of optimum pH and temperature

values, making them suitable in various conditions and in many applications. They act exclusively on D-xylose-containing substrates. They usually have a low pI and they have two catalytic glutamate residues in the active site cleft. The structure of the GH11 xylanases is known as a β -jelly roll fold structure and looks somewhat like a hand. The active site (the palm of the hand) is highly conserved and is ~9Å deep, ~4Å wide and ~30 Å long, which is enough to fit 5-6 xylose residues of the xylan backbone. GH11 shows no activity on short-chain xylan (DP2-3), little activity on DP4 and increasing activity on DP5-9. Even acetylation of the xylose significantly reduces the hydrolysis rate. All of these characteristics result from the structure of the active site and substrate-binding pocket described above. The highly conserved nature of the substrate-binding pocket make it difficult to modify the specificity.

Xylanases of family GH10 and GH5 show very similar structures, known as an $(\alpha/\beta)_8$ TIM barrel structure. Some members of this family also have a Carbohydrate-binding motif (CBM). GH10 (and GH5) members display much lower substrate specificity as well as lower catalytic efficiency, they tend to be larger in size (~35-50 kD) and have a higher pI. They are often more acidic. Importantly they are able to act on a more diverse range of substrates, even substituted xylan with substitutions on xylose residue close to or adjacent to the cleaved backbone bond. In addition, because the binding to substrate is less specific, the GH10 xylanases can also cleave smaller xylan oligomers, resulting in dimer and monomer formation.

This is explained by the fact that GH10 (and GH5) xylanases have a relatively wide and shallow cleft with the catalytic dyad located in the middle, so that they show much lower specificity for the substrate and are able to fit substituted xylan (while there is simply no room for that in the cleft of GH11 xylanases).

GH8 xylanases have only recently been characterized in more detail and they show even greater specificity towards unsubstituted xylan than GH11 xylanases. They have an $(\alpha/\alpha)_6$ barrel structure with a deep binding pocket.

Xylanase inhibitors

Three structurally distinct classes of proteins have been shown to inhibit xylanases (All three have been isolated from various cereals). They are thought to be defence mechanisms of plants against fungal attack. Inhibitors generally do not inhibit plant xylanases.

- xylanase inhibitor protein (XIP) type. XIP-1 inhibits families GH10 and GH11 fungal xylanases but is not active against bacterial xylanases. Most commonly found inhibitor.
- *Triticum aestivum* xylanase inhibitor (TAXI) type. TAXI-type inhibitors have demonstrated inhibition exclusively against both fungal and bacterial GH11 xylanases, but have no activity against family GH10 xylanases.
- Thaumatin-Like Xylanase Inhibitor (TLXI) type. TLXI is a competitive inhibitor and acts on GH11 but not on GH10 xylanase. Unlike the other two, TLXI actually binds outside the active site.

So far there is little evidence of any of these three inhibitors working on family GH8 xylanases, possibly because of the high specificity of GH8 xylanases. Also, although an inhibitor might act on all GH10 xylanases (for example) it might act more on some than others.

Industrial uses of xylanases in animal feed

For the industrial use of xylanases the specific application conditions and substrates are relevant as well as the physiological effect that should be achieved. A number of properties may be relevant in the case of xylanases used in animal feed applications, including activity on soluble and insoluble substrate, activity on substituted and unsubstituted xylan and arabinoxylan, pH optimum, temperature optimum, temperature stability, ability to reduce viscosity, type of product, inhibition by xylanase inhibitors in the feed and, linked to that, ability to assay the enzyme activity in the feed.

Which of these is most relevant and which are not will depend on the processing (e.g., high-temperature pelleting) as well as the application and mode of action and the substrate(s). Thus different substrates may require different enzymes and the enzymes required will be different if they are used for viscosity reduction than if they are used for generating pre-biotics. Additionally, the types of inhibitors present in the substrate will play a role as well, as some xylanases are more sensitive than others to specific types of inhibitors.

In general wild-type mesophilic xylanases show a steep loss of activity, as a result of inactivation, above a certain temperature, typically ~50°C for *Trichoderma* and *Aspergillus* xylanases, though this will vary with the water content and incubation time. However, these xylanases may show a better activity at lower temperatures, such as the ~41°C found in the chicken digestive tract. Xylanase enzymes from other sources, such as thermotolerant fungi and bacteria, may be able to sustain much higher temperatures but will possibly be less active at the lower temperatures in the application.

For viscosity reduction GH11 xylanases may show a higher specific activity, for a given member of this family, but may not be effective on a particular substrate because the substrate is highly substituted. Bacterial xylanases may be less affected by inhibitors than fungal xylanases if the substrate contains significant amounts of XIP-type inhibitors. This may also play a significant role in determining the xylanase activity on the animal feed preparation as not all xylanases can be detected equally in all types of feed, mainly because of inhibition. While this may not be so relevant for the effectiveness in the application, it may be a critical factor for selling xylanases.

Commercial xylanases

The market for commercial xylanases in feed is highly fragmented with multiple products from all the main enzyme suppliers as well as a number of smaller players. Most commercial feed xylanases are produced in fungal systems but there are mesophilic and thermostable enzymes, mixtures of

different enzymes activities (e.g., xylanase and endoglucanase) and classical products containing many different enzyme activities (possibly multiple xylanases as well as other enzyme activities). Such products will either be used in lower temperature granulation or will require a coating technology to make them thermostable. Classical strains have many or all of the side activities for complete hydrolysis, whereas genetically modified production strains will generally have one main activity. This will allow more control of the process unless total hydrolysis is actually the target. Which side activities play a role is understood in principle but how this is relevant in the application is not well understood. Indeed, the fact that there are so many different commercial xylanase products suggests that different products may be more suitable for different applications, depending on the substrate, the process and the application.

Next generation xylanase

While the key method(s) of action of xylanases may not yet be fully understood, a number of parameters are likely to be required for any successful commercial xylanase in the future:

- Thermostability for Pelleting Process with greater than 90% recovery at 90°C
- Broad temperature optimum to retain activity in the animal digestive tract (37-41°C)
- Resistance to pepsin degradation (which is often linked to thermostability)
- pH optimum in the range of 4-7
- High specific activity for reducing costs

- High productivity in microbial production host, for cost reduction

However, these are needed for the enzyme to be active in the application and with the processing conditions used, but the key component is the effect that actually is beneficial in the application, whether this be viscosity reduction or production of pre-biotic xylooligomers or some other mechanism. In order to test this more reliably in the future, the type and source of xylanase used should be noted as well as the dosage. Ideally, mono-activity xylanases would be used for direct comparison of different enzymes in the application, to remove any side activities and to allow a direct comparison.

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NSPases as a solution to the problem of fibre

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The two faces of fibre

Fibre is the least digestible organic component in the diet of monogastric animals. Fibre, however, does not refer to a chemical entity. There are various terms used to describe "fibre", including Crude Fibre (CF), Neutral Detergent Fibre (NDF), Acid Detergent Fibre (ADF) and Dietary Fibre (DF). CF refers to the remnants of plant material after extraction with acid and alkali and includes variable portions of the insoluble non-starch polysaccharides (NSPs). NDF, by and large, represents the insoluble portion of NSPs plus lignin, and ADF refers to lignin plus a portion of insoluble NSPs comprised largely, but not exclusively, of cellulose. These terms can be confusing to nutritionists who still use CF as a criterion in feed formulation, underestimating the true fibre content of feed by two to three folds. DF is used mostly in human nutrition to describe the sum of NSPs plus lignin. DF is in fact an accurate representation of the true fibre fraction of feed.

Cereal grains and vegetable protein sources – the bulk of a monogastric animal's diet – contain between 10-75% of NSPs. NSPs in cereals form part of the cell wall structure and in vegetable proteins, such as legumes, may also play a role as an energy storage material. As far as monogastric animal nutrition is concerned, NSPs are either

poorly digested or anti-nutritive. The most significant role that NSPases have played in animal nutrition relates to their ability to neutralise the anti-nutritive effects of soluble NSPs on nutrient digestibility and hence impact positively on animal performance.

Insoluble NSPs, on the other hand, are not anti-nutritive and are regarded as nutrient diluents in monogastric feed. However, over the past decade, the role of insoluble NSPs as part of structural components, i.e., coarse non-digestible organic constituents, has been extensively studied (Hetland and Svihus 2001; Hetland *et al.*, 2003; Svihus *et al.*, 2004). In relation to the use of NSPase in poultry diets, insoluble NSPs are regarded as constituents of cell walls encapsulating nutrients, hindering the access of digestive enzymes to their substrates in the gastrointestinal tract.

Viscosity reduction

As far back as 1966, Burnett eloquently elucidated the relationship between the viscous nature of β -glucans and the nutritive value of barley for chickens. This pioneering work demonstrated the use of enzymes to lower gut viscosity, thereby alleviating the negative impact of β -glucans on nutrient digestibility. Pettersson and Åman (1987) showed the close relationship between extract viscosity and soluble arabinoxylans

in rye, wheat and triticale, further elucidating the importance of solubility and hence viscosity in the nutritive value of viscous grains for poultry.

There have been numerous studies confirming this finding with greater details in different viscous cereals (Campbell *et al.*, 1989; Bedford *et al.*, 1990; Bedford and Classen 1992; Bengtsson *et al.*, 1992). Choct and Annison (1992) isolated arabinoxylans from wheat and then depolymerised them with a microbial xylanase in the laboratory. The average molecular weight of the original

arabinoxylans was 758 kdaltons and it was reduced to 194 kdaltons after treatment with xylanase (Annison *et al.*, 1991). They added 3.5% (equivalent to 3% arabinoxylans) of the two isolates to a sorghum-based broiler diet. The high molecular weight isolates increased digesta viscosity by over three folds whereas the low molecular weight isolate increased it by two folds. Interestingly, partial depolymerisation of the polysaccharides had a disproportionately large effect on AME and FCR as shown in Table 1.

Table 1. Effects of adding a high-molecular weight (High-MW) and partially depolymerised (Low-MW) wheat arabinoxylans to a sorghum-based broiler diet on weight gain, FCR, feed intake and AME for da 21-28 (n=8)

DIET	WEIGHT GAIN (G/WEEK)	FCR	FEED INTAKE (G/WEEK)	AME (MJ/KG DM)
Control	430 ^a	1.59 ^b	681 ^a	16.13 ^a
High-MW	325 ^b	1.96 ^a	622 ^a	14.53 ^b
Low-MW	404 ^c	1.65 ^b	661 ^a	15.74 ^a
Pentoses	394 ^c	1.70 ^b	658 ^a	16.23 ^a

^{abc} Means sharing the same superscripts within a column do not differ significantly at P <0.05
Adapted from Choct and Annison (1992)

The results demonstrate that: a) soluble NSPs have anti-nutritive activities in poultry diets that can be deactivated via partial degradation, and (b) a complete depolymerisation of arabinoxylans has little or no benefit due to the poor absorptive capacity of chickens for pentose sugars. Indeed viscosity reduction is a key mechanism for the efficacy of enzymes added to poultry, to a lesser extent, of pig diets containing large amounts of viscous grains. However, the benefits of NSPase supplementation in diets based on non-

viscous grains, such as corn and sorghum, are related to other mechanisms, such as the breakdown of cell wall architecture to release encapsulated nutrients and the production of bioactive components, *i.e.*, prebiotics.

Releasing encapsulated nutrients

In relation to use of enzymes in monogastric diets based on non-viscous grains like corn and sorghum, it is the cell wall structure and the rate of starch digestion, rather than viscosity, that are important considerations.



It is thought that nutrients, such as starch and lipids, are encapsulated within the complex structure of cell walls, thereby preventing them from mixing with the endogenous enzymes. Thus, if cell walls are broken down by enzymes, nutrients are released early in the digestive tract to allow sufficient time for mixing of substrates and endogenous enzymes, leading to enhanced availability of energy and nutrients to the animal. Again, there have been numerous studies demonstrating the effects of NSPase on non-viscous grains.

This raises the question of whether NSP enzymes that can reduce viscosity and break down insoluble cell walls would be superior to enzymes that do only one part of the job? Choct *et al.*, (2004) examined three different xylanases having different substrate affinities (a. viscosity-reducing; b. cell wall degrading, and c. reducing viscosity as well as degrading cell walls) for their effects in broiler chickens fed a low or normal ME diet. The study showed that the enzyme that had affinities for both soluble and insoluble NSPs was more efficacious than the other two enzymes. However, it is important to stress that this study was done under experimental conditions with specially selected wheats. Under practical conditions, such a clear cut result would be difficult to demonstrate.

Modulation of gut microbiota

Choct *et al.*, (1996) used xylanase in a broiler diet with added soluble NSPs. The study showed that the enzyme reduced fermentation in the ileum but increased it in the caeca. These results coincided with a

reduced digesta viscosity and an increased digestibility of nutrients (starch, protein and that fat). Traditionally, the small intestine of poultry was considered to have no fermentative activity.

The significance of the study in question is not the detection of fermentation in the ileum, nor the amount of metabolisable energy arising from it. It is the physiological and microbiota changes that small intestinal fermentation may bring about for the bird. On the other hand, change of the gut microbiota in response to NSPase supplementation is not really a surprise because when the substrates are modified, the organisms relying on them for their existence will have to adapt. Diet-dependent variation in gut flora (Wagner and Thomas 1987) is another indication of the highly dynamic nature of more than 600 species of organisms harboured in the gut.

The complexity of the change is, however, difficult to understand. For instance, Bedford and Apajalahti (2002) showed a marked reduction in certain microbes in chickens by enzyme supplementation, leading to a significant reduction in the total number of microbes in the gut. The hypothesis is that the removal of substrates from the gut may leave the organisms to "starve", leading to a reduction in numbers. Another thought is that the production of certain oligomers in situ, such as xylo-oligosaccharides, provides prebiotics to the gut ecosystem and hence selectively stimulates the beneficial organisms whilst suppressing the growth of undesirable organisms. In a separate study, Choct *et al.*, (2006) lent some support

to this hypothesis where supplemental xylanase reduced the number of *Clostridium perfringens*, the causative agent for necrotic enteritis, to a non-detectable level in both the ileum and the caeca within a short period of time.

The production of specific substrates in situ through the use of enzymes to tailor for their prebiotic activities in animal diets will require a lot more work. The latest progress on the topic is also covered in another section of the proceedings.

Conclusions

The short term goal for the feed enzyme industry with regards to carbohydrases is to have the ability to match enzyme activities with animal performance criteria

under typical commercial situations. This will require work on enzyme-substrate relationships as well as on standardisation of in-feed enzyme assays. In the medium term, however, more work is needed to understand the types of low-molecular weight carbohydrates produced in situ and whether this process can be fine-tuned to encourage the proliferation of the organisms of choice. In the long term, the ultimate frontier for NSPases remains to be the ability to reach a complete depolymerisation of insoluble NSPs like cellulose and pectins to use them as energy sources for monogastric animals. Such a breakthrough will also pave the pathway for sustainable biofuel production in the future, which will alleviate the competition for grains used as feed and food.



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Arabinoxylan-oligosaccharides produced with the use of xylanases: What are their benefits to health?

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Arabinoxylans (AX) from cereals are cell wall components that constitute an important part of the fibre intake in humans and animals. They strongly impact the technological and nutritional performance of cereals or cereal derived products due to their properties. As non-digestible cell wall constituents, AX are part of a physical barrier that could prevent interaction between cereal components or nutrient uptake. Native water extractable AX have high molecular weight and lead to viscosity build-up. Water unextractable AX have a high water holding capacity and increase faecal bulking.

Enzymatic hydrolysis of AX by xylanases yields arabinoxylan-oligosaccharides (AXOS). This reaction takes place in the production of AXOS and of cereal-derived food and feed products, as well as in the gastrointestinal tract upon ingestion of AX in combination with xylanases. More and more scientific evidence becomes available that AXOS exert prebiotic effects in the colon of humans and animals through selective stimulation of beneficial intestinal microbiota. An increasing number of *in vitro* experiments and *in vivo* intervention studies on animals or humans have investigated potential health-related effects resulting from dietary intake of AX, AXOS or XOS. Both aspects are discussed here.

The first question is whether AXOS are

prebiotic. The definition by Gibson *et al.*, (2004) states that "*prebiotics are non-digestible food components that affect the host in a beneficial way by selectively stimulating growth and/or activity of one or a limited number of bacteria in the colon such as Bifidobacteria or Lactobacilli*". AXOS do show resistance to gastric acidity, to hydrolysis by host digestive enzymes and to gastrointestinal absorption. They are furthermore fermented by colon microbiota yielding short chain fatty acids such as butyrate, propionate and acetate, either as such or through cross-feeding. They show selective stimulation of growth and/or activity of colon bacteria which are today considered as beneficial to health. Whether such bacteria continue to be considered beneficial is not clear given the advances in microbiome analysis through next generation sequencing on the one hand, and the difficulties encountered in establishing a clear link between specific microbiota and health on the other. We can hence state that AXOS are prebiotic, but the key question remains: which benefits do they bring to the host and what are the mechanisms involved.

Possible end points for establishing such beneficial effect, ranging from weaker to stronger are ease of fermentation, production of butyrate and other short chain fatty acids, extended saccharolytic fermentation at the disadvantage of proteolytic fermentation,

improvement of gut barrier function, reduction of colon cancer risk.

Several of these end points were established in a number of studies. Analysis of the structure-function relation of AXOS in rats (Van Craeyveld *et al.*, 2008) showed that addition of the smallest AXOS yielded the highest increases in butyrate production. Reduction of proteolytic fermentation was more outspoken with the larger, somewhat more complex AXOS structures. Analysis of the effect of ingestion of AXOS on colonic bacterial metabolism in healthy human volunteers showed that nitrogen excretion through faeces increased upon ingestion of AXOS, while urinary excretion went down (Cloetens *et al.*, 2008), suggesting a stronger incorporation of nitrogen in bacterial mass. When looking at the impact of AXOS on preneoplastic lesions in the colon of rats treated with colon cancer inducing 1,2-dimethylhydrazine, less preneoplastic lesions in the distal colon of rats through the administration of AXOS was observed (Femia *et al.*, 2010). When the impact of AXOS on Salmonella colonization in broilers was studied, a strong reduction in Salmonella in the gut and of Salmonella translocation to the spleen upon administration of AXOS was observed (Eeckhaut *et al.*, 2008). Is this due to a pH effect, lowering Salmonella numbers in the gut, or to an improved barrier function of the gut wall? The study by Neyrinck *et al.*, (2012) on the effect of AXOS on metabolic endotoxemia in diet-induced obese mice suggests the latter. Plasma lipopolysaccharides levels returned back to control levels when mice were fed a high fat diet supplemented with AXOS

compared to a high fat diet without AXOS. Zonula occludens 1 expression in the colon in mice fed the AXOS supplemented high fat diet was increased compared to the high fat diet only.

What does all of this mean for the zootechnical performance of broilers? What is, for example, the impact of AXOS on feed conversion rate in broilers? A dosage of 0.5% AXOS was shown to lead to a reduction in feed conversion rate (kg feed/kg body weight) of 5% in a wheat-based diet. A similar reduction as was observed in a xylanase complemented diet (Courtin *et al.*, 2008). The observation that the broiler gut bifidobacteria levels were ten times higher for the animals receiving the AXOS supplemented diet compared to the xylanase diet suggests, however, that the feed conversion rate improving mechanisms for AXOS and xylanases are not entirely the same. This complexity is further illustrated by Damen *et al.*, (2011), who studied the fate of AXOS, water-extractable AX and water-unextractable AX and several combinations of these components in rats. In the latter cases, synergy was observed.

In summary, we can say that AXOS are prebiotic components, showing a bifidogenic effect. Improvement of gut barrier function is one of the suggested mechanisms for better animal performance and health effects. Proper xylanases can produce these AXOS and by doing so 'functionalise' AX. Probably xylanases with a selectivity for water-extractable AX are preferred. Knowledge on the enzymes used in feed and the mechanisms by which they work is crucial to understand their functionality.

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The influence of fermentation on gut hormones and consequences – the PYY story?

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Two mechanisms for the mode of action of exogenous xylanase in non-ruminant diets are prominent in the literature with a third recently coming to light. The first is the long held understanding that xylanases can degrade soluble non-starch polysaccharides (NSP), thus reducing digesta viscosity and improving nutrient digestibility. However, studies have shown xylanases to be effective in improving feed efficiency in diet containing low viscosity wheats and also maize. Therefore, the second mechanism, of direct cell wall destruction, is suggested. This involves the digestion of cell wall material by xylanase, directly liberating the contents of the cell for digestion by endogenous enzymes. Micrographs of feed material taken from the ileum of a broiler fed diets with or without a xylanase show the effect of xylanase on this cell wall material. However, *in vitro*, under conditions of the gastrointestinal tract, this process has been shown to take upto 24 hours – more time than feed material takes to pass through the tract of a broiler for example. This brings to light the suggestion of a third mechanism – a cecal prebiotic effect stimulated by the production of arabinoxylo-oligosaccharides (AXOS) with the use of a dietary xylanase.

Cereal based diets contain appreciable amounts of xylan material. Wheat for example may contain 8% arabinoxylan

(on a fresh basis) and maize as much as 5% (Choct *et al.*, 1997). Digestion of this material will release AXOS in the small intestine because some mechanical digestion has already occurred in the gastric phase and also most dietary xylanases are active within this region. Commercial mono component xylanase products are particularly efficient in the production of AXOS as they should contain minimal xylosidase side activities. This superfluous activity may reduce AXOS production by reducing the xylan chain length and liberating free xylose. The characteristics of AXOS produced by xylanase activity are also dependant on the specific xylanase employed and the dose and time for the reaction to occur (Damen *et al.*, 2012), and the substrate on which it is acting. The fermentation of AXOS within the hind gut of most species, including broilers and pigs, is known to modulate the microbiome within those regions. For example, Eeckhaut *et al.*, (2008) demonstrated a decrease in the time taken for Salmonella to be cleared from the small intestine of the chicken with addition of AXOS to the diet. Furthermore, it is known that well performing broiler chickens, those with low FCR, have a distinct and determinable pattern of caecal microflora compared to those that perform less well (Apajahlahti *et al.*, 2004). Therefore developing a 'good' microflora

may be a target mechanism for an efficacious enzyme. The caecal population can be shifted from one pattern to the other with the use of a xylanase (Murphy *et al.*, 2004). Several studies have also reported dramatic positive changes in fermentation and fermentation products in the caeca of chickens fed xylanase which were highly correlated with improved performance (Cowieson and Masey O'Neill 2013, Masey O'Neill *et al.*, 2014). Further, Courtin *et al.*, (2008) have shown that the addition of AXOS to the diet of broiler chickens, produced *in vitro* by digestion of what flour with xylanase, can significantly improve the feed conversion ratio to the same extent as the addition of xylanase directly to the diet. In summary, it appears that the production of fermentable AXOS in the gut of the non-ruminant can have beneficial effects. But what is the mechanism?

A large body of scientific work in the late 1980's described the hormone Peptide YY (PYY) in humans and in rodents. This gut hormone belongs in a group with various others such as cholecystokinin and enteroglucagon, which are all involved in the modulation of digestion and the regulation of appetite. Peptide YY has been shown to respond particularly well to fermentable fibre (Goodlad 1987). In fact, when short chain fatty acids, which are the products of fermentation, are infused into the colon of a rat, PYY is released into the blood. However, it has also been shown to be released in response to dietary fat (Chaudrhi *et al.*, 2006). Peptide YY is released from the L cells of the gastro-intestinal tract; they are present in various regions but are particularly

prevalent in the hind gut. Peptide YY has been implicated in modulating gastric retention time and particularly in slowing gastric emptying (ref). We hypothesise that this will improve gastric digestion. Initial work has shown that in broilers, PYY does respond to the use of a xylanase (Singh *et al.*, 2012) and potentially, in a dose and diet dependant manner. However, another study did not see a xylanase dependant response in plasma PYY (Masey O'Neill, Scholey and Burton, unpub.) but noted two other important factors. Firstly, xylanase appeared to reduce the between bird variation in performance. Secondly, PYY is significantly positively correlated with feed conversion ratio and feed intake, particularly in mature birds. Although correlation does not imply causation it is a potential target, via microfloral and fermentative changes, for the action of NSP enzymes. The suggestion that PYY is implicated in stimulating FI is contrary to the mammalian literature, which implicates PYY as a satiety signal (Batterham *et al.*, 2002). But, as evidence suggest with other gut hormones, it is possible that PYY exerts opposite effects in chickens as would be expected in mammals.

Importantly, what does this mean for the use of NSP enzymes? Can we optimise non-ruminant diets with this new knowledge? Holo-analysis is a method of comprehensive data capture, including all available information on a topic, followed by predictive statistical modelling. A recent such analysis of all the available broiler data on the use of a xylanase, Econase XT (Masey O'Neill *et al.*, 2011) has shown that, amongst



other things, there are two factors that are important in getting a good response to a xylanase; the amount of time the broiler is fed the enzyme and dietary fat inclusion. It was seen that the longer the broiler is fed the enzyme the greater the eventual response. This agrees with an earlier, similar but more comprehensive review (Rosen 2002). In that study, feeding an enzyme from day of age was suggested to carry significant benefit. The cecal prebiotic effect suggested above probably takes time to occur. The modulation of the bacteria is not instantaneous and in a near-sterile hatchling will take time to develop. Likewise in a piglet where the diet has just undergone a radical change, microflora need time to become established and respond to new ingredients. From our holo-analysis we can also see that a certain level of fat is necessary to promote the response to a xylanase. This is possibly because, particularly in the young animal, where the hind gut microflora is underdeveloped, PYY is not adequately stimulated by fermentation, but can respond to fat. In one study, it was shown that fat removal from a starter diet decreased amino acid digestibility, probably because fat promotes gastric retention, improving overall gastric protein digestion. Up until 21 days, xylanase did not result in any improvement in amino acid digestibility. However, at 42 days, with continued feeding of xylanase, amino acid digestibility was regained and in fact was better than that of the positive control suggesting by this point, fermentation could overtake fat in stimulating the PYY response. Similar results have been shown in piglets, whereby there was no response to xylanase in low fat diets but a significant

($P < 0.1$) improvement in FCR on a higher fat diet. Bearing these factors in mind, caution should be exercised when assigning a large energy matrix to xylanase in early diet formulation. This is probably particularly true in maize-based diets where viscosity is not an issue. However, later on in life, a more aggressive energy matrix could be applied. A small amino acids matrix may be appropriate but if these are not considered it is likely the benefit will be seen in weight gain.

If the generation of PYY via production of specific VFAs in the caeca does prove to be a significant mode of action of NSPases, then the argument regarding single component versus multi component enzymes may become more transparent. It would not matter how the VFAs are generated, as long as the correct VFAs are generated in the correct quantity. Thus the focus would be on generation on the right amount / type of oligomer prebiotics. It may well be that such a target could equally be achieved through production of a significant amount of a single oligomer or lesser amounts of multiple oligomers. Clearly this field lends itself to a great deal more research before this mechanism is fully understood.

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NSP-degrading enzymes in non-viscous diets for poultry

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Introduction

The use of non-starch polysaccharide (NSP) degrading enzymes in diets based on viscous-grains is almost ubiquitous. The pertinent example in this case is xylanase for rye/triticale/wheat- and gluconase for barley-based diets where the positive response to enzyme supplementation is explained, predominantly, in terms of the reduced digesta viscosity brought about by a partial hydrolysis of the soluble NSP fraction (Choct & Annison, 1990; Bedford & Schulze, 1998; Gao *et al.*, 2007). Contrary to that, commercial poultry diets based on non-viscous grains like corn or sorghum generally attract less penetration of the NSP degrading enzymes. This appears to stem from the lack of consensus regarding the most pertinent enzyme activity or set of activities, and the mode of action through which these enzymes are expected to exert beneficial effects. This paper reviews the published literature on NSP degrading enzymes with specific reference to corn-soy diets. It highlights, and attempts to clarify, some areas that are not well defined and hence add a factor of perplexity on the subject matter. It also shares insight on the evolving mode of action that may help explain the positive effect of NSP degrading enzymes in non-viscous diets, with the idea that a better understanding in this area may help make correct choices and a wider

acceptance of the NSP enzymes by the commercial feed industry.

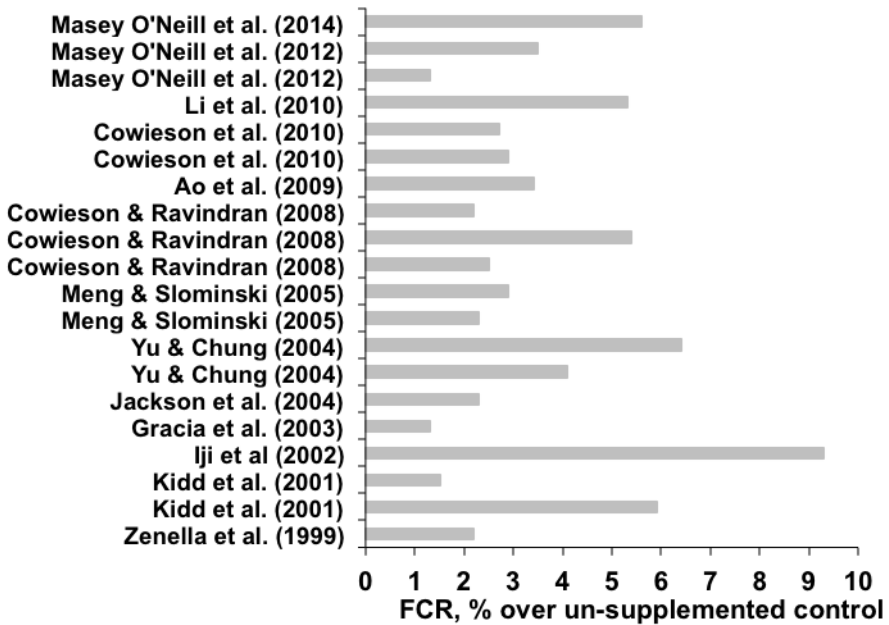
The research focus

NSP enzymes have been shown to offer significant improvements in the energy and nutrient digestibility and growth performance of broilers fed corn-soy diets (Aftab, 2012). Figure 1 summarizes the findings of different reports, demonstrating an improvement in FCR by supplementation of NSP enzymes. The response appears to average at about 3.6% improvement over the un-supplemented control, although quite a large variation in the responses has been observed across different reports (2 up to 10% improvement). Unlike the case with the viscous grains where the research is focused on distinct, single, enzyme activity, the responses shown in the Figure 1 have been recorded with preparations displaying single- to multiple-enzyme activities, hence causing a split in the scientific opinion regarding the question of the most pertinent enzyme activity, or the set of activities, in relation to the corn-soy diets. Broadly speaking, one opinion is that a 'complex substrate' offered by this dietary model can best be dealt by offering a 'complex enzyme' displaying a range of enzyme activities and thus to be able to widen the scope and extent of substrate hydrolysis (Yu & Chung, 2004; Meng *et al.*, 2005;

Meng & Slominski, 2005; Slominski, 2011). The alternative view is that a controlled and targeted hydrolysis of the substrate is needed for a predictable enzyme response and this can best be achieved by using a single enzyme activity at precise dosage (Bedford & Graham, 2008; Zou *et al.*, 2006; Liu *et al.* 2011; O'Neill *et al.*,

2012a,b; 2014). The following discussion takes a few examples from the published research involving multiple-enzyme, and questions some of the interpretations and conclusions that appear to have been made without support of defensible experimental methods.

Figure 1. FCR response to the supplementation of NSP enzymes in corn-soy broiler diets



The number of enzyme activities

The data in Table 1 is one from the series of experiments conducted with an objective of establishing the 'most appropriate blend' of NSP enzymes for complex diets, including those based on corn and soybean

meal (Meng *et al.*, 2005). The results appear to fit well to the hypothesis in that a combination of Cellulase, Pectinase, Xylanase, Glucanase, Mannanase and Cellulase (C+P+XG+MC) resulted in the FCR that was significantly better than the rest

of the treatments involving 'less-complete' combinations of the component activities. Looking into the data with a bit more detail, however, questions this conclusion. From the first three treatments, it is apparent that the FCR of the control was improved with supplementation of each of the C+P, C+XG or C+P+XG. The absence of a treatment with single activity makes it difficult to reach firm conclusion regarding the most pertinent enzyme activity, though one can argue there could only be two possible interpretations to the data; 1) C being a common factor in all was the one to elicit the response, and hence P and XG were not needed or 2) it was P and XG that caused the response, and C was not needed. The data, by no means, suggests a need for C+P+XG

since no further improvement in FCR was observed with the combination of all these activities. The final treatment, addition of another increment of C, combined with M, resulted in a further improvement in the FCR that led the authors to conclude the most appropriate combination of enzymes was C+P+XG+MC. Nothing excludes one to draw an alternative conclusion, that the recorded response was all due to the C alone (from the first three treatments) and when the activity of C was increased in the fourth treatment (as MC) it improved the FCR further simply as a function of the greater C dose. To make a sense out of that, one may need to know the precise activity of C, since C and G are very closely related.

Table 1. Effect of different enzyme combinations on the performance of broilers

Enzyme*	Feed intake, g	Gain, g	FCR	AME, kcal per kg
None	668	436 ^b	1.53 ^a	2902 ^b
C+P	687	459 ^a	1.50 ^b	2997 ^a
C+XG	695	470 ^a	1.48 ^b	3004 ^a
C+P+XG	678	456 ^a	1.49 ^b	3001 ^a
C+P+XG+MC	676	466 ^a	1.45 ^c	3046 ^a

a-c, P<0.05; *each enzyme preparation added @ 0.1 g per kg of diet

C+P, Cellulase 340 units per g, Pectinase 10,000 units per g

XG, Xylanase 63,600 units per g, Glucanase, 48,300 units per g

MC, Mannanase, 10,900 units per g, Cellulase, 600 units per g

Source: Meng et al., 2005

The matter of a rather vague description or definition of the enzyme activity is apparent in literature. Table 2 refers to a paper where the terms "amylase" and "xylanase" have been used for two different enzyme

preparations, but with inconsistent definition of the activity (Yu & Chung, 2004). In one case, the amylase and xylanase has been described as 'units' and in the others it refers to 'KNU' for amylase and 'FXU' for

xylanase. This makes it impossible for the reader to understand what these numbers are in relative terms. Indeed, while a common assay method to define the *in-vitro* activity is not expected to give all the information regarding the relative *in-vivo* value of the enzymes, it may at least provide a foundation to assess the comparative value of different enzyme preparations and perhaps a better understanding of the dose-response relationships. Just looking at the data in Table 2, the first logical choice is likely to be the one with the best FCR response with the least number of enzyme activities i.e. the amylase + glucanase. If one had to further guess the relative contribution

of either of these activities in improving the FCR, it would probably be the glucanase since the studies on the amylase as the sole activity appear not to support the notion that the endogenous supply of amylase needs to be augmented in broilers, except, perhaps for first few days of age (Gracia *et al.*, 2003; 2009). One conclusion, however, is that simply increasing the number of activities failed to elicit a greater response. The above two examples suggest that need for a 'complex enzyme solution' has not been convincingly demonstrated, and can well be questioned simply based on the lack of accurate experimental methods.

Table 2. Effect of various enzyme combinations on FCR of broilers

Treatment group	FCR
Positive control	1.76 ^b
Negative control, less 100 kcal ME	1.88 ^a
Protease, 6000 U; Amylase, 2000 U; Xylanase, 800 U	1.81 ^{ab}
Amylase, 30 KNU; Glucanase, 100 GBU	1.76 ^b
Amylase, 30 KNU; Glucanase, 100 GBU; Xylanase, 100 FXU	1.76 ^b
Amylase, 22.5 KNU; Glucanase, 75 GBU; Xylanase, 100 FXU	1.79 ^b
Amylase, 15 KNU; Glucanase, 50 GBU; Xylanase, 100 FXU	1.86 ^{ab}

a-c, P<0.05

Source: Yu and Chung, 2004

The defensible way of testing the idea of single- versus multiple-activities would have been the one adopted by Cowieson *et al.*, (2010). This study employs all possible combinations of activities and dosage,

hence serving the basic prerequisite if one has to reach definitive conclusions regarding the relative, and combined, value of different enzyme activities. What it shows is an improvement in the basal FCR with



the supplementation of either xylanase or glucanase; combination of both, at any of the doses tested, failed to bring a further improvement in the FCR – suggestive of a non-additive nature of the responses when it comes to combining more than one enzyme activity. It appears that one of the largest determinants of the enzyme response is the digestibility of the control diet – higher control digestibility simply leaves lesser room for an improvement or vice versa (Cowieson *et al.*, 2010; Rutherford *et al.*, 2007). What this implies is that the presence of an enzyme, or some other pro-nutrients in the basal diet, would likely alter the magnitude of the response to the other enzymes. One of the most relevant examples in this context would be the phytase. An increase in the basal energy and amino acid digestibility by phytase (Rutherford *et al.*, 2004; Cowieson *et al.*, 2006; Ravindran *et al.*, 2006, 2008; Rutherford *et al.* 2012) would likely mute the response to the NSP enzyme that is often considered a second additive in corn-soy diets. This raises a question on the significance of a vast majority of the published literature where the positive effect of NSP enzymes has been confirmed in isolation, rather than with diets already containing a phytase.

Also important in this context would be to discuss one example on the NSP-phytase cocktail. The experimental design would typically involve a simultaneous down-specification of Ca, P, energy and AA to a positive control diet, to which the enzyme cocktail is supplemented and response compared with that of the positive control diet e.g. Francesh & Geraert, (2009).

The role of the phytase has been well established and is being utilized in majority of the commercial diets today, the important question needs to be answered is if the NSP enzymes improve the energy (and nutrient) availability in a corn-soy based diet in the presence of a phytase, or is there any additive or synergistic effect between NSP and phytase enzymes? Unfortunately, the experimental design in the current study does not address these questions since the P/Ca/ME/AA are reduced simultaneously in the negative control diets. With the finding that the negative control diet caused a significant depression in the growth performance and that this effect was ameliorated by the supplementation of a phytase-NSP cocktail, it is not possible to define the relative contribution of phytase versus the NSP fraction in the improvement noted. In this context, such studies are not expected to add to our current knowledge on the application of feed enzymes *per se*.

Maximizing the scope and extent of hydrolysis

It appears that an increased degree of the NSP hydrolysis, *in-vitro* or *in-vivo*, is not a good predictor of an enzyme response. For example, a high dose of galactosidase may double the digestibility of oligosaccharides in the gut but this does not appear to translate into an improved performance (Slominski *et al.*, 2006). There could have been instances where an extreme influx of some of these sugars may even prove detrimental, as shown for high doses of mannanase supplementation (Vahjen *et al.*, 2005) or high levels of xylose and arabinose (Schutte, 1990). On the contrary, the work

of Nian *et al.* (2011) suggests an improved ileal digestibility of energy ($P < 0.05$) and AME ($P = 0.07$) with the supplementation of xylanase, despite no meaningful increase in the hemicellulose digestibility being observed. Similarly, caution should be practiced while attempting to widen the scope of NSP hydrolysis by increasing the number of enzyme activities. In many published studies the choice of the candidate enzyme activities is based on the *in-vitro* hydrolysis potential, as was done by Vahjen *et al.*, (2005). Their data showed that combining the galactanase and mannanase increased the *in-vitro* release of the sugars from soybean meal samples but when fed to broilers, the combination significantly ($P < 0.05$) depressed feed intake and gain (Vahjen *et al.*, 2005). It is important to note numeric improvement in the FCR when galactanase was offered as the only activity. In this context, it appears likely that offering a multi-activity solution may end up with zero net effects, as the positives of some activity(s) are countered by the negatives of the other(s).

Specificity is the key

The provision of oligosaccharides has been proposed as one of the mechanisms to explain the positive effects of NSP enzymes, especially in the diets based on non-viscous grains. The main highlight of the research in this area is that certain oligosaccharides may have a potential to act as prebiotics and hence may mediate an improved nutrient assimilation and growth performance of the subject animals. Studies involving a number of potential candidates conclude that only some specific oligosaccharides, at

a specific dosage, can exert these positive effects (Biggs *et al.*, 2007; Biggs & Parsons, 2007). Those studied include inulin/fructo-oligosaccharides, mannan-oligosaccharides (MOS), and xylo- (XOS) and arabino-xylo oligosaccharides (AXOS). Inulin/fructo-oligosaccharides appear to be not relevant to the NSP enzyme supplementation; the MOS, although linked to the mannanase-soybean galactomannan, needs more elucidation since the majority of research in this area involved MOS (or more likely mannans) from yeast cell walls. XOS and AXOS appear to relate to the xylanases supplementation, but use of the generic terms for the products of hydrolysis and the enzyme appear not sufficient in view of the findings that specific characteristics of the XOS or AXOS, including the chain length, degree of polymerization, and the relative proportions of the water-extractable versus non-extractable arabinoxylans, were important determinants of the effectiveness of these oligosaccharides (Van Craeyveld *et al.*, 2008; Eeckhaut *et al.*, 2008). Clearly, this necessitates need to apply specific xylanase at specific dosage to be able to produce the AXOS of biological significance. Since different xylanases possess variable degree of affinity towards the substrate (Choct *et al.*, 2004) and different feed ingredients vary in the content and association of the substrate with the other cell wall components, it may well be that the precise dosage of enzyme may need to be re-established if a major change in the diet composition is observed.

The value of the negative responses

Another factor adding a bias to the published literature is the fact that the

negative responses rarely get published. For academic journals, a negative response or no response is not worth an invention, and for the commercial producers it is not something they would like to mention. It has been realised, however, that an analysis of these negative responses may potentially give some very interesting insights into the subject matter that could have been possible if just the positives responses were considered. A holo-analysis of 133 all-inclusive data points revealed that the various factors, including dietary level of Ca, Na, age of the bird, level of the supplemental fat, etc., may influence the FCR response to xylanase supplementation in corn-based broiler diets (Masey O'Neill *et al.*, 2011). Validation of these factors by means of direct/formal studies would likely yield more predictable enzyme responses.

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Efficacy of feed enzymes – Laying hens

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Introduction

Generally, the nutritive value of a feedstuff for poultry is defined by its chemical composition and digestibility, and can be limited by their content of dietary fibre (Non-starch polysaccharides (NSP) + lignin). The mechanism behind the negative effects of NSP on the conditions in the intestinal system of poultry is very complex. The antinutritive effects of NSP in diets for poultry seem to be multifactorial and cannot solely be explained by the increase in intestinal viscosity caused by soluble NSP (Iji, 1999; Choct *et al.*, 1999.). In addition to the direct effect of soluble NSP on intestinal viscosity, it has been suggested that soluble NSP have an indirect effect attributable to microbial activity (Choct *et al.*, 1996; Smits *et al.*, 1998; Langhout, 1999). The primary purpose of using exogenous enzymes in poultry diets is to improve the nutritive value of the feedstuffs, where β -glucanase and xylanase have been developed specifically to degrade the arabinoxlan (AX) and β -glucans present in cereals, which are the main source of these NSP components, where the highest content are found in wheat, rye and barley (Bach Knudsen, 1997). The application of exogenous enzymes in diets for poultry have to some extent attributed to an improved understanding of the negative effects cell wall NSP have on the digestive

processes in the intestinal system of poultry fed cereal based diets. Diets for organic layers are often high in fibre due to inclusion of alternative ingredients and access to foraging material, however only few studies has been reported on the effect of enzyme supplementation to organic layer diets.

Laying hens and cereal based diets

Consistent improvements in performance owing to NSP degrading enzymes have been found in many studies for broiler chickens fed diets based on wheat, barley or rye, where the content of especially water soluble arabinoxylans in the endosperm of wheat and rye and β -glucan in barley, are responsible for increasing digesta viscosity and consequently reduction in the absorption of nutrients (Bedford and Classen, 1992; Choct *et al.*, 1996; Brufau *et al.*, 2006). Since the effect of soluble NSP on intestinal viscosity have the most negative effect in young birds and decrease with age, the improvements in performance with enzyme supplemented diets seem to be less pronounced in older birds (Salih *et al.*, 1991; Almirall *et al.*, 1995), probably because the effect of soluble NSP are better tolerated by adult birds with a more developed digestive tract with a higher digestive capacity and an increased production of enzymes and bile acids.

The effect of enzyme supplementation in different studies with layers has varied to some extent, influenced by the main cereal used in the diets, the different parameters measured, hen age and type and concentration of the enzyme. Wheat and barley are important feed cereals globally and included in poultry diets to a high extent as energy source. However, the nutritive value of both wheat and barley can vary considerably dependant on cultivar, harvest year and locality, which can influence the AME, nutrient digestibility and performance (Mollah *et al.*, 1983; Choct *et al.*, 1995; Francesch *et al.*, 1994; Choct *et al.*, 1999; Steinfeldt, 2001; Svihus and Gullord,

2002; Lazaro *et al.*, 2003).

In a recent paper by Mirzaie *et al.*, (2012) increasing levels of the wheat cultivar Pishtaz in layer diets decreased egg weight and egg mass and increased ileal viscosity. The specific wheat was selected due to its higher content of NSP to study the effect of supplementation with xylanase on layer performance, ileal viscosity, nutrient retention, intestinal pH and enzyme activity. The xylanase enzyme had a positive effect on several parameters such as egg production, egg mass and FCR. Further, enzyme addition decreased viscosity, which probably was responsible for the improved fat digestibility observed (Table 1).

Table 1. Example of enzyme supplementation (xylanase) to wheat based layer diets and effect on selected parameters (Mirzaie *et al.*, 2012)

PARAMETERS	WHEAT (%)				XYLANSE (%)	
	0	23	46	69	-	+
Performance*:						
Egg production (%)	89.5	89.3	89.8	88.1	88.5 ^b	89.8 ^a
Egg weight (g)	58.5 ^a	58.8 ^a	57.7 ^{ab}	56.8 ^b	57.7	58.8
Egg mass (g/d)	52.4 ^a	52.6 ^a	51.9 ^{ab}	50.1 ^b	51.1 ^b	52.3 ^a
FCR (g/g)	1.832 ^{ab}	1.813 ^b	1.831 ^{ab}	1.882 ^a	1.859 ^a	1.819 ^b
Viscosity (cps):						
33wk	3.6 ^c	3.9 ^c	4.8 ^b	6.4 ^a	5.2 ^a	4.1 ^b
47wk	4.0 ^c	5.2 ^b	5.8 ^b	10.9 ^a	7.1 ^a	5.8 ^b
Fat digestibility (%)						
33wk	80.6 ^a	79.7 ^a	79.4 ^{ab}	76.2 ^b	78.0 ^b	79.9 ^a
47wk	79.0	78.7	78.6	77.4	78.0	78.8

*25-47wk. ^{ab}: Means with the same row with different superscripts differ significantly (P<0.05)

Different mechanisms are influenced by increasing digesta viscosity, since viscous polysaccharides can reduce absorption due to a decrease in the convective transport of nutrients (Edwards *et al.*, 1988) and probably cause a thickening of the unstirred water layer. Therefore, a decrease in viscosity due to xylanase supplementation enhances the probability for a successful

interaction between substrate and digestive enzymes at the mucosal surface. Positive effect of enzyme supplementation has also been found by Lazaro *et al.*, (2003), where an enzyme complex with both β -glucanase and xylanase activity to wheat-, barley- and rye based diets improved egg production and FCR as well as the digestibility of fat and AME_n (Table 2).

Table 2. Example of enzyme supplementation to wheat, barley and rye based diets for layers and effect on selected parameters (Lazaro *et al.*, 2003)

PARAMETERS	WHEAT (50%)			BARLEY (50%)			RYE (35%)			PROBABILITY	
	0	250	1250	0	250	1250	0	250	1250	C	E
Performance:											
Egg production (%)	83.6	84.1	86.2	81.2	84.6	85.2	82.7	84.3	84.4	NS	*
Egg weight (g)	58.4	57.6	58.1	58.7	58.1	57.9	58.3	58.0	58.7	NS	NS
Egg mass (g/d)	50.1	48.8	51.0	48.4	48.8	49.8	48.1	49.3	50.1	NS	NS
FCR (g/g)	2.18	2.22	2.09	2.20	2.25	2.10	2.27	2.23	2.19	NS	*
Viscosity (cP)	11.1	6.7	6.3	33.2	9.8	5.8	112	91.6	65.7	***	***
Fat digestibility (%)	85	85	87	85	86	88	83	87	88	NS	***
AME _n (MJ/kg)	11.4	11.3	11.4	10.8	11.5	11.5	11.5	11.8	11.7	***	***

Performance: 20-44wk; Viscosity, fat digestibility and AME_n: 45wk.

Enzymes: β -glucanase and xylanase (0, 250, 1250mg/kg). C: cereal, E: enzyme

Wheat, barley and rye substituted maize in diets and differences in viscosity was measured, however, without any effect on performance indicating that adult birds are more tolerant to viscous polysaccharides in wheat, barley and rye. However, enzyme addition improved performance and nutrient digestibility showing that even though viscosity seems to have a minor importance in layers it has a profound effect on different parameters (Lazaro *et al.*, 2003). The

highest content of soluble arabinoxylans are present in rye and the effect of enzyme supplementation was least pronounced with the rye diets, which induced substantial viscosity in spite of addition with xylanase.

Results from a study by Smulikowska (1997) also showed that intestinal viscosity was higher in hens fed diets based on 30% whole rye compared to wheat based diets, even though both diets were supplemented with beta-glucanase and pentosanase. However,

egg production was improved by enzyme supplementation to the rye based diets and not different from the wheat based control which indicated that rye can substitute wheat to a certain extent in layer diets. In some European countries rye is considered a potential alternative to other cereals due to a lower price and the use of NSP enzyme can contribute positively to the economy.

In other studies similar positive effects of enzyme addition on performance has been found with barley based diets (Coon *et al.*, 1988; Brufau *et al.*, 1994; Francesch *et al.*, 1994) and rye based diets (Campbell and Campbell, 1989; Pettersson and Aman, 1989; Smulikowska *et al.*, 1997). In some studies no effect have been seen with enzyme supplementation on performance and egg quality (Ciftci *et al.*, 2003; Mathlouthi *et al.*, 2003; Roberts and Choct, 2006), and the explanation can be due to the cereal cultivar used, hen genotype and age during the experimental period.

Overall, it can be concluded that enzyme supplementation for layers diets in most cases has positive effects on performance and nutrient digestibility and one other important factor observed in some studies is the reduction in the incidence of dirty eggs (Francesch *et al.*, 1995; Lazaro *et al.*, 2003), which can become a serious economic problem for the individual egg producer.

Organic egg production and use of enzymes

Research in the effect of NSP enzymes in organic poultry production is very scarce and only few studies have been published.

Buchanan et al. (2007) studied the effect of NSP enzymes (β -glucanase, pentosanase, hemicellulase) on performance of organic broilers with the purpose of increasing forage digestibility and found that the enzyme enhanced performance when broilers were foraging in the spring month, where the quality of the pasture was highest. Since the organic egg production is increasing in Europe (Magedelaine, 2010) and the implementation of 100% organic feed are approaching (January 2015; EU, 2012), supplementation of specific enzymes would be valuable, since reduced excretion of nutrients would be beneficial both for the bird and the environment. Use of exogenous enzyme in organic poultry production is limited due to EU legislation, where the use of GMO produced enzyme is forbidden in organic diets (EU, 2007, EU, 2012). Since synthetic amino acids is not permitted in organic production the supply of sufficient levels of amino acids in organic egg production is often achieved by feeding diets with higher protein content than required by the birds and supplementation with appropriate enzymes could probably improve nutrient digestibility and eventually reduce the protein content in diets and thereby reduce the excretion of nitrogen to the environment.

With their ability to grow in temperate climate, the use of faba beans, peas and lupine could increase the amounts of home-grown protein sources and improve self-sufficiency instead of being dependent on imported soya, where organic and GMO-free soya is becoming increasingly difficult to obtain. Lupin contains high amounts of

protein (Petterson, 2000) and could be a fine supplement to soya beans, which presently are imported to northern Europe and used in organic poultry feed as the main vegetable protein source. However, like most legume proteins, the content of methionine and cystine are low (Alloui *et al.*, 1994; Petterson, 2000). Further, a high level of the non-starch polysaccharides (NSP), which is almost twice as high in lupins as in other protein-rich plants (Daveby and Aman, 1993; Bach-Knudsen, 1997) could be another factor restricting the use of lupin in poultry diets (Perez-Maldonado *et al.*, 1999; Hammershøj and Steinfeldt, 2005). Enzyme supplementation to lupine based diets for laying hens could reduce the negative effect of NSP in lupins as seen with broilers (Hughes *et al.*, 2000; Kocher *et al.*, 2000; Steinfeldt *et al.*, 2003), however, only few studies have been reported on effect of enzymes to lupin based diets for layers. Williams *et al.*, (2005) found positive effect of pectinase addition to layer diets with whole and dehulled lupins on performance and found a decreased viscosity and reduction of wet droppings in diets with

20% lupins. However, more research could be relevant in order to study the possibility to improve the nutritive value of lupin and other legumes for laying hens.

Organic egg production requires access to outdoor pasture with either good quality grass/herbs or supplement forage material such as fresh hay, silages or vegetables. The consumption of foraging material can be considerable and reach a daily intake in the range between 60-120g/hen/day (Hammershøj and Steinfeldt, 2005; Steinfeldt *et al.*, 2007; Horsted *et al.*, 2007), and it can be expected that good quality foraging material could contribute to the nutrient requirement of organic layers to some extent. It has been reported that the consumption of foraging material can reduce the feed intake by up to 20% (Blair 2008), indicating that digestion and absorption of the foraging material take place to some extent. The chemical composition of different silages and vegetables is presented in Table 3 (Hammershøj and Steinfeldt, 2005; Steinfeldt *et al.*, 2007; Hammershøj and Steinfeldt, 2012).



Table 3. Chemical composition of silages and vegetables

CONSTITUENTS	MAIZE SILAGE	BARLEY-PEA SILAGE	ALFALFA SILAGE	LUPINE ⁴ SILAGE	CARROTS	BEETROOT ⁴	KALE
Dry matter	32.5	23.3	25.9	45.9	9.7	10.9	15.0
Ash	3.8	7.8	13.6	11.9	7.1	9.2	9.8
Protein ¹	9.2	14.4	22.9	16.0	7.5	15.7	20.5
Methionine	1.5	1.6	3.0	1.2	0.8	0.7	3.6
Cystine	1.2	1.5	1.3	1.3	0.7	1.0	2.8
Lysine	3.1	7.4	4.5	6.5	2.6	3.8	12.9
Threonine	3.3	4.8	3.3	5.1	2.2	2.7	8.9
Starch	29.2	13.7	0.5	0.4	†	†	7.2
Cellulose	17.3	19.0	19.8	17.5	7.3	5.2	7.3
Soluble-NSP	1.9	4.6	8.7	5.9	10.9	7.3	17.3
Insoluble-NSP	34.0	33.0	30.8	30.2	10.0	11.3	12.9
Total NSP ²	35.9	37.6	39.5	36.1	20.9	18.6	30.2
Lignin	8.0	10.1	10.6	10.8	1.9	1.6	3.3
Dietary fibre ³	43.9	47.7	50.1	46.9	22.8	20.2	33.5

Values given represent average analyses from different batches. ¹N*6.25. ²NSP=Non-starch polysaccharides. ³Dietary fibre = Total NSP + lignin. ⁴Unpublished data. Constituent in: g/100g dry matter. For amino acid: g/kg dry matter. The NSP content and composition of foraging material differ to a large extent and addition of cell wall degrading enzyme could be beneficial in order to increase the utilisation of potential nutrient in foraging material.

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Enzyme efficacy and cereal quality

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Introduction

It is often assumed that 'cereal quality' affects the animal performance enzyme response, which is supposed to manifest itself through a reduced response to enzyme addition when cereal quality is good. Whilst theoretically this may well be valid, under practical and research conditions this effect is not easily demonstrated. This paper will look at some of the available data in this respect.

First of all, what is meant by 'cereal quality' needs to be defined. The nutritionist has an array of analytical possibilities to define 'quality', ranging from the very easy parameters defined at grain intake, such as bushel weight and dry matter to much more costly parameters like mycotoxin content, endosperm structure etc. Commonly, people work with a set of standard analytes comprising dry matter (DM), fat, protein, ash and fibre. Starch analysis is seldom done, due to cost and variability, even though starch is the main source of energy from the cereal. The starch, fat and protein could be considered the nutrients delivering the value of the cereal, and in fact the protein present in the cereal can have quite an impact on the value of cereals, something which is often forgotten. In a way the amount of protein, fat and starch in a cereal determines the potential nutrient value of the cereal,

which can then be reduced due to factors in the cereal that reduce the availability of these nutrients to the animal. The potential for enzymes logically lies in the reduction of the negative effect of those factors, but maybe that is not the only area of potential improvement. Factors that negatively affect availability of nutrients are numerous, such as phytate, extract viscosity, fibre and mycotoxins. Mycotoxins fall outside of the scope of this paper. Phytate can be destroyed by phytase, but for the purpose of this paper we will focus on viscosity and fibre (or non-starch polysaccharides (NSP)), as those are areas where we can expect positive effects from the use of NSP enzymes.

Viscous cereals

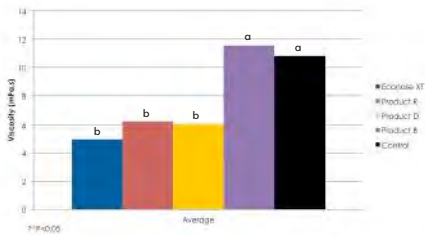
The effects of NSP-degrading enzymes in diets based on viscous cereals are well known, and it is also well known that, for instance, wheat can vary substantially in terms of the viscosity in the animal. The hypothesis would be that in a highly viscous diet the enzyme effect would be larger than in a diet made with a less viscous wheat. Often people test enzyme efficacy by using some rye mixed into the diet to mimic the use of a batch of high viscosity wheat, which seems a valid approach. In one of those tests (AB Vista internal data, 2010), it was shown that indeed viscosity was higher

in the diets containing some rye and that addition of xylanase (Econase XT, 16000 BXU/kg) reduced viscosity of all diets to a common level. So it could be argued that if viscosity is part of the 'cereal quality' definition, the use of xylanase can certainly help to reduce the effect of variable cereal quality. Interestingly, it has been shown that xylanase can reduce viscosity across a range of diets; even in a full barley diet the use of this xylanase had a significant effect on *in vivo* viscosity (Santos *et al.*, 2013),

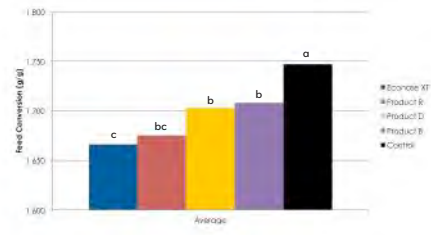
whilst β -glucanase only had a positive effect on viscosity if barley was present in the diet. Xylanases can differ in their ability to reduce viscosity, as demonstrated in a trial with a wheat/rye diet performed in 2010 (AB Vista trial report 206). Interestingly, even though one of the xylanases gave no benefit at all in terms of viscosity, this product still had some positive effect on feed conversion ratio (FCR). The best FCR was achieved by Econase XT, which also showed the lowest viscosity in the birds (Figure 1).

Figure 1. The influence of 4 different xylanase-based commercial enzyme products on broiler digesta viscosity and feed conversion efficiency when fed a wheat-rye based diet (AB Vista trial report 206)

Viscosity (mPa.s) in 22d broilers fed a wheat-rye diet



Better Feed Conversion (42d, AH broiler) with low viscosity



With viscous cereals there is often debate about a so-called new crop effect, which is hypothesized to be related to the viscosity of the cereal. As such, the use of NSP enzymes is considered as a potential remedy for the negative impact of feeding new crop cereals. Part of the debate is whether it is required to increase the dose level of NSP enzymes already in use during the new crop cereal period. To an extent this will depend on the ability of the particular enzyme to reduce viscosity, and the dose level that is routinely used. Some data

was recently published (Soares, 2014) to try and look at this. The data showed no difference in *in vitro* viscosity between old crop and new crop wheat, and showed that three commercial enzyme products differed in terms of their effect on viscosity, but there was no difference between the enzyme effect on old crop wheat or new crop wheat. This suggests that as long as an enzyme product is chosen that is effective in reducing viscosity, there may not be a need to increase the dose level during the new crop period. But, given the difficulty in

doing proper research into this subject and the relatively low cost of a slightly higher inclusion of NSP enzyme, it is likely that practical nutritionists will continue using higher doses during a period post-harvest, so it is important to choose a xylanase that will not have a negative impact if used at higher dose levels.

Non-viscous cereals

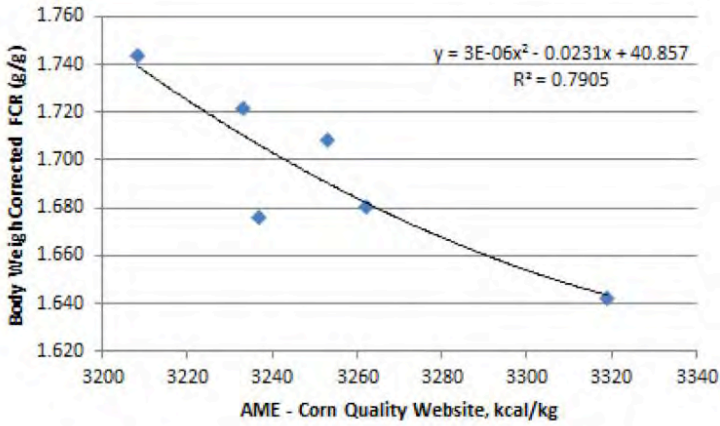
When viscosity is no longer so relevant, for example for diets based on corn (maize) or sorghum, then the use of NSP degrading enzymes has taken longer to develop into routine commercial use across the world. Variability in corn quality, as defined by the AME content of the corn, has been shown, for instance, by Collins & Moran (1998). Coupled with variability in AME found in individual birds (as shown by Hughes & Choct, 1997), this is often used as an argument in favour of the use of NSP enzymes in corn based diets. And it is true that with practical use of NSP enzymes, it is often observed that variability improves, as illustrated by Gomez (2012). Also, one can argue that if an ingredient is extremely digestible then the potential to improve digestibility further diminishes accordingly (Cowieson & Bedford, 2009). However, does this mean that variation in corn quality can indicate whether the use of xylanase will give a benefit? Corn quality definition and subsequent measurement should be such that the resulting parameters can be used to predict animal performance. AB Vista has developed a combination of measurements in the Corn Quality Service, the result of which is expressed as a predicted AME value. This predicted value combines analysed main

nutrient values with parameters indicative of protein solubility and endosperm structure, using a proprietary formula. Gehring *et al.*, (2012) showed that corns with similar proximate analysis but varying in protein solubility index (PSI) resulted in different performance.

In a recent trial (AB Vista internal data) corns ranked using this method were fed to birds, with performance (FCR) measured as well as Ileal Digestible Energy (IDE). Whilst the correlation between measured IDE and FCR was poor, there was quite a good correlation between Corn Quality Service predicted AME and FCR (Figure 2). When these same corns were fed with or without xylanase (Econase XT, 16000 BXU/kg), there was a tendency for a bigger improvement in IDE for low IDE corns, but no interaction between corn and enzyme dosing for FCR. This data tells us two things; first of all that the Corn Quality Service gives a worthwhile prediction that correlates well with actual animal performance. Secondly, it shows that the addition of enzyme does improve performance, irrespective of whether a good or mediocre corn is being used. Similar trials have been performed in other parts of the world, with similar responses. Whilst the theoretically expected reduction in enzyme benefit may well be real, so far we have not been able to demonstrate this in our trial work. An explanation for this could be that the enzyme has a beneficial effect at diet level, not just affecting the cereal, as shown by Masey O'Neill (2014) during the Inspire Forum.



Figure 2. Correlation between predicted AME and broiler 21-day FCR when fed corn-based diets



Conclusions

Depending on particular purpose of the NSP enzyme chosen, the interaction between cereal quality and enzyme use appears to be limited. For viscous diets there can be an impact of cereal quality in terms of viscosity,

and this can interact with the ability of the NSP enzyme to reduce intestinal viscosity. For non-viscous diets, it appears that the use of a xylanase (Econase XT) can have a positive impact on animal performance, irrespective of the cereal quality.

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Efficacy of NSP'ases: interactions with other enzymes with specific reference to phytase and protease

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Fibre-degrading enzymes (NSP'ases) are very commonly used in commercial pig and poultry diets and yet their value, as determined in trials reported in the scientific literature, may be incorrectly estimated. Phytase is almost ubiquitous in monogastric diets, whereas proteases are used only in a small proportion of diets. Nevertheless, it is unlikely that an NSP'ase will be used in the absence of one or both phytase and protease. As such, the value of an NSP'ase needs to be considered in the presence of phytase at least, and perhaps even protease, but the vast majority of research to date on NSP'ases has focussed on diets which contain neither. This paper will review the recent data available which investigates the effects NSP'ases in the presence and absence of phytase and to a lesser extent protease, and attempt to conclude whether there is an error in estimation of their value when used commercially as a result of the context in which their matrices have been derived.

Combinations of individual enzymes may be synergistic, additive, sub additive or even detrimental. If the matrices of each enzyme are taken at face value, then mistakes in their employment will accrue unless their combination is shown to be additive, as is generally assumed. This is not commonly found to be the case, with the result that

the value of the NSP'ase may be under or over-estimated, depending upon the effect of their combination.

A recent review of the literature indicated that the whilst xylanases (19 studies) and phytases (16 studies) both improved ileal digestibility of amino acids when applied in isolation, when the combination were used the effects were sub-additive (5 studies), suggesting that simple addition of the amino acid matrices of both would over-estimate the value achieved (Cowieson and Bedford, 2009). This work established that the NSP'ases effectively recovered a fixed proportion of the undigested amino acids present in the diet, and that co-administration of a phytase effectively reduced the content of undigested amino acids and thus reduces the substrate on which the NSP'ase can act. Nevertheless, this review considered digestibility of amino acids and not the effect of the enzymes on performance; i.e. how these effects translate into rate and efficiency of gain, which is much more relevant for the commercial world.

A confounding factor to consider is that in much of the literature, when phytases and xylanases are combined factorially, this is done in diets which are deficient in P in order to guarantee a response to the phytase. Low P diets would be expected to

respond to a phytase more so than xylanase since the former specifically releases P and the latter is assumed to release primarily energy. The response to the xylanase in the absence of a phytase may be compromised as it will be releasing nutrients which are not growth limiting. Commercially, however, the xylanase will be employed in a diet, containing a phytase, which is not limiting in P, and thus the literature may not represent commercial reality.

A review of the literature regarding the effect of a combination of xylanases and phytases on animal performance was conducted by Rosen in 2004, in which he commented on the points raised above (Rosen, 2004). At that point in time only 11 publications provided a total of 17 2x2 factorial combinations of the two enzymes. He found that in general the effects of xylanase and phytase were at best additive as far as FCR was concerned, although this was only true in wheat based diets, which constituted the bulk of the trials investigated (15 out of 17 tests), and sub-additive for gain. He also noted that the extent of the response was dependent upon the performance of the control diets; the poorer the control performance, the bigger the response to both enzymes. Given the control performance was often dictated

by the AvP level of the diet; i.e. P deficient diets resulting in poor control performance, it suggests that even the xylanase may be improving performance in low P diets. This counter-intuitive consideration was not addressed in the work of Rosen.

In the present work, 13 publications were collected between 2005 and the present day where NSP'ases and phytase were factorially combined and fed to broilers, 12 of which provided performance data. From these 12 papers, there were 41 tests where the NSP'ases were added to a diet in which a phytase was either present or not such that the effect of the presence of phytase on the NSP'ase response could be determined. The average age of birds used was 23d with the vast majority of trials being 1-21d, with one trial going to 35d of age. Ca and P levels averaged 0.84% and 0.30%, respectively, and ranged from 1.67-0.64% and 0.45-0.15%, respectively, with ME averaging 3017 kcals/kg but ranging from 3140-2868 kcal/kg. All except two of the trials used male birds (the others being mixed sex and undeclared!) and all except four studies fed mash diets, the rest cold pellet (2), 70°C pellet (1) or not declared (1). Of the 41 data points, 24 were maize-based diets with the balance being wheat-based.

Table 1. Percentage response of broilers to the addition of a NSP'ase overall, in the presence and absence of a phytase, compared with the relevant control

	AVERAGE	NO PHYTASE	PHYTASE PRESENT
Gain response	2.5%	3.0%	1.9%
Intake response	1.0%	1.9%	-0.1%
FCR response	-2.3 %	-2.9%	-1.68%
N	41	19	22

The average response to NSP'ase across the whole data set was a 2.5% response in gain and a 2.3% reduction in FCR (Table 1). When the xylanase was used in isolation, the gain response was marginally larger, at 3%, and FCR approximately the same. This was surprising given the fact that in such diets P was most often the limiting nutrient. This suggests that xylanase can release some P and thus improve performance, but it must be noted that in such P deficient diets the response to phytase was more than twice that recorded for xylanases. In the presence of phytase, the effect of the NSP'ase on FCR and gain was weakened, suggesting sub-additivity and thus some cross-over in mechanism. It must be noted, however, that when the NSP'ase was placed in a diet containing the phytase, the weights of the "control" (ie the diet containing the phytase alone) were greater than that of the control diet containing no phytase (555g ave vs 523g ave). Thus the final weights of the birds fed the combination of enzymes were on average greater than those fed either enzyme in isolation.

Given the data presented here, it seems that the combination of xylanases and phytases will overlap to a degree, and as a result there may be a need to reduce the matrices

of each enzyme when both are fed in combination. These conclusions are based on the average of the responses from the scientific literature, but clearly in some trials there is no additivity at all (Wu *et al.*, 2004). In such cases it may simply be a case of the diet being relatively nutrient dense such that either of the two enzymes fed in isolation can release the rate-limiting nutrients. Such observations are also evident for single enzymes when employed in nutrient rich diets, and highlights the need to use appropriate diets to exploit the benefits of the enzymes employed.

Mechanistically, any synergy between the two enzymes may well be due to the proposal that NSP'ases delay gastric emptying, and as a result allow the phytase to work longer in an environment conducive to phytate degradation – i.e. low pH. More work is needed to confirm such hypothesis but, if found to be correct, the use of these two enzymes in combination may prove to be as beneficial commercially as has been suggested from the literature.

Conclusion

It was clear when reviewing the literature that there was far more diligence and concern in measuring the in-feed activity levels of

phytase than for xylanase. Nine papers reported phytase activity versus seven for the NSP'ase, and, more alarmingly, the phytase levels were found to be within 75-125% of target whereas the NSP'ase varied from 55-255% of target. Given there is ample evidence that the response to the NSP'ase is dose dependent, it seems incredible that such short shrift is given to discussion of the massive variation in actual activity from the targeted level of the NSP'ase. It is clear that there is a lot of confusion in the NSP'ase literature regarding efficacy of different enzymes, but perhaps a fundamental reason for such apparent confusion is the absolute disregard of the relationship between dose and efficacy. Often the recovery of excess activity is due to endogenous feed xylanases swamping the assay whereas lower than expected


activity is due to pelleting losses or inhibition by one of the recognised three inhibitors of xylanase activity in feeds. Until the literature recognises that such explanations need to be rejected and a true estimate of the added activity recorded, the interpretation of the information available will be equivocal. Improvements in the assay and use of enzymes which are both inert to the inhibitors and more thermostable will allow for much more consistent administration of the activity in question and only then can it be judged whether the response to such products is as variable as is currently considered. The lack of ability to consistently prove accurate administration of the targeted dose of a product hampers its development, and makes comparison of products an almost impossible and virtually random process.

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