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Water-soluble short-chain arabinoxylans produced by enzymatic treatment of wheat grain improve growth performance and intestinal health of broilers

JURY

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List of abbreviations

1-DE: single SDS-PAGE electrophoresis

16S rRNA: 16S ribosomal ribonucleic acid

^{1}H NMR: proton nuclear magnetic resonance

2-DE: 2-dimensional gel electrophoresis

A: arabinose

AA: amino acid

AGP: arabinogalactan-proteins

AME: apparent metabolizable energy

AMP: antimicrobial peptides

ANOVA: analysis of variance

AX: arabinoxylans

AXOS: arabino-xylo-oligosaccharides

AZCL: azurine-crosslinked

BG3: tri-saccharide β-glucan

BG4: tetra-saccharide β-glucan

BWG: body weight gain

C: carbon

CAZy: carbohydrate active enzymes

CBM: carbohydrate-binding module

CD3: cluster of differentiation 3

CD4: cluster of differentiation 4

CD8: cluster of differentiation 8

CE: carbohydrate esterases

CTAB: hexadecyltrimethylammonium bromide

DF: Dietary fiber

DHAP: dihydroxyacetonephospate

DNA: deoxyribonucleic acid

DNS: dinitrosalicylic acid

DP: degree of polymerization

dXyl: di-substituted xylose

EC: Enzyme Commission

EFSA: European Food Safety Authority

EIF: ethanol-insoluble fraction

EIS: ethanol insoluble solids

EU: European Union

F: ferulic acid

FCR: feed conversion ratio

FDA: Food and Drug Administration

FI: feed intake

FOS: fructo-oligosaccharides

G: galactose

Ga: glucuronic acid

GALT: gut-associated lymphoid tissue

GH: glycoside hydrolases

GIT: gastrointestinal tract

GLC: gas liquid chromatography

GLP-1: glucagon-like-peptide-1

GLP-2: glucagon-like-peptide-2

GOS: galacto-oligosaccharides

GT: glycosyltransferases

H': Shannon index

HBSS: Hank's Buffered salt solution

HMW-GS: high molecular weight glutenin

HMw: High molecular weight

HPLC: high-performance liquid chromatography

HPSEC: high-performance size-exclusion chromatography

HX: heteroxylans

Hyp: hydroxyproline

Ig: immunoglobulins

IL: interleukin

InhU: inhibitor unit

kDa: kilo dalton

LA: lactic acid

LMW-GS: low molecular weight glutenin

LMw: Low molecular weight

MAMP: microbe-associated molecular patterns

ME: metabolizable energy

MEP: multi-enzyme preparation

MHC: histocompatibility complex

MLG: mixed-linked β-glucan

Mw: molecular weight

NC: negative control

NK-cells: natural-killer cells

NSP: non-starch polysaccharides

OTU: operational taxonomic units

PBS: Phosphate-buffered saline

PCR: polymerase chain reaction

PEG: poly-ethylene-glycol

PEP: phosphoenolpyruvate.

pI: isoelectric point

PITC: Phenyl isothiocyanate

PL: polysaccharide lyases

PP: Peyer's patches

RI: refractive index

RNA: Ribonucleic acid

SB: sodium butyrate

SC-AX: short-chain arabinoxylan

SCFA: short chain fatty acids

SD: standard deviation

SDS-PAGE: sodium dodecyl sulfate-Polyacrylamide gel electrophoresis

TAXI *: Triticum aestivum* xylanase inhibitor

TCR: T-cell Receptor

TEA: triethylamine

TIM-1: TNO gastrointestinal model-1

USA: United States of America

v/v: volume/volume

w/v: weight/volume

w/w: weight/weight

WC: wheat control

WE: wheat+enzyme

WS: water-soluble

WSF: water-soluble fraction

X: xylose

XIP: xylanase inhibitor protein

XOS: xylo-oligosaccharides

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General introduction

General introduction

Cell wall polysaccharides-degrading enzymes are used routinely for many years in poultry feed to enhance feed digestibility. The literature suggests that cereal-based diets, including wheat, induce inflammation of the chicken digestive tract. Reducing this inflammatory process in the intestine is increasingly seen as the key to digestive health of animals and would have significant consequences on animal performance. The digestive health problems in poultry production represent significant economic losses, especially since the ban of the use of antibiotics as growth promoter. A safe alternative solution is essential to prevent intestinal problems and ensure a similar level of production. The use of curative antibiotics increased significantly during the last years (Persoons et al., 2012). As already widely reported by the authorities, the excessive curative use of antibiotics should be reduced to avoid appearances of antibiotic resistance. Different solutions are possible to prevent and/or cure digestive health problems of animals. One of the alternatives is the use of cell wall polysaccharides-degrading enzymes that would improve the inflammatory status of the digestive tract. Their effect is likely multifactorial. However, to date the mechanisms of action of exogenous enzymes are not fully understood. Though, it is suggested that these enzymes favor the production of oligosaccharides in the animal digestive tract, which are favorable for intestinal health. During the last decade, there was a huge breakthrough of scientific research on using oligosaccharides to improve digestive health in humans and animals. These oligosaccharides, qualified as “prebiotics”, are molecules resistant to gastric acidity, to hydrolysis by the endogenous digestive enzymes and the gastrointestinal absorption, which are fermented by the intestinal microbiota and selectively stimulate the growth of beneficial bacteria. Nevertheless, the connection between enzymes/ prebiotics/ effects on digestive health has not been fully established. Adisseo is a world's leading company producing feed additives, including enzyme preparations under the brand Rovabio®. The Rovabio®, has a large number of glycoside hydrolases (Guais et al., 2008). This enzyme cocktail is particularly rich in xylanase and β-glucanase activities. These activities allow it producing a wide range of oligosaccharides from cereal cell walls, as it has been demonstrated (Maisonnier-Grenier et al., 2006; Lafond et al., 2011a). Experiments on chickens and pigs showed microbiota changes but also physiological and immune parameter changes that suggest an effect of enzymes on digestive health (Devillard et al. 2008, Willamil et al. 2009a and 2009b).

The objective of this PhD work was to investigate the mechanisms of action of this exogenous enzyme cocktail on a wheat-based diet and establish the link between the enzyme preparation, the potential release of prebiotic components and the effect on animal performance and digestive health.

This PhD work was co-funded by Adisseo S.A.S France and the Agence Nationale de la Recherche et de la Technologie (ANRT) by an industrial agreement for training through research (Convention Industrielle de Formation par la recherche- CIFRE, convention N° 2012/0893). This is a joint research project between the research unit Biopolymers Interactions and Assembly (UR 1268 BIA) belonging to the National Institute of Agronomic Research (Institut National de la Recherche Agronomique- INRA) in Nantes (France) and the department of Pathology, Bacteriology and Avian Diseases from the faculty of veterinary medicine in Ghent (Belgium). Both research teams were highly complementary to carry out this project. INRA team is specialized in plant cell wall structure and physicochemical properties as well as cell wall-degrading enzymes. In particular, they developed many characterization methods of the hydrolysis products to diagnose the mechanisms of action of enzymes. Ghent team is specialized in the characterization and culture of avian intestinal microbiota and has developed *in vitro* and *in vivo* models to investigate the mechanisms and interactions between feed, microbiota and intestinal health.

The manuscript is organized as follows:

The first chapter is a bibliographic review of the different topics relevant for this study. It starts with a general description of the avian intestinal tract anatomy, physiology and the associated immune system. It is followed by a review of wheat grain structure and chemical composition and its link to animal nutrition. Finally, exogenous enzymes used as feed additives and their effects are described.

The second chapter describes the water-soluble fractions obtained with or without enzymatic degradation of wheat and their effect on the cecal microbiota *in vitro.*

In **the third chapter** wheat enzymatic degradation at a pilot scale was setup to produce large amounts of the wheat fractions that will be used *in vivo.*

The fourth chapter investigates the effects of the wheat fractions produced with or without enzymatic degradation of wheat, on performance, intestinal health and gut microbiota of broilers fed a wheat-based diet.

The fifth chapter is the general discussion of the results obtained during this thesis and ends with the **Conclusions and future perspectives**.

The thesis led to the following publications:

Publications in international journals:

Publication 1: N. Yacoubi, F. Van Immerseel, R. Ducatelle, L. Rhayat, E. Bonnin, and L. Saulnier. 2016. Water-soluble fractions obtained by enzymatic treatment of wheat grains promote short chain fatty acids production by broiler cecal microbiota. Anim. Feed Sci. Technol. 218:110–119. http://dx.doi.org/10.1016/j.anifeedsci.2016.05.016

Publication 2: N. Yacoubi, L. Saulnier, E. Bonnin, E. Devillard, V. Eeckhaut, L. Rhayat, R. Ducatelle and F. Van Immerseel. Short-chain arabinoxylans prepared from enzymatically treated wheat grain exert prebiotic effects during the broiler starter period. Submitted for publication in the British Journal of Nutrition (BJN-RA-16-0675: June 7th 2016).

Oral presentation in international conferences (speaker name underlined)

Nadia Yacoubi, Luc Saulnier, Estelle Bonnin, Estelle Devillard, Richard Ducatelle and Filip Van Immerseel. Effects of enzymatic degradation of wheat grain cell walls on the digestive health of poultry. The 14th European Poultry Conference, June 23-27, 2014 in Stavanger, Norway.

Nadia Yacoubi, Luc Saulnier, Estelle Bonnin, Lamya Rhayat, Richard Ducatelle and Filip Van Immerseel. Degradation products of multi-enzyme preparations improve the performance and digestive health of wheat-fed broilers. Poultry Science Association104th Annual Meeting. Student Competition: Metabolism and Nutrition: Feed Additives I (abstract N°24), July 27-30, 2015 in Louisville, USA.

Nadia Yacoubi. Effects of Rovabio® on broilers performance and potential intestinal health benefits. The feedase conference, June 22-23, 2016 in Bruges, Belgium.

Nadia Yacoubi, Luc Saulnier, Estelle Bonnin, Estelle Devillard, Lamya Rhayat, Richard Ducatelle, Filip Van Immerseel. Wheat-arabinoxylan produced by enzymatic pretreatment improve the performance and digestive health of wheat-fed broilers. Poultry Science Association105th Annual Meeting, Metabolism and Nutrition, Enzymes I (abstract N°168). July 11-14, 2016 in New Orleans, USA.

Posters on international meetings (attendee's name underlined)

Nadia Yacoubi, Luc Saulnier, Estelle Bonnin, Richard Ducatelle, Filip Van Immerseel. A multi-enzyme preparation degrades wheat to butyrogenic substrates and improves broiler digestive health. P-129 (ID 248). The 20th European Symposium on Poultry Nutrition ESPN 2015, August 24-27, 2015 in Prague, Czech Republic.

Nadia Yacoubi, Luc Saulnier, Estelle Bonnin, Estelle Devillard, Lamya Rhayat, Richard Ducatelle, Filip Van Immerseel. Enzymatic treatment of wheat favours butyrate-producing bacteria in the broiler ceca, stimulating gut health. Session 5: Impact of diet on gut microbiota. The 10th INRA-Rowett symposium on gut microbiology. June 20-23, 2016 in Clermont Ferrand, France.

Nadia Yacoubi, Luc Saulnier, Estelle Bonnin, Estelle Devillard, Lamya Rhayat, Richard Ducatelle, Filip Van Immerseel. Designing arabinoxylan structure by enzymatic degradation to improve the performance and the digestive health of wheat-fed broilers. Nutrition and Feeds session. The 25th World's Poultry Congress, September 5-9, 2016 in Beijing, China.

Chapter I

Bibliographic Review

I.1- Broiler digestive system

Understanding the anatomical and functional characteristics of the broilers digestive system is essential to develop and adapt the best economic and nutritional strategy to improve performance of these animals. In the following part, the anatomy and the physiology of the digestive system, as well as the mutualistic relationship between the broiler and the intestinal microbiota are described.

I.1.1- Anatomy and physiology of the broiler digestive system

The digestive system, as described in figure I-1, comprises different segments.

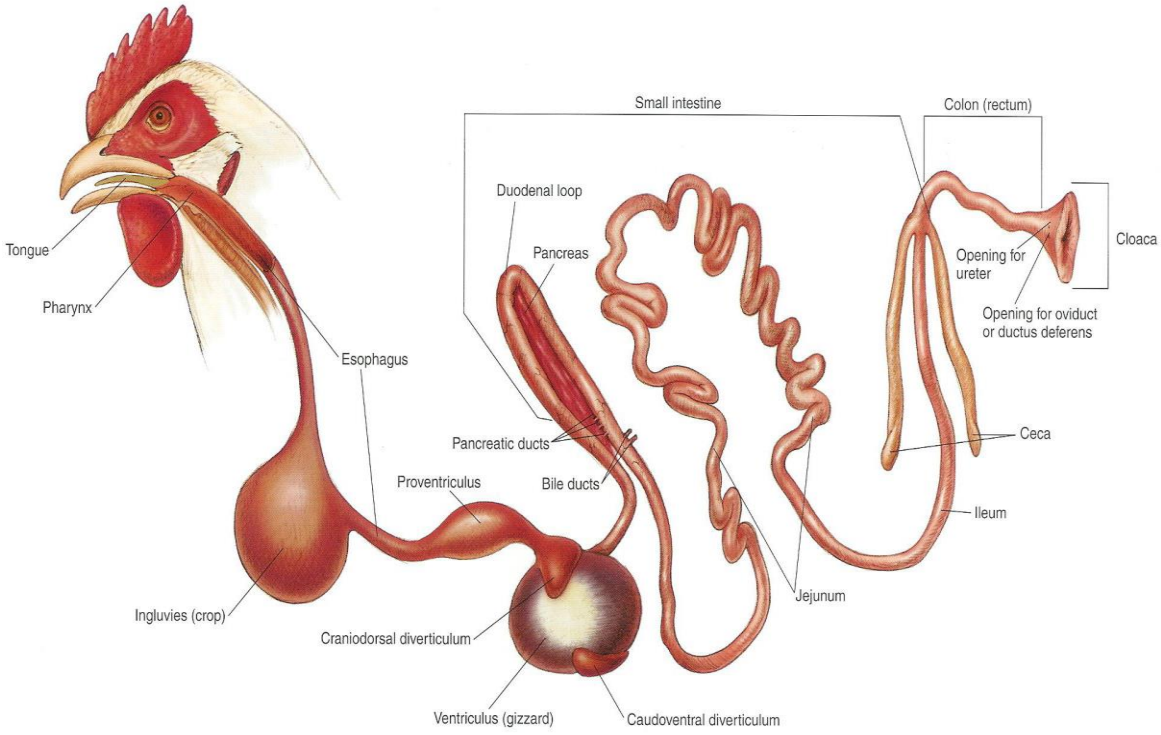

Figure I-1: Digestive tract of a chicken (www.uspoultry.org).

a) The esophagus is a long, tight and straight tube that prolongs from the glottis at the posterior end of the pharynx, through the neck and thorax to join with the proventriculus (Nasrin et al., 2012).
b) The crop (ingluvies) is an expansion of the esophagus that represents a storage organ of water and feed. Little digestion can occur by amylases coming from salivary secretions or by exogenous enzymes (Denbow, 2015).

c) The proventriculus, also named glandular stomach is the site where the digestion begins. The glands shape the thickness of the organ and are responsible for the production of the mucus and the gastric juice (Denbow, 2015). The last is constituted of hydrochloric acid and digestive enzymes such as pepsin (Gelis, 2013).

d) The ventriculus or gizzard is responsible for the mechanical breakdown of the feed and allows the gastric juice to exert its hydrolytic action on smaller feed particles. The gizzard consists of two pairs of opposing muscles: 2 thin muscle layers, *i.e*, the caudoventral and craniodorsal and 2 thick muscle layers, *i.e*, caudodorsal and cranioventral (Farner and King, 2013). The mechanical contractions of the thick layers are responsible for the grinding and mixing of the feed (Klasing, 1999). The gizzard is coated by the koilin layer, a cuticle layer that acts as a grinding surface and protects the underlying mucosa from the gastric juice (Gelis, 2013). The ventriculus is separated from the small intestine by a small pyloric fold that adjusts the passage of feed into the small intestine by slowing the movement of the large particles (Vergara et al., 1989).

e) The small intestine is composed of 3 parts: duodenum, jejunum and ileum. The duodenum starts from the pylorus and forms a ring that encircles the bulk of the pancreas and receives the pancreatic and hepatic ducts (Gelis, 2013). These conduct the exocrine pancreas secretions to the small intestine. The exocrine pancreas contains a mix of enzymes such as amylases, lipases, trypsin and chymotrypsin, carboxypeptidases (A, B and C), deoxyribonucleases, ribonucleases and elastases (Klasing, 1999; Pandol, 2011), as well as bicarbonate that buffers the intestinal pH (Gelis, 2013). In combination with the enzymes produced by the intestinal wall mucosa (amylases, maltases, sucrases, enterokinases, lipases…) the exocrine pancreas is responsible for the enzymatic degradation of feed components (Denbow, 2015). Meckel's diverticulum is considered as a landmark of the end of jejunum and the start of the ileum since these are often difficult to differentiate anatomically (Denbow, 2015). Meckel's diverticulum is the remnant of the yolk stalk that originally connected the yolk sac and the gut of the embryo.

As shown in figure I-2, the structure of the intestinal wall, starting from the exterior, is as follows:

- serosa, a serous membrane on the outside surface of the intestine.
- muscularis externa composed of a layer of longitudinal muscle that runs longitudinally along the intestine, and a three time thicker layer of circular muscle.

- sub-mucosa, situated below the two muscle layers and containing blood vessels, lymph vessels and a network of nerve fibers.
- muscularis mucosae is a thin layer of muscle, located outside the lamina propria mucosae and separating it from the submucosa.
- mucosa or mucous membrane consisting of the epithelium and the lamina propria. The small intestine epithelium contains villi and crypts (Sklan, 2001). The epithelial cells on the villi have about 10^5 microvilli per mm^2 on their apical surface increasing the absorption area (Klasing, 1999). The villi include a capillary bed that transfers the absorbed nutrients to the portal blood vessels. The lamina propria is constituted of loose connective tissue allowing it to contain a large panel of varied cells including, fibroblasts, lymphocytes, plasma cells, macrophages, eosinophilic leukocytes, and mast cells (Tizard, 2013).

f) The ceca are two small pouches at the junction of the ileum and the colon. They are composed of three morphologically different regions: the proximal region (*basis ceci)*, with well-developed villi, the medial region (*corpus ceci)*, with longitudinal folds and small villi, and the distal region (*apex ceci*), with longitudinal and transverse folds and small villi (Ferrer et al., 1991). The ceca play an important role in the fermentation of the undigested feed components. They are filled by the rectal anti-peristaltic and ileal peristaltic movements (Denbow, 2015). Nevertheless, only water and small particles are able to enter the ceca due to the architecture of the ileum-colon junction. The cecal fluid is composed of 87-97 % urine (Björnhag, 1989).

g) The colon is also named the large intestine, though it is shorter than the small intestine. Its main role is the absorption of water and nutrients at the exit of the ileum and ceca (Denbow, 2015).

h) The cloaca is a common opening on the exterior of the intestinal and the urogenital tract (Denbow, 2015). It consists on 3 compartments: the copradaeum (a continuation of the colon-rectum), the urodaeum (the ureters and genital ducts openings) and the proctodaeum (opens to the exterior of the vent).

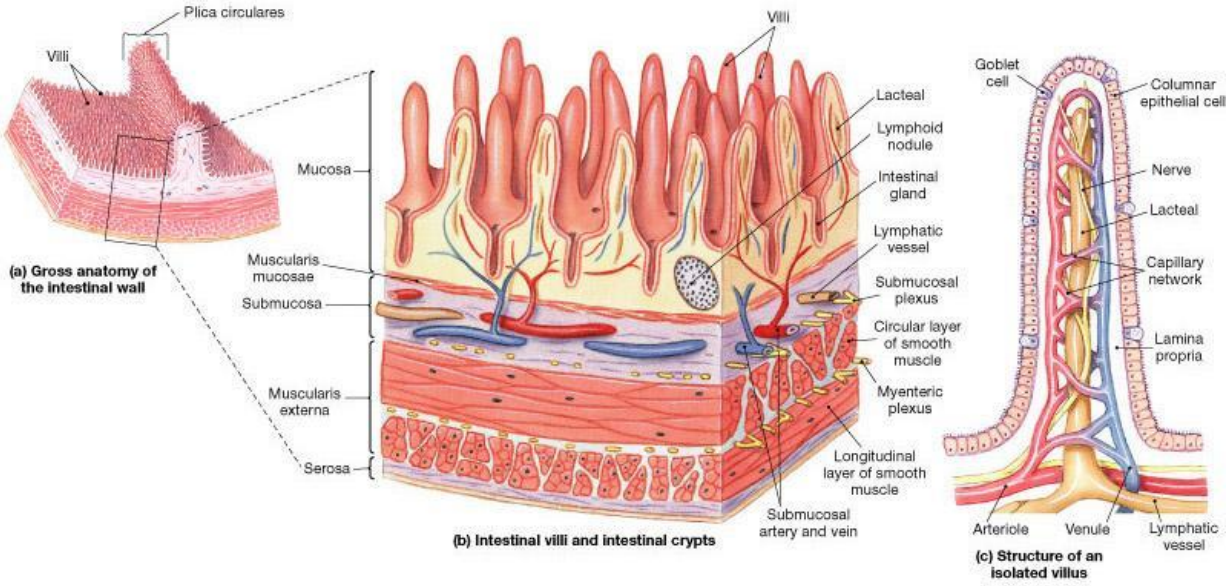

Figure I-2: Structure of the intestinal epithelium (www.austincc.edu).

I.1.2- Intestinal microbiota

At hatch, the gastrointestinal tract (GIT) is germ-free and is colonized by exogenous microorganisms immediately after (Uni et al., 1999). After one day post-hatch the bacterial density reaches 10^8 and 10^{10} cells/g digesta in the ileum and ceca respectively (Apajalahti et al., 2004). Until recently, studies were based on *in vitro* culture techniques, which are not suitable to determine the complex composition of the microbiota. Amit-Romach et al. (2004) studied the evolution of the microbiota of chickens fed a commercial diet using molecular techniques. They showed that in the ceca of 4 days old chicken, the relative proportion of Lactobacilli was about 25 % of the total examined bacteria and that no *Bifidobacterium* was detected. About 35 % of the cecal bacteria consisted of *E. coli* and *Clostridium* species. The microbiota becomes diverse and reaches a relative stable dynamic state at 2 weeks post-hatch (Pan and Yu, 2014). The microbiota is then mainly composed of strictly anaerobic bacteria, which is one of the reasons why only 5-10 % of the species diversity was described with the culture techniques till the arrival of pyrosequencing methods (Bedford and Cowieson, 2012).

The intestinal microbiota is in constant interaction with the host, the diet and the environment. In poultry, the GIT tract is shorter than in mammals resulting in a short transit time (average of 3.5 h for broilers, Hughes, 2008) and in a natural selection of fast growing bacteria that can adhere to the mucosal layer. In contrast, the ceca, which have a slow transit rate allowing a more diversified microbiota.

I.1.2.1- Microbiota composition

Depending on the DNA extraction methods, sequencing technology and bio-informatics analysis pipelines, the relative abundance of the different taxonomic levels differs. The 16S ribosomal ribonucleic acid (rRNA) sequencing is a common amplicon sequencing method used to identify and compare bacteria present within a given sample. 16S rRNA gene sequencing is a well-established method for studying phylogeny and taxonomy of samples from complex microbiota by analyzing the prokaryotic 16S rRNA. Wei et al. (2013) generated a phylogenetic diversity census of the broilers intestinal bacteria, by using high quality 16S rRNA gene sequences (3184) collected from databases. The analyses allowed identifying 915 clusters belonging to 13 phyla. In cecal samples, only 10 known bacterial phyla were identified and they account for 92.8 % of the chicken cecal sequences (Figure I-3). The *Firmicutes* and *Bacteroidetes* are the most abundant phyla and account for approximately 78 and 11 % of the total cecal sequences, respectively. In the chicken ceca the most common families within the order *Clostridiales* are *Lachnospiraceae* and *Ruminococcaceae. Clostridium, Ruminococcus, Lactobacillus*, and *Bacteroides* were the most abundant genera of the 117 genera identified in the chicken ceca. Archaea were only represented by the *Euryarchaeota* phylum with a very small number of sequences validating the low abundance of methanogens in the gut of chickens (Saengkerdsub et al., 2007).

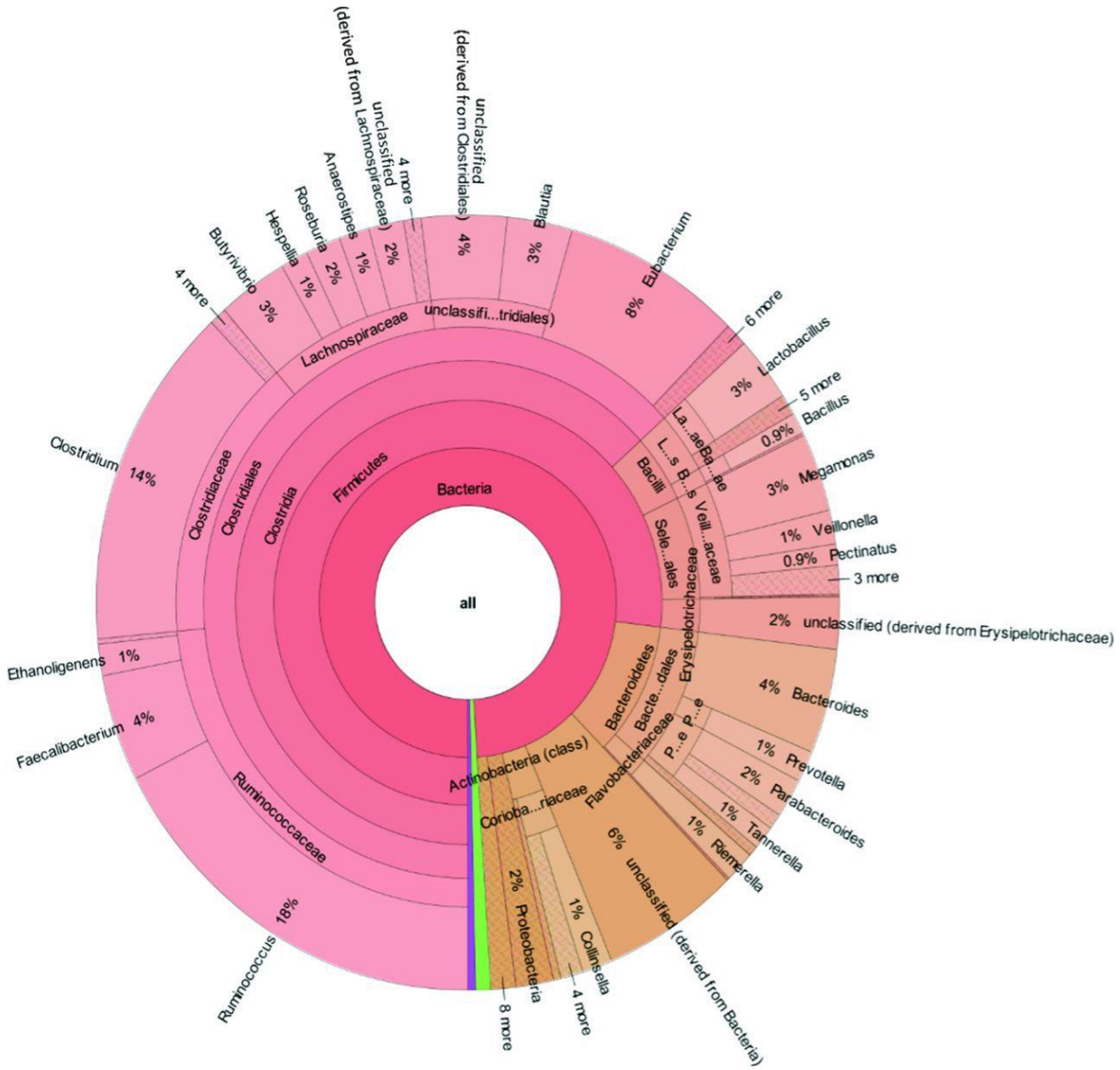

Figure I-3: Krona chart of bacteria from chicken cecum (from 919 of the 972 chicken cecal sequences) (Wei et al., 2013).

Stanley et al. (2014) found that *Lactobacillus spp*. dominate the duodenum and ileum, together with *Streptococcus*, *Enterobacteriaceae* and various *Clostridiaceae*. The microbiota composition between ileum and cecum (Figure I-4) differs according to the different functions of the corresponding segment (Torok et al., 2011; Apajalahti and Vienola, 2016). However, most of the studies investigating the intestinal microbiota are based on cecal or fecal samples, and only few focused on ileum.

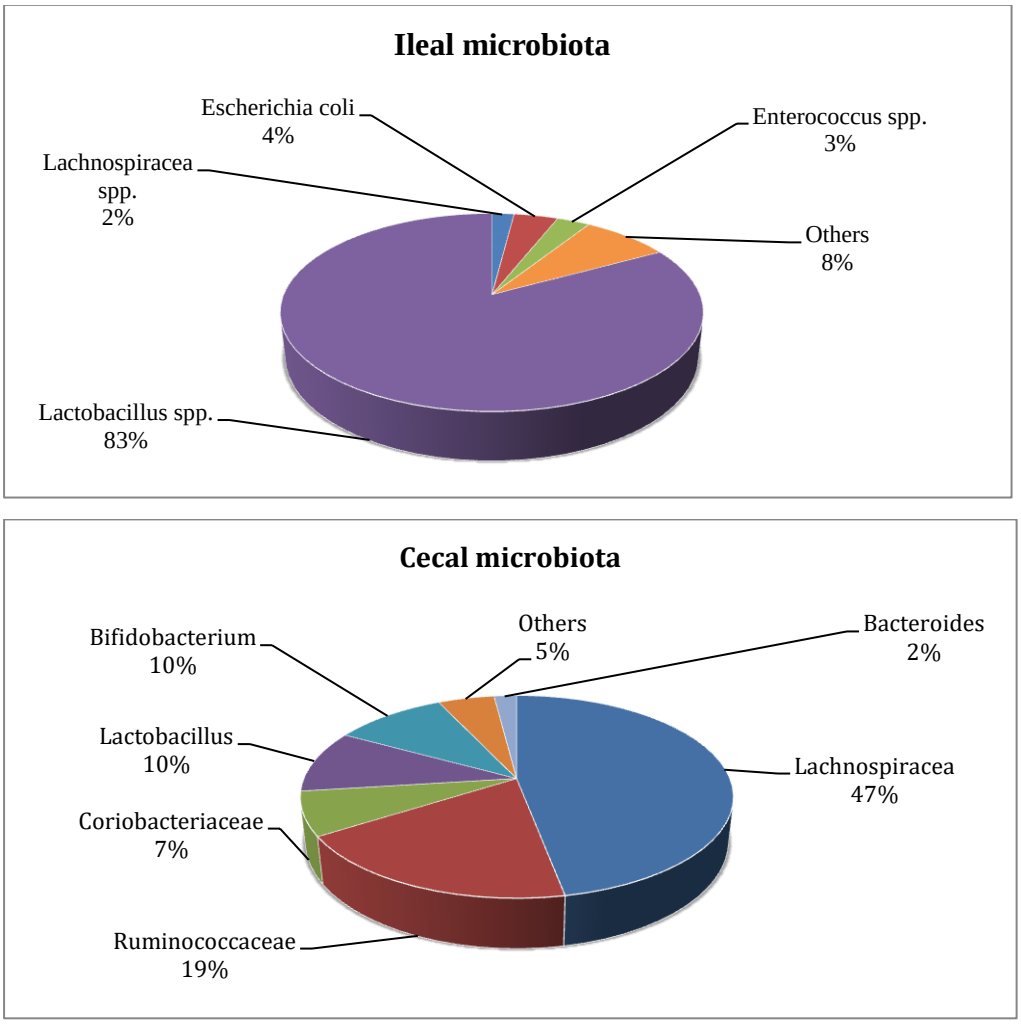

Figure I-4: Average microbiota composition on commercial broiler chicken farms. The diagrams show the average microbiota composition (%) in the ileum and cecum for 10 European farms (Apajalahti and Vienola, 2016).

The microbiota is influenced by different factors, of which the diet has the biggest impact, as described in the next section.

I.1.2.2- Factors influencing the microbiota

Dietary components that are not digested and not absorbed by the host, act as a substrate for the intestinal bacteria. Wheat-based diets contain non-starch polysaccharides (NSP) that favor the proliferation of *Clostridium perfringens*, responsible for necrotic enteritis (Annett et al., 2002; Jia et al., 2009). A similar effect was observed when the diet contained a high level of animal fat (Knarreborg et al., 2002). The source and the level of protein may also alter the microbiota. For example, fermented cottonseed meal (505 g/kg of

crude proteins and 90 g/kg of crude fibers) increases *lactobacilli* and decreases the coliforms in the broilers cecum (Sun et al., 2012).

Feed additives influence the gut microbiota considerably. Antibiotics have been used as growth promoters in Europe till 2006 and are still used in other countries. They affect the intestinal microbiota by inhibiting enteric pathogens and favor the proliferation of beneficial species (Gunal et al., 2006). An alternative to antibiotics are prebiotics, that are indigestible feed ingredients and serve as a substrate for the microbiota in favor of beneficial bacteria over others (Candela et al., 2010; Licht et al., 2012). Prebiotics such as galacto-oligosaccharides (GOS) have been reported to stimulate the growth of bifidobacteria in the intestinal tract of broilers (Jung et al., 2008). Fructo-oligosaccharides (FOS) decrease *Salmonella* Enteritidis in the cecum of laying hens (Donalson et al., 2008) and *Clostridium perfringens* and *Escherichia coli* in the gut of broilers (Kim et al., 2011). Many authors studied the effects of adding arabino-xylo-oligosaccharides (AXOS) or xylo-oligosaccharides (XOS) to monogastric diets and found that oligosaccharides with a degree of polymerization lower than 5 contribute to the proliferation of beneficial bacteria and improve microbial diversity (Van Craeyveld et al., 2008; Sanchez et al., 2009; Damen et al., 2011). Thus, both can be considered as prebiotic candidate.

Exogenous enzymes (detailed in section I.4) used as feed additives might also influence the intestinal microbiota. Glycoside hydrolases such xylanases and β-glucanases increase intestinal lactic acid bacteria and decrease *Escherichia coli* occurrence (Rodríguez et al., 2012).

The interactions between the different bacteria within the microbiota influence its composition. There is for example competition between some bacterial species for nutrients such as dietary zinc, which is an essential trace element, required by both eukaryote and prokaryote cells for many cellular functions (Berg and Shi, 1996; Gielda and DiRita, 2012). The competition may also be for attachment sites. Healthy birds have a layer of commensal bacteria that colonize the intestinal mucosa and act as barrier against enteric pathogens invasion (Lan et al., 2005; Lawley and Walker, 2013). The competition between the commensal microbiota and exogenous pathogens could be accomplished by the production of bacteriostatic and bactericidal substances.

I.1.2.3- Short chain fatty acids and organic acid

The intestinal microbiota possess a wide range of hydrolytic enzymes able to depolymerize the substrate in monosaccharides, that are the main substrates fermented by the microorganisms (Macfarlane and Macfarlane, 2003). The major end products of the bacterial metabolism in the intestinal tract are the short chain fatty acids (SCFA): acetate, propionate, butyrate, and the organic acid lactate.

Lactate is the major fermentation product of lactic acid bacteria, including *Lactobacilli, Bifidobacteria, Enterococci* and *Streptococci* and can also be produced by strict anaerobes such as *Eubacterium spp.* (Macfarlane and Gibson,1997). However it is an intermediate metabolite that is, if not rapidly absorbed, used in cross-feeding for butyrate producers (Bernalier et al., 1999; Duncan et al., 2004; Belenguer et al., 2006).

Acetate is the most abundant SCFA, which is produced by the strict anaerobic acetogenic bacteria from the genera *Ruminococcus, Blautia, Clostridium* or *Streptococcus* (Macfarlane and Macfarlane, 2003; Drake et al., 2008). Acetate improves ileal motility and is suggested to have a trophic effect on the colonic epithelium by local action and by increasing mucosal blood flow (Scheppach, 1994; Van der Wielen et al., 2000). It is generally recognized that acetate is also an intermediate consumed by the major butyrate producing bacteria (Louis and Flint, 2009).

Propionate has been shown to inhibit hepatic cholesterol synthesis in humans (Bugaut and Bentéjac, 1993) and to play an essential role in the regulation of feed intake in non-ruminants. Adding propionate to feed during a week increased fecal mass and *Bifidobacteria* (Hosseini et al., 2011). It was also shown that it influences both fat and carbohydrate metabolism by depressing the catabolism of substrates like oleate and lactate (Arora et al., 2011).

Butyrate is considered as the most important SCFA in the intestinal tract due to its numerous beneficial effects. Butyrate provides epithelial cells with energy and increases their proliferation and differentiation (Cook and Sellin, 1998; Mariadason et al., 1999). In the small intestine, butyrate enhances epithelial proliferation, differentiation and maturation, and reduces apoptosis of normal epithelial cells (Sengupta et al., 2006; Guilloteau et al., 2010;). Low concentrations of butyrate reduce the proliferation of macrophages and T-lymphocytes in the intestinal tract but induce apoptosis only in activated T-lymphocytes and non-differentiated macrophage cell lines (Bailón et al., 2010).

Oral administration of butyrate can improve growth performance (Zhang et al., 2011). Butyrate improves weight gain and feed conversion ratio, and increases villus length (Vercauteren et al., 2010). Hu and Guo, (2007) found that the ratio of villus height to crypt depth and the *Lactobacillus* count increased linearly with the increase of dietary sodium butyrate (SB) supplementation ($P \leq 0.01$).

As reviewed by Guilloteau et al. (2010), the effective dose of SB is between 1 and 4 % of dry matter intake and efficacy is improved by microencapsulation in lipid matrix. A slow release of butyrate from a lipid matrix prevents its rapid metabolization in the stomach and upper small intestine (Claus et al., 2007). Butyrate was found to have widespread positive effects on growth, digestibility and feed efficiency through the modulation of cell proliferation, differentiation and function in the GIT, especially mucosal epithelial cells, and on defense systems (barrier function, antimicrobial potency, immune system) in healthy and diseased animals (Kyner, 1976; Segain, 2000; Manzanilla et al., 2006; Mátis et al., 2015).

Acetate and lactate are intermediate molecules in the fermentation process and they are associated with the cross-feeding between the different species of the microbiota (Bernalier et al., 1999). As described by Macfarlane and Macfarlane (2003) and Louis et al. (2014) intestinal bacteria use different source of carbon (C) as substrate for fermentation (Figure I-5). Acetate can be produced by many enteric bacteria from pyruvate via acetyl-CoA and also via the Wood–Ljungdahl pathway by acetogens, such as *Blautia hydrogenotrophica*. Butyrate is formed from two molecules of acetyl-CoA by several Firmicutes, and the butyryl-CoA:acetate CoA-transferase is usually used to catalyze the last enzymatic step (Louis et al., 2004). The main propionate production pathway is the succinate pathway, which is used by *Bacteroidetes* to generate propionate from carbohydrates and by some *Firmicutes* to produce propionate from lactate or succinate. Two other propionate biosynthesis pathways are found in some gut bacteria: the acrylate pathway, which uses lactate, and the propanediol pathway, which uses deoxyhexose sugars (such as fucose and rhamnose) (Reichardt et al., 2014). Pathways that are involved in hydrogen metabolism and ethanol production were also shown. The bacterial species that are shown are based on studies of cultured isolates of dominant species and metagenomic analyses and are thus not exhaustive.

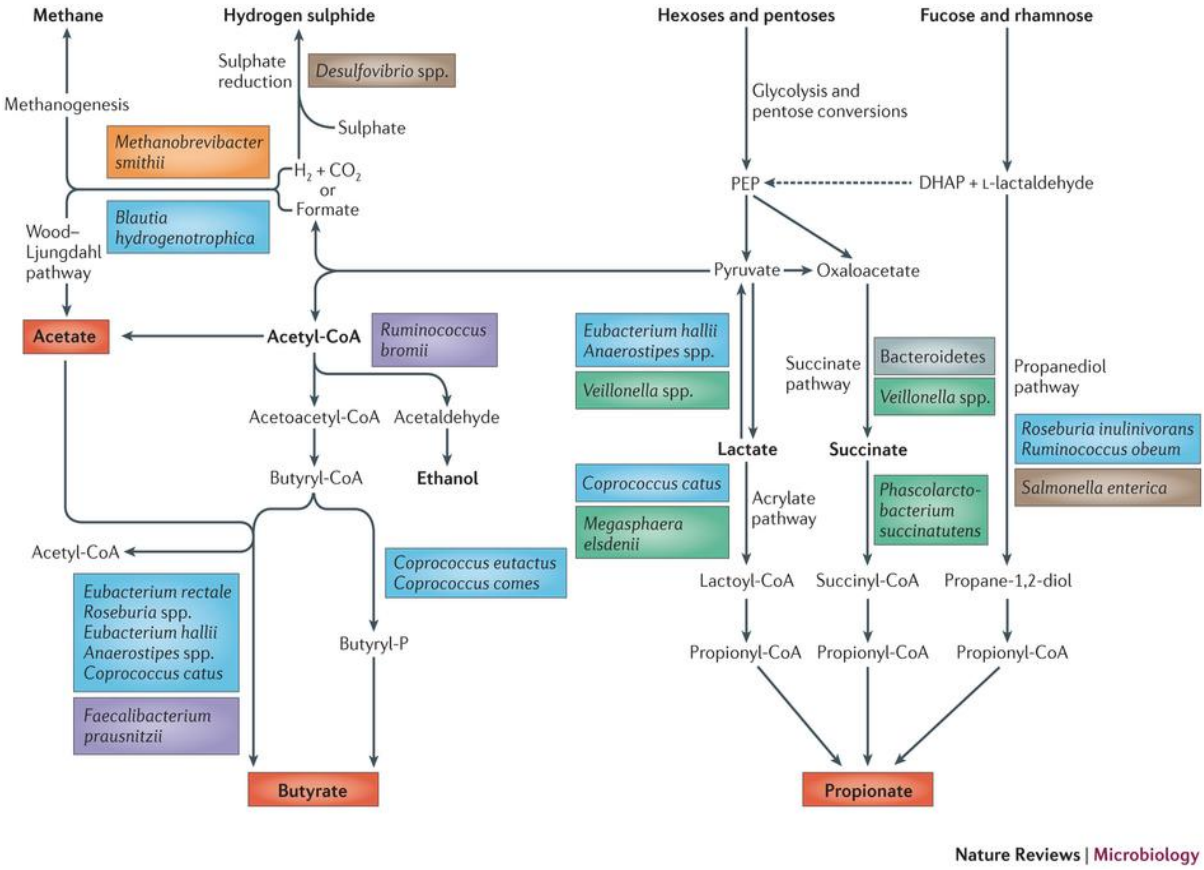

Figure I-5: Acetate, propionate and butyrate pathways (Louis et al., 2014), Archaea are shown in orange, *Bacteroidetes* are shown in grey, *Lachnospiraceae* (Firmicutes) are shown in blue, *Ruminococcaceae* (Firmicutes) are shown in purple, *Negativicutes* (Firmicutes) are shown in green and Proteobacteria are shown in brown. DHAP, dihydroxyacetonephospate; PEP, phosphoenolpyruvate.

I.1.3- Intestinal L-cells

SCFA, mainly butyrate, stimulate the differentiation of precursor cells into the glucagon like peptide-1 (GLP-1) and -2 (GLP-2) producing L-cells (Dubé and Brubaker, 2007). L-cells are endocrine-cells located mainly in the mucosa of the ileum and colon (Larsson et al., 1975). The intestinal L-cells are open-type cells and reach the intestinal lumen via a slender apical process (Eissele et al., 1992). They occur in all parts of the crypts and villi in the ileum.

GLP-1 is a 37 or 36-amino acid peptide depending on the cleavage of the preproglucagon during post-translational processing. It is now known to be a potent glucose-dependent insulinotropic hormone, which has important actions on gastric motility, on the suppression of plasma glucagon levels, and possibly on the promotion of satiety and stimulation of glucose disposal in peripheral tissues independent of the actions of insulin (Kieffer and Habener, 1999).

GLP-2 is a 33-amino acid peptide derived from the tissue-specific, post-translational processing of the pro-glucagon gene expressed in L-cells, which are located predominantly in the distal small intestine and colon. GLP-2 is a pleiotropic hormone that affects multiple facets of intestinal physiology, including growth, barrier function, digestion, absorption, motility, and blood flow (Dubé and Brubaker, 2007). The GLP-2 receptor is a G protein-coupled receptor expressed in discrete sets of intestinal cells, including endocrine cells, subepithelial myofibroblasts, and enteric neurons (Kieffer and Habener, 1999). GLP-2 increases intestinal epithelial growth by enhancing crypt cell proliferation and inhibiting apoptosis (Shousha et al., 2007; Drucker, 2015). The injection of GLP-2 to pharmacologically stressed broiler reversed the negative effect of stress on the weight, the morphology and the absorptive function of small bowel and improved the animal health and performance (Hu et al., 2010).

The integrity of the animal health is assured by the different component of the immune system especially the gastrointestinal one, which represents the first barrier against pathogens.

I.2- Avian gastrointestinal mucosal immune system

The avian immune system is similar to the human one in several aspects. First, the bird has a wide range of physical and chemical barriers that act like a shield to avoid exogenous invaders to penetrate the system. Once these barriers fail to counter the threat, the animal

mobilizes cellular and soluble components to eliminate it. This innate immunity is not able to eliminate and control all the pathogens and diseases, so a third line of adaptive immunity is mobilized.

I.2.1- Development of the Immune system

The development of the GIT fully takes off on embryonic day 15 by increasing the intestinal relative weight, epithelial morphology and activities (Uni et al., 2003). Despite the fact that the intestinal mucosa is structurally developed at hatch, there is much change in structure with age, especially over the first 7 days post hatch (Iji et al., 2001). The secretion of digestive enzymes in the post-hatched chick is considered as one of the limiting factor in digestion and subsequently in food intake and growth (Nitsan et al., 1991). Chicks forage an adult-type diet immediately post-hatch, inducing a rapid adaptation of the intestinal tract to digest and absorb nutrients and to face colonization by exogenous microbiota (Sklan, 2001; Geyra et al., 2007). The challenge is how to maintain the system integrity while the gut-associated lymphoid tissue (GALT) system is not fully developed at hatch. In fact, an adaptive immunity in the GALT appears only after 14 days post hatch (Bar-Shira et al., 2003). Therefore, newly hatched birds leaving the sterile environment of the egg require temporary immunological assistance provided by maternal antibodies (Shawky et al., 1994; Kaspers et al., 1996). Immunoglobulins (Ig) are actively transported from the hen's serum to the yolk while still in the ovary (Uni et al., 1999; West et al., 2004). In the fluid phase of the egg yolk, IgY are therefore found at a level equal to that of the hen's serum. In addition, as fertilized *ova* pass down the oviduct IgM and IgA from oviduct secretions are accumulated in the albumen (Figure I-6). As the chick develops *in ovo* it absorbs the yolk IgY, which then appear in its circulation. At the same time, the IgM and IgA from the albumen diffuse into the amniotic fluid and are swallowed by the embryo. Thus when a chick hatches it possesses maternal IgY in its serum and IgM and IgA in the intestine (Tizard, 2013).

Chick stem cells arise in the yolk sac membrane and migrate to the thymus and bursa at day 5 to 7 of incubation. These cells differentiate within the bursa and follicles develop by day 12. Lymphocytes with surface IgM can be detected in the bursa by day 14 and lymphocytes with surface IgY develop on day 21, thus around the time of hatching. IgA-positive cells first appear in the intestine 3 to 7 days after hatch.

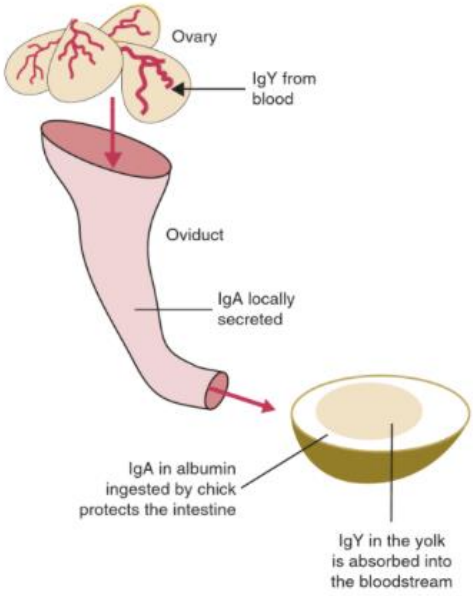

Figure I-6: Passive transfer of immunity from the hen to the chick (Tizard, 2013).

I.2.2- Physical and chemical barriers

I.2.2.1- Anatomic features

The mucous membranes coating the digestive tract provide an effective way to avoid invasion by microorganisms, unless they are damaged (Tizard, 2013). Many antigens cannot penetrate or are entrapped in the mucus secretions. Some nutritional deficiencies (such as biotin deficiency), injury or infectious diseases compromise the integrity of the body mucosae, which allows penetration of pathogens (Poultry Cooperative Research Centre).

I.2.2.2- Normal microbiota

The GIT usually maintains a resident, stable microbial population. This stable microbiota prevents invasion by other bacteria and fungi. Inadequate use of antibiotics or poor sanitation can disrupt the balance of the microbiota (Tizard, 2013). The role of the microbiota is important in the gut, where it additionally plays an important role on the digestion of non-digested feed particles and producing SCFA, as described before in section I.1.2.

I.2.3- Innate immunity

Innate immunity includes the natural or inherited ability to resist invasion and to avoid infections and the development of diseases. It consists of a non-specific disease response mechanism involving preexisting or rapidly responding chemical and cellular defense mechanisms (Tizard, 2013). It recognizes pathogens in a generic way but it doesn’t confer a

long-lasting protection to the host (Alberts et al., 2002). The innate immunity actors are macrophages, dendritic cells, neutrophils, natural-killer cells (NK-cells) and antimicrobial molecules (Pulendran and Ahmed, 2006).

I.2.4- The acquired immunity

The acquired immunity is able to respond to a large panel of foreign antigens, recognized by its main actors, the lymphocytes. All lymphocytes have antigen receptors on their surface that allow them to recognize and respond to antigens. They are round cells with a diameter ranging from 7 to 15 µm, with a round nucleus surrounded by a thin cytoplasm containing some mitochondria, free ribosomes and a small Golgi apparatus (Tizard, 2013). Lymphocytes are composed of 3 categories: NK-cells, T-cells and B-cells. They are all differentiated from the common lymphoid progenitor (Revillard, 2001).

NK-cells are identified by their cytoplasmic CD3+ receptor. The NK cells have the ability to recognize stressed cells in the absence of antibodies and major histocompatibility complex (MHC), allowing for a much faster immune reaction (Vivier et al., 2011). NK cells are large granular cells that differentiate and mature in the bone marrow, lymph nodes, spleen, tonsils, and thymus, where they then enter into the circulation.

B-cells are a subtype of the lymphocytes, and function in the humoral immunity component of the adaptive immune system by secreting antibodies (Murphy, 2014). The B-cells present antigen (they are also classified as antigen-presenting cells) and secrete cytokines (Murphy, 2014). B-cells develop from hematopoietic stem cells that originate from bone marrow in mammals, but in birds the B-cells mature in the Bursa of Fabricus (Cooper, 2015).

T-cells are the third subtype of the lymphocytes and they mature in the thymus. T-cells are divided into sub-sets according to their specific receptor (TCR). All T-cells possess accessory molecules associated to the TCR called cluster of differentiation 3 complex (CD3) (Tizard, 2013). The helper T-cells are equipped with the CD4 proteins on their surfaces and bind only the MHC class II molecules. The cytotoxic T-cells, also known as CD8 T cells, recognize their targets by binding antigen associated with MHC class I molecules mainly virus (Murphy, 2014). The memory T-cells are a subset of antigen-specific T-cells that persist long-term after an infection has been resolved (Tizard, 2013). The suppressor T-cells are crucial for the maintenance of immunological tolerance. Their major role is to shut down T-

cell-mediated immunity toward the end of an immune reaction and to suppress auto-reactive T-cells that escaped the process of negative selection in the thymus (Guyton and Hall, 2006).

I.2.5- The gut associated lymphoid tissue

The gut associated lymphoid tissue (GALT) is part of the common mucosal immune system of the chicken. The GALT comprises all lymphoid cells and tissues along the alimentary tract from the pharynx to cloaca. Apart from organized lymphoid tissues, lymphoid aggregates and single cells in the lamina propria and epithelium are also included in the GALT.

I.2.5.1- Post-hatch GALT anatomy

The GALT is the largest and most active immune organ of the body. It contains up to 80 % of the immune cells (Tizard, 2013). The question remains how the GALT is able to differentiate normal microbiota and feed antigens from pathogens. The gut epithelium acts as a barrier to invading organisms, while at the same time permitting the absorption of nutrients. This function is granted by its complex architecture.

Organized lymphoid tissues are sometimes located at the tip of the pharynx and further in Meckel's diverticulum, Peyer's patches, cecal tonsils and bursa of Fabricius (Tizard, 2013).

The GALT is composed of 3 parts each with its own clear and specific immune elements and reactions.

I.2.5.1.1-Peyer's patches (PP)

They are organized lymphoid aggregates located in the wall of the small intestine. PP are organized in 3 domains: The follicular area, the interfollicular area and the follicule-associated epithelium (Neutra et al., 2001). PP lymphoid follicles have a germinal center containing proliferating B-lymphocytes, follicular dendritic cells and macrophages. The follicular area is surrounded by the corona and contains mixed-cells including B-cells, T-cells, macrophages and dendritic cells. PP are connected to the body by lymphatic vessels and high endothelial venules (Neutra et al., 2001; Tsuji et al., 2008; Jung et al., 2010).

I.2.5.1.2- Lamina propria

The lamina propria is a meshwork of connective tissue underlying the gut epithelium that contains a broad spectrum of myeloid and lymphoid cells, especially immunoglobulin (Ig) A plasmablasts, CD4 T cells, dendritic cells, and mast cells.

I.2.5.1.3- Intraepithelial leukocyte spaces

These are the spaces between intestinal epithelial cells and above the basement membrane that are populated by a variety of small, round cells, especially natural killer cells and many CD8 T-cell subsets (Cebra et al., 1998; Ogra et al., 2012). The status of these 3 compartments with respect to the numbers and activation states of their cellular elements seems to depend on stimulation by gut microbial antigens.

I.2.5.2- The GALT development

The GALT system is relatively similar for all the vertebrates (Figure I-7). The differentiation starts at the pre-hatch stage by the development of secondary lymphoid tissues (PPs and mesenteric lymph nodes) and cryptopatches. Intraepithelial lymphocytes seed the epithelium before hatch by the recruitment of dendritic cells, T-cells and B-cells in preparation for the immune response to the microbiota (Tsuji et al., 2008). Post-hatch, bacteria colonize the intestine immediately, initiating the development or functional maturation of the mucosa and the GALT. Microbe-associated molecular patterns (MAMP) sensed by pattern-recognition receptors on intestinal epithelial cells and dendritic cells adjacent to cryptopatches stimulate further recruitment of B-cells and T-cells, causing the cryptopatches to develop into mature isolated lymphoid follicles. Microbes also cross the epithelium and enter PP through M cells, from which they are endocytosed by dendritic cells in the sub-epithelial dome. Dendritic cells containing antigen interact with local lymphocytes to induce T-cell differentiation and B-cell maturation in the germinal centers. The mature B-cells release dimeric IgA into the lamina propria. The MAMP stimulates the proliferation of intestinal epithelial cells in crypts, resulting in their increased depth (Tsuji et al., 2008; Jung et al., 2010).

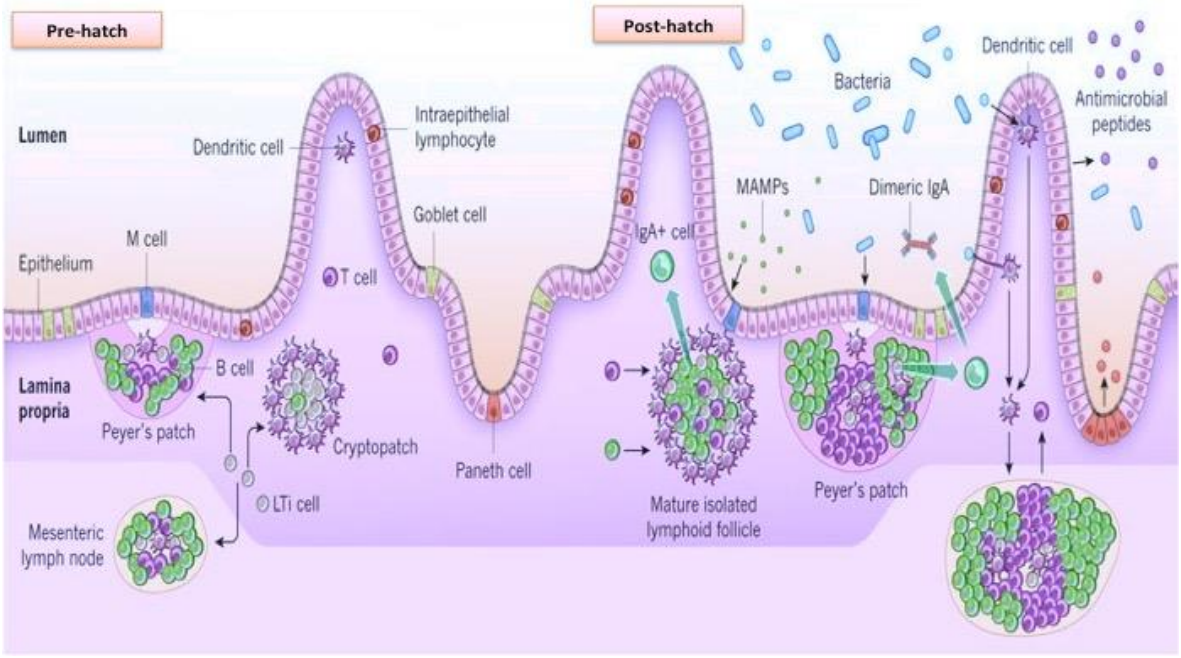

Figure I-7: The gut-associated lymphoid tissue establishes pre- and post-hatch microbiota mutualism in the intestine (adapted from Tsuji et al., 2008).

Diet is the main factor affecting the digestive tract, its anatomy and physiology as well as the associated immune response. The broiler diet is mainly composed of cereals, principally corn or wheat. Wheat is the leading cereal used in poultry nutrition in Western Europe, Australia, New Zealand and some provinces in Canada. In the next section wheat grain characteristics and use in animal nutrition will be described.

I.3- Wheat Grain

Wheat (*Triticum aestivum)* is an allohexaploid (2n= 42) from the genus *Triticum* that belongs to the family of *Poaceae*. In 2013, wheat was the 3rd most produced cereal after maize and rice, with 713 million tons in the world (FAO, 2013). *Triticum aestivum* represents 95 % of the cultivated wheat worldwide, while *Triticum durum* represents the other 5 % (Shewry, 2009).

Wheat grain is botanically a caryopse. At maturity, it is 7-9 mm long, 3-4 mm large and 4-5 mm high. The average weight varies between 20 and 50 mg and the density between 1.3 and 1.4 (Angus et al., 2011). The kernel (Figure I-8) has on one end, the germ or the embryo (the future plant) and on the other end, a bundle of hair also called beard or brush.

I.3.1- Histology of wheat grain

Wheat grain has a complex structure composed of different tissues (Figure I-8), with different functions during the grain development. They are therefore characterized by distinct structures and compositions.

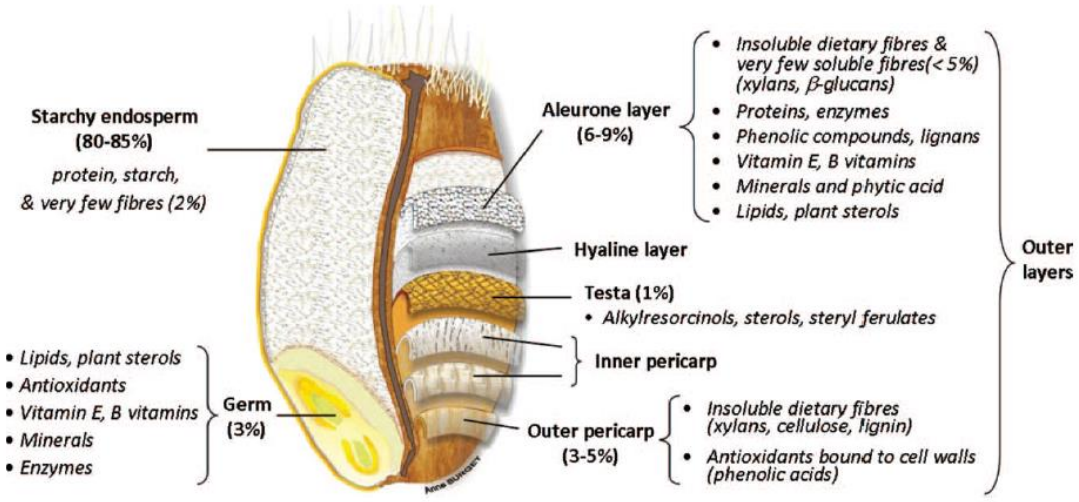

Figure I-8: Histological structure of wheat grain. Adapted from Surget and Barron (2005) by Brouns et al. (2012).

I.3.1.1- The outer-layers or bran

The outer layers represent 12-18 % of the total weight of the whole grain and are composed of the pericarp, testa, hyaline layer (*i.e* nucellar epidermis) and the aleurone layer (Figure I-9).

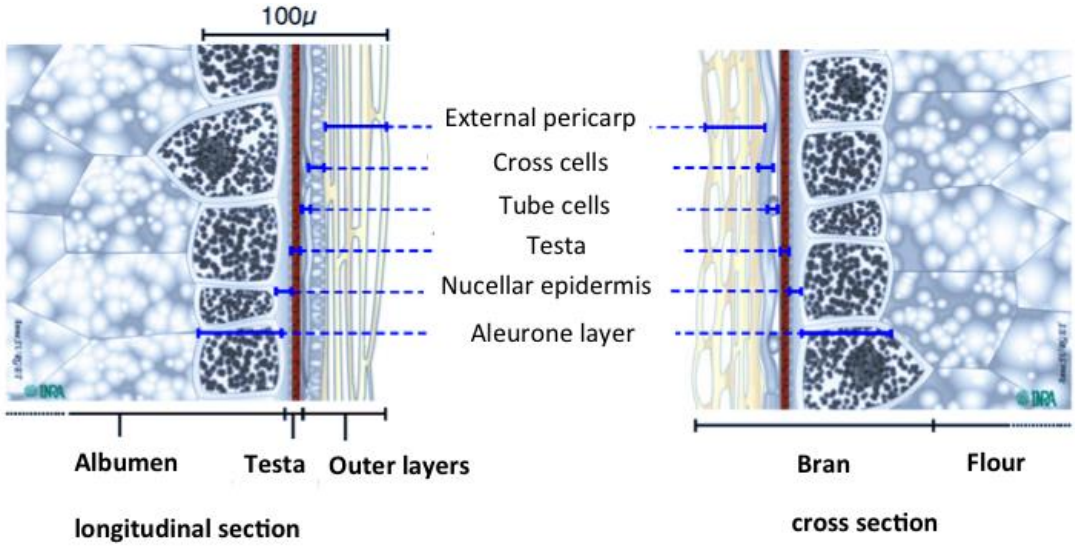

Figure I-9: Schematic view of the wheat grain histology, adapted from Surget and Barron, 2005.

I.3.1.2- The pericarp

The pericarp of the mature grain is composed of empty cells and is organized in two parts (Figure I-8):

- the external pericarp is composed of the epidermis (the outer layer) 15-20 µm thick and covers the entire grain except the rachis attachment point (Shewry, 2009), the hypodermis with a similar structure as the epidermis to which it sticks closely, and intermediate cells localized only at the grain edge and ensuring the linkage between the layers (Bradbury et al., 1956).
- the internal pericarp is constituted of two layers: the mesocarp composed of cross cells and the endocarp constituted of tubular cells.

I.3.1.3- The testa

It is a hydrophobic layer rich in lignin and lipidic compounds such as alkylresorcinols. It is considered as the seed coat covering the entire grain. The testa (Figure I-9) is composed of cellular layer rich in catechin interposed between 2 thin layers of cutin (Bradbury et al., 1956). The thickness of the testa ranges between 5-8 µm.

I.3.1.4- The nucellar epidermis (*i.e.* hyaline layer)

The nucellar epidermis is a compressed cellular layer between the pericarp and the aleurone layer (I-Figure 9) and closely united both (Lutz, 2007). It is 12-20 µm thick and covers the entire grain except the embryo that is partially exposed.

I.3.1.5- The aleurone layer

The wheat aleurone layer is composed of a single layer of living cells (I-Figure 9), whose diameter is 20–75 µm (Stevens, 1973). It represents up to 50 % of the wheat bran mass. The aleurone cell is composed of the cytoplasm or intracellular medium, surrounded by thick non-lignified cell walls, which represent up to 35 % of the cell volume (Fulcher et al., 1972), and more than 40 % of the layer in mass (Hemery et al., 2009). Aleurone cell walls contain 25 % of mixed-linked β-D-glucans, up to 18 % proteins, and 60 % of arabinoxylans (Bacic and Stone, 1981; Rhodes and Stone, 2002; Saulnier et al., 2007). The intracellular medium of aleurone cells contains many spherical particles (2–4 µm diameter) called aleurone granules, which are either phytate inclusions (composed of phytic acid crystals), or niacin inclusions (composed of niacin and proteins), each granule being surrounded by a fine layer of lipidic droplets (Morrison et al., 1975).

I.3.1.6- The starchy endosperm

The endosperm represents 80-85 % of the grain dry matter. It is mostly composed of starch and proteins (Pomeranz, 1988; Barron et al., 2007). Three types of cells can be differentiated depending on their size, form and localization in the endosperm (Fincher, 1989). The first type is the sub-aleuronic cells, having a cubic shape and ~60 µm side. The second type is the prismatic cells, having an elongate shape of ~150 x 50 µm. The third type is the central cells, located in the center of the grain and having heterogeneous shapes and size varying from 72-144 µm length and 69-120 µm large (Fincher, 1989).

The starchy endosperm is composed of 82 % of starch, 12 % of protein and 2 % of lipids (Feillet, 2000). Cell walls represent 2-7 % of the tissue; they are hydrophilic and mainly composed of two polysaccharides: arabinoxylan (AX) and mixed-linked β-glucan (MLG) (Saulnier et al., 2007). The ratio of MLG/AX in the endosperm is ~22/78 (w/w) (Mares and Stone, 1973a; b). Other polymers were reported in lower amounts such as glucomannan (2-7 % of cell walls) and cellulose (2-4 % of cell walls) (Barron et al., 2007). In

addition, wheat endosperm contains water-soluble arabinogalactan-proteins (AGP; 0.23-0.33 % w/w flour) (Loosveld, 1998), that are possibly associated to cell walls.

I.3.1.7- The embryo

The embryo occupies a dorsi-ventral position in the proximal portion of the grain (Bradbury et al., 1956). It comprises an embryonic axis and the scutellum. The scutellum consists of thin walled parenchyma cells. The embryonic axis consists of the shoot protected by the coleoptile, the mesocotyl, and the root, protected by the coleorhiza. (Saulnier et al., 2007)

The wheat germ is the reproductive part of the grain and is rich in protein (31 %) and lipids (12 %) (Feillet, 2000). It is a condensed source of several essential nutrients including vitamin E, folic acid, phosphorus, thiamin, zinc, and magnesium, as well as essential fatty acids and fatty alcohols (Feillet, 2000; Cohen, 2003). It also contains up to 52 % of carbohydrates including 15 % of dietary fibers (United States Department of Agriculture, 2015).

I.3.2- Composition of wheat grain

I.3.2.1- Starch

Starch is the main component of the wheat grain. Depending on the varieties, it represents up to 70-80 % of the total weight of the grain. It consists on two homopolymers of glucose, amylose and amylopectin (Figure I-10).

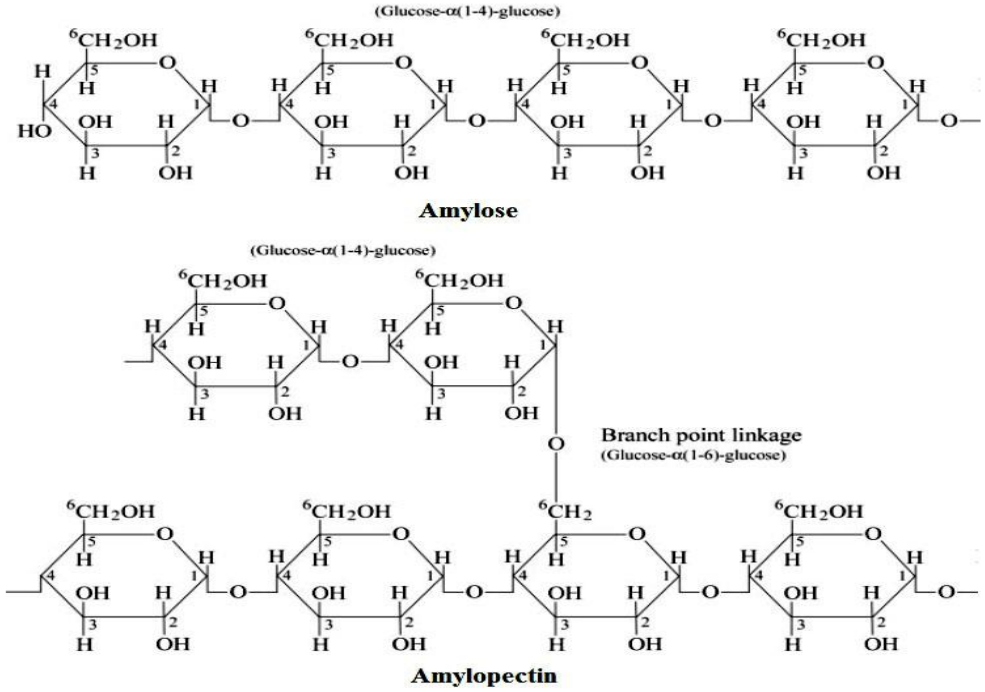

Figure I-10: Chemical structure of amylose and amylopectin (Buléon et al., 1998).

Amylose is a linear α-1,4-D-glucose chains and represent up to 20-30 % of wheat starch. Amylopectin is a highly branched polysaccharide that consists of α-1,4-D-glucose chains connected by α-1,6 branch points that occur each 20-30 glucose units. Amylopectin represents 70-80 % of the wheat starch (Raeker et al., 1998; Thompson, 2001; Zaefarian et al., 2015). Starch digestibility ranges between 0.79-0.98 in poultry depending on the bird age (Zaefarian et al., 2015). Starch digestibility rate is negatively correlated with the amylose content. Indeed amylose chains form with lysophospholipids complexes that are hardly degraded by digestive amylase (Svihus et al., 2005).

I.3.2.2- Proteins

Mature wheat grain contains 9-16 % of proteins, mainly the gluten proteins that constitute up to 80-85 % of total flour proteins (Figure I-11) (Kuktaitė, 2004). Aleurone provides up to 15 % of the total wheat protein but also up to 30 % of the total lysine (the first limiting essential amino acids in wheat) (Pomeranz, 1988). At least 80 % of total niacin in wheat is found in the aleurone layer which also contain a considerable amount of other B vitamins (Brouns et al., 2012). The vast majority of gluten proteins belongs to prolamins, which were initially defined based on their solubility in 60-70 % (v/v) ethanol. The non-gluten wheat proteins are the albumins that are water-soluble, and the globulins that are

soluble in salt-solutions (Singh et al., 2001). The non-gluten proteins represent up to 20 % of the wheat proteins. Alpha-amylase/trypsin, serpins and purothionins are predominant in albumins and globulins. They also contain a high proportion of the two essential amino acids lysine and methionine (Lasztity, 1995).

Gluten proteins form a continuous matrix around the starch granules in the endosperm of mature grain, and are formed of gliadins and glutenins.

Gliadins are monomeric proteins that consist of single chain polypeptides and constitute from 30 to 40 % of total flour protein content. Gliadins are polymorphic mixture of proteins soluble in 70 % alcohol (Anderson and Greene, 1997). Gliadins are rich in proline and glutamine and have a low level of charged amino acids (Shewry et al., 2002). They are classified into four groups α, β, γ and ω and have a molecular weight in the range of 30-80 kDa (Shewry et al., 1986).

Glutenins are the polymeric proteins of wheat gluten and are extractable in dilute acetic acid (Field et al., 1983). Glutenins and gliadins have very similar amino acid composition, with high levels of glutamine and proline and low levels of charged amino acids (Goesaert et al., 2005). Two classes of glutenin subunits are distinguishable according to their high molecular weight (HMW-GS) or low molecular weight (LMW-GS) and they are separated at the reduction of disulphide bonds (Malik, 2009). HMW-GS have molecular weights between 65 and 90 kDa, based on amino acid sequences, and between 80 and 130 kDa based on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). LMW-GS have molecular weights of 30-51 kDa and 30-40 kDa according to the two methods, respectively (Malik, 2009).

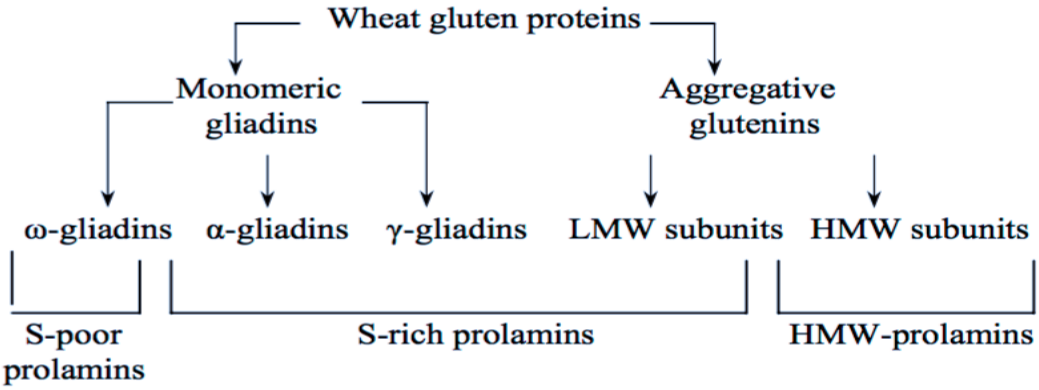

Figure I-11: Wheat gluten proteins classification (Shewry et al., 1995, 2002; Kuktaitė, 2004).

I.3.2.3- Lipids

Lipids represent around 2 % of the total weight of the wheat grain and are distributed in the whole grain but with different proportions: 63 % are located in the starchy endosperm, 13 % in the germ, 24 % in the aleurone layer (Feillet, 2000; Brouns et al., 2012).

Lipids in wheat grains display large structural diversity and comprise neutral (acylglycerols and free fatty acids) and polar (glycolipids and phospholipids) components (González-Thuillier et al., 2015). Triacylglycerols are the main storage lipids that are essentially found in the germ and are located in subcellular organelles called oil bodies (Morrison, 1994). Glyco- and phospho-lipids are structural lipids essentially found in the starchy endosperm where they are partly associated to starch granules.

I.3.2.4- Dietary fibers and non-starch polysaccharides

Dietary fiber is the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the small intestine with complete or partial fermentation in the large intestine. Dietary fibers include polysaccharides, oligosaccharides, lignin, and associated plant substances. Dietary fibers promote beneficial physiological effects including laxation, and/or blood cholesterol attenuation, and/or blood glucose attenuation (American Association of Cereal Chemists (AACC), 2001). This definition was adopted on June 2009 by the Codex Alimentarius Commission (Howlett et al., 2010). However, this definition include oligosaccharides with the DP between 3 and 9 as DF and “physiological effect(s) of benefit to health” remain undefined (Howlett et al., 2010).

In animal nutrition, dietary fibers (DF) are considered to consist mainly of non-starch polysaccharides, resistant starches, and lignin that can be provided by whole grains, legumes, vegetables, nuts and seeds (Turner and Lupton, 2011). DF are derived from plant cell wall constituents that are not digestible by endogenous enzymes and can be fermented by the intestinal microbiota (Theander et al., 1993; Turner and Lupton, 2011). In wheat, DF are composed of cell-wall polysaccharides mainly of arabinoxylans, mixed-linked β-glucans and arabinogalactan-proteins.

I.3.2.4.1- Arabinoxylans

Arabinoxylans (AX) represent 5-6 % of the whole grain dry weight (Ordaz Ortiz, 2005; Saulnier et al., 2012). The structure of AX depends on the tissue: aleurone layer, starchy endosperm, pericarp or testa (Figure I-12).

AX represent up to 2-3 % of white flour (mainly endosperm tissue) where they are partly water-soluble (25-30 % of total wheat flour AX) (Faurot et al., 1995; Dervilly et al., 2000; Dervilly-Pinel et al., 2001; Saulnier et al., 2007). They are formed of a linear backbone of β-(1,4)-linked-D-xylopyranosyl residues substituted with α-L-arabinofuranose residues (Izydorczyk and Biliaderis, 1995). Arabinose residues are mainly present as single side-chain units as mono-substitutions on position *O*-3 (21 %), or di-substitutions (dXyl) on position *O*-2 and *O*-3 (13 %) of the xylosyl residues of the backbone. On average, 66 % of xylosyl residues of the backbone are unsubstituted (Saulnier et al., 2007). The typical arabinose to xylose ratio (A/X) of wheat endosperm AX ranges from 0.5 to 0.6 (Faurot et al., 1995; Dervilly et al., 2000). The A/X ratio ranges from 0.47 to 0.58 in the water-soluble fraction and from 0.51 to 0.67 in the water-insoluble fraction (Hoffmann et al., 1991, 1992; Izydorczyk and Biliaderis, 1994, 1995). Though, many studies showed that water-soluble AX are heterogeneous in their structure depending on the cultivars and the extraction/fractionation methods used (Gruppen et al., 1992; Ordaz Ortiz, 2005; Saulnier et al., 2007). They are large polymers, as indicated by their intrinsic viscosity that ranges between 200 and 600mL/g according to extraction procedures and varieties (Dervilly-Pinel et al., 2001). Due to these macromolecular features, water-soluble AX exhibit good thickening properties.

In addition to arabinose and xylose, AX contain some ferulic acid ester-linked to the *O*-5 of the arabinofuranosyl units. The presence of ferulic acid linked to AX is a common feature of grass cell walls. This component plays an important role in AX interactions and cell wall properties, as they can dimerize under oxidizing conditions and acts as bridging agent interconnecting xylan chains together. As a consequence, the water-soluble/insoluble character of AX in cereal endosperm seems to be linked to the level of cross-linking through diferulic bridges (Saulnier et al., 2012). The amount of ferulic acid linked to AX in starchy endosperm is very low and represents 0.2-0.4 % of water-soluble AX (w/w) and 0.6-0.9 % of water-insoluble AX (Saulnier et al., 2007). In the aleurone layer, AX are structurally related to endosperm AX, but are totally water-insoluble and exhibit a lower A/X ratio. They are also more heavily esterified by ferulic acid and dehydrodiferulic acids that represent about 3.2 % and 0.45 % of aleurone AX, respectively (Antoine et al., 2003; Parker et al., 2005).

AX isolated from wheat bran have more complex structures than those of the endosperm, and they are described as heteroxylans (HX). The level of substitution of the xylan backbone is very high (80 %) with high proportion of dXyl (25-35 %) (Hoffmann et

al., 1991; Kormelink et al., 1993). The A/X ratio is around 0.8-1 and arabinose residues are mainly found as terminal side chains. Glucuronic acid and xylose residues are also found as terminal residues. In addition, short side chains constituted by arabinose, xylose and galactose have been isolated from these HX. The amount of ferulic acid linked to AX represents 0.9 % (w/w) in the pericarp from wheat and very similar amounts are observed for the dehydrodimers (Antoine et al., 2003). As pointed out for endosperm AX, ferulic acid plays an important role in xylan interactions and cell wall properties, and acts as bridging agent interconnecting xylan chains together and also with lignins that are present in pericarp tissues. The HX found in pericarp tissues are totally water insoluble.

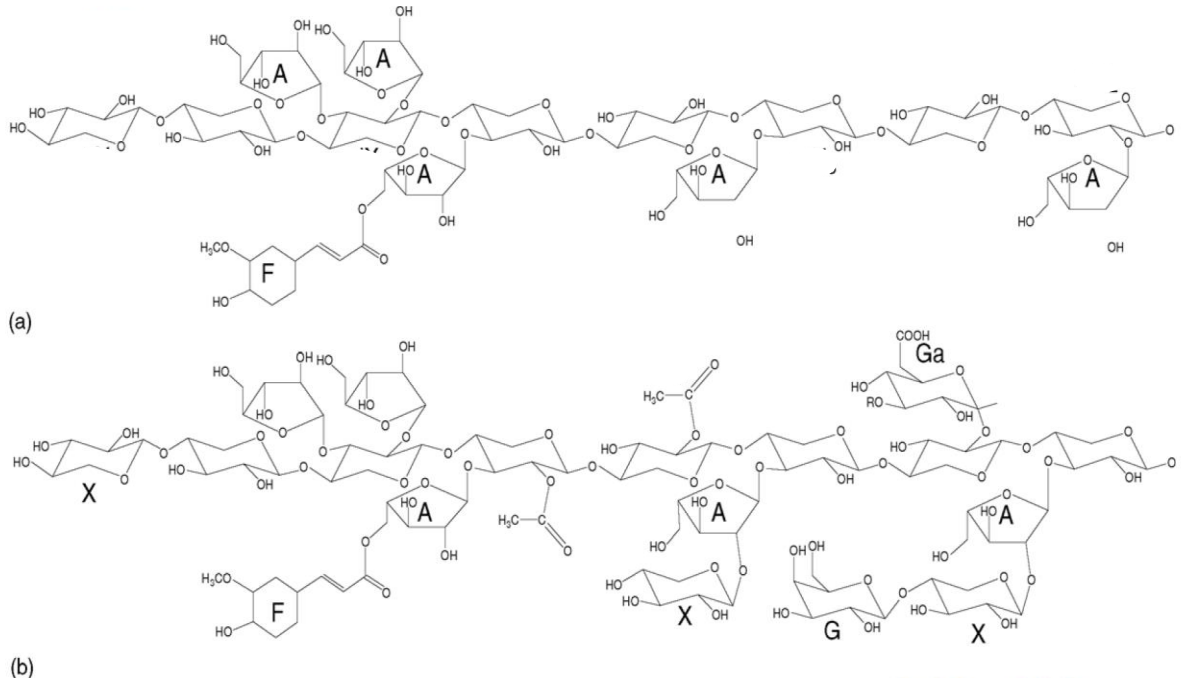

Figure I-12: Main structural features of AX from endosperm (a) and outer tissues (b) of cereal grains A: arabinose; X: xylose; G: galactose; Ga: glucuronic acid; F: ferulic acid (Saulnier et al., 2007).

I.3.2.4.2- Mixed-linked β-glucan

Mixed-linked β-glucans (MLG) represent 0.60-0.65 % of the whole wheat grain on the dry matter basis, 0.24-0.36 % of the endosperm (Henry, 1987) in which they represent up to 22 % of the cell-walls (Saulnier et al., 2012). MLG are linear homopolymers of D-glucopyranose arranged as blocks of consecutive 1,4-linked β-D-glucose residues separated by single 1,3-linkages. The polysaccharide backbone consists of β-1,3-linked cellotriosyl (58-72 %) and cellotetraosyl (20-34 %) blocks; however, minor amount of β-(1→4)-linked

cellulosic blocks with more than four residues and up to 14 was reported (Cui et al., 2000; Virkki et al., 2005; Li et al., 2006) (Figure I-13). Cereal MLG are characterized by differences in the trisaccharide-to-tetrasaccharide ratio (BG3/BG4), which varies in the range of 4.2-4.5 in wheat, 2.8-3.3 in barley and 2.0-2.4 in oat (Cui et al., 2000). The average molecular weight (Mw) of the cereal MLG is generally high but ranges from 43 to 758 kDa (Li et al., 2006). Similarly to endosperm AX, MLG are partly water-soluble and exert thickening properties. High BG3/BG4 ratio seems to favor chain-chain interactions and together with high Mw might be the cause of the low water solubility and the fast gelation of wheat β-glucans (Cui et al., 2000).

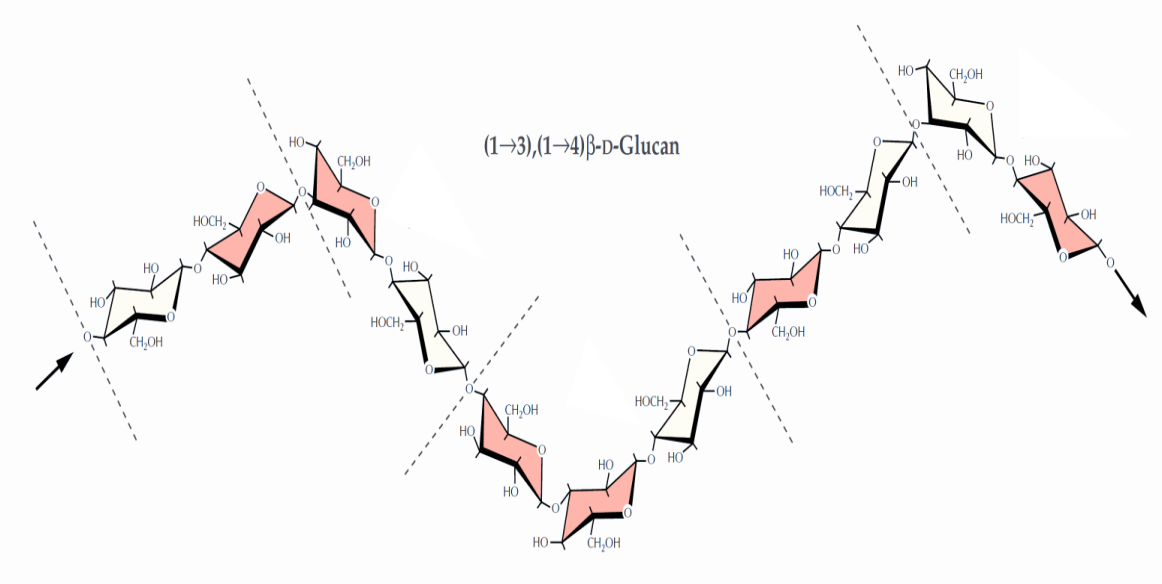

Figure I-13: The mixed-linkage 1,3-1,4-β-D-glucan. (Carpita and McCann, 2000).

I.3.2.4.3- Arabinogalactan-proteins

Arabinogalactan-proteins (AGP) represent about 0.14 % of the wheat grain (Van den Bulck et al., 2005). AGP are structurally complex large branched polysaccharides, attached to hydroxyproline (Hyp) residues of cell wall polypeptides (Tryfona et al., 2010). Arabinogalactan domain represents 90 % of the AGP (Fincher and Stone, 1974). The wheat AGP (Figure I-14), consist of β -1,3 or β -1,6 or β -1,3-1,6-linked D-galactopyranosyl backbone substituted with L-arabinofuranosyl at C-3 and occasionally also L-rhamnosyl, L-fucosyl and D-glucuronosyl (with or without *4-O*-methylation) residues (Fincher et al., 1974; Tryfona et al., 2010). The A/G ratio ranges from 0.66-0.73 (Fincher et al., 1974; Loosveld, 1998).

The peptide part of the wheat AGP represents 8-9 % of the AGP and is composed of 15–20 amino acids with Hyp, glutamic acid/glutamine, alanine, serine, threonine, and valine as the most important ones (Fincher and Stone, 1974; Fincher et al., 1974; Loosveld et al., 1997; Tryfona et al., 2010). The average Mw of the AGP is 23.5 kDa (Fincher et al., 1974; Loosveld, 1998).

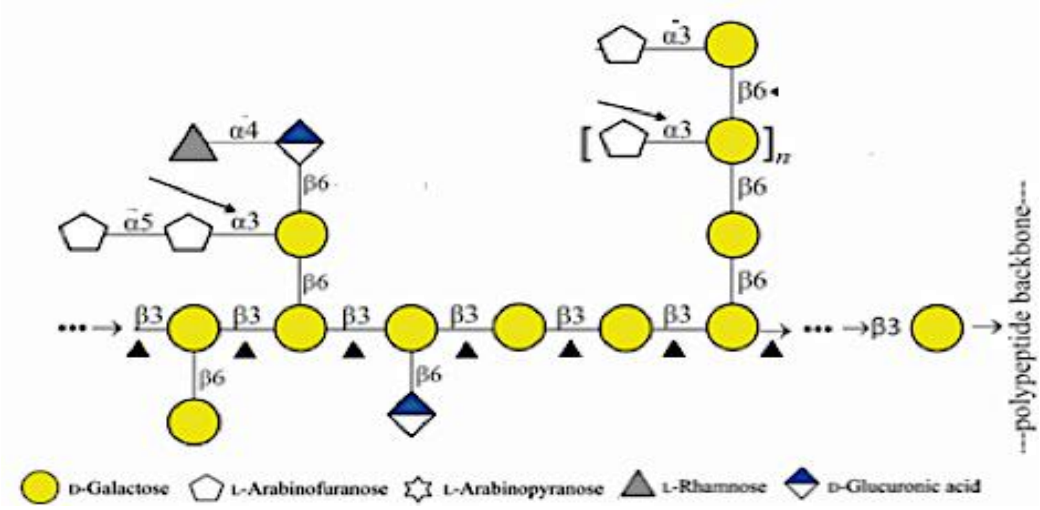

Figure I-14: Schematic representation of the type II arabinogalactan (adapted from Tan et al. (2010); Tryfona et al. (2010); Gille et al. (2013); Dilokpimol et al. (2014) and Knoch et al. (2014)).

I.3.3- Wheat in animal nutrition

Wheat represent up to 50 % of the chickens diet. Depending on the age, the sex, the type of production (meat or eggs) and the nutritional value of the feedstuff the diet composition should be adapted to cover the animal needs. Wheat is the major source of energy in the diet. Wheat can account for up to 70 % of the metabolizable energy (ME) and 35 % of the protein requirements of broilers (Gutierrez del Alamo et al., 2008; Amerah, 2015). The ME content of wheat grains varies according to varieties, environmental condition and storage conditions (Ciccoritti et al., 2011). In Canada, wheat ME varies between 2945 and 3960 Kcal/kg while it varies between 2880 and 3220 Kcal/kg in the USA. The ME of European wheat grains ranges between 2900 and 3120 Kcal/kg (Blair and Paulson, 1997). The ME depends on the content and digestibility of starch, protein and lipids (McCracken and Quintin, 2000; Svihus and Gullord, 2002; Wiseman, 2006; Carré et al., 2007). The starch digestibility, the main energy source, depends on starch structure, amylose to amylopectin ratio and interactions with lipids and proteins of the endosperm as well as the processing conditions (Svihus et al., 2005;

Amerah, 2015). The range of digestibility variations is about 15 % for lipids, 10 % for starch and 3 % for proteins between broilers fed wheat based diets (50 % wheat in the diet) (Carré et al., 2007). Digestion of wheat lipid is negatively correlated with grain viscosity, and high levels of water-soluble NSP result in a reduction in fatty acids absorption (Carré et al., 2007). Wheat also contains phytate, which varies depending on the cultivars and on the growing conditions. It ranges from 1.35 g/kg to 3.20 g/kg with an average of 2.20 g/kg (Selle et al., 2003) and has an antinutritional effect (Selle and Ravindran, 2007).

As reviewed by Montagne et al. (2003), soluble DF increase intestinal transit time, delay gastric emptying and glucose absorption, increase pancreatic secretion, and slow absorption, whereas insoluble DF decrease transit time, enhance water holding capacity and assist faecal bulking in non-ruminant animals. DF increase feed conversion, moisture and organic matter in faeces, and reduce quality of the litter (Choct and Annison, 1992). Nevertheless, the negative effect of DF depends on the physico-chemical characteristics of the DF, their source and content in the diet and the animal species and age (Montagne et al., 2003). Cereal grains are broadly classified into two major categories, viscous and non-viscous cereals. For example, rye, barley, oat, wheat and triticale contain considerable amounts of soluble NSP and are classified as “viscous grains”, whereas corn, sorghum, millet and rice contain negligible amounts of soluble NSP and are known as “non-viscous cereals” (Choct, 2006). In wheat, NSP could be soluble or insoluble and consequently have different properties and different effects on digestion in poultry. Most of the AX in wheat and other cereal grains are insoluble because they are anchored to the cell walls by alkali-labile ester cross links (Choct, 1997). However, wheat and rye contain soluble AX, which are responsible for the viscosity increase in digesta, the decrease of the nutrient digestion in the gut and the decline of the animal performance (Choct and Annison, 1992; Choct et al., 1995; Choct, 2006). In barley and oat, water-soluble MLG are more abundant than AX and therefore drive the viscose effect of these cereals. The viscosity induced by water-soluble NSP (AX and/or MLG) is considered as the main mechanism that explains the negative effect of NSP on nutrient utilization and birds performance. The encapsulation of starch and proteins in the cell wall network is frequently proposed as a mechanism that could reduce the digestibility of these macronutrients by animals (Classen and Bedford, 1991; Bedford and Schulze, 1998). However, contrary to legume seeds, cell walls of cereal grains are very thin and they are disrupted during the processing of grains into flour. The anti-nutritional effects of wheat have been largely overcome by the use of appropriate feed enzymes. The different exogenous

enzymes used in animal nutrition, their mechanisms of action and their effects on animal performances are discussed in the next section.

I.4-Enzymes

I.4.1- Exogenous enzymes in animal nutrition

The use of exogenous enzymes in animal nutrition, especially in poultry nutrition, increased considerably in the last decades. This increase is partially due to the ban of the use of antibiotics as growth promoters in Europe in 2006 (European Union, 2006) and to the strategy to reduce their use for medical purposes. This ban was part of the EU food and public health safety strategies after the development of "anti-microbial resistance". The antibiotics are still used as feed additives in other countries. However, consumer protection organizations and lobbies are making pressure to ban the use of antibiotics as growth promoter especially in the USA. For this reason, in 2013 the Food and Drug Administration (FDA) updated their regulation by limiting the use of antibiotics as growth promoters (The Food and Drug Administration, 2013). In June 2015, the FDA announced in the new Veterinary Feed Directive that the use of antibiotics will be exclusively under veterinary supervision so that they are used only when necessary for ensuring animal health (The Food and Drug Administration, 2015).

One of the effective and safe alternatives to antibiotics is the supplementation by exogenous enzymes as they allow reducing the anti-nutritional effects of viscous cereal grain. However, the majority of the researches investigating these feed additives focused mainly on their effects on animal performances and nutrient digestibility, and their mechanisms of action are still not fully understood nor their effects on health.

The International Union of Biochemistry and Molecular Biology have developed a nomenclature for enzymes, the Enzyme Commission numbers; each enzyme is described by a sequence of four numbers preceded by 'EC'. The first number classifies the enzyme based on the reaction catalyzed (www.chem.qmul.ac.uk):

- EC 1, Oxidoreductases: catalyze oxidation/reduction reactions
- EC 2, Transferases: transfer a functional group (e.g. a methyl or phosphate group)
- EC 3, Hydrolases: catalyze the hydrolysis of various bonds
- EC 4, Lyases: cleave various bonds by means other than hydrolysis and oxidation
- EC 5, Isomerases: catalyze isomerization changes within a single molecule
- EC 6, Ligases: join two molecules with covalent bonds.

The second number defined the subclasses according to the targeted linkage. As an example, EC 3.1 are hydrolases acting on ester bonds, while EC 3.2 are hydrolases acting on glycosydic bonds. The third number indicates more precisely the type of linkage to be processed. EC 3.2.1 hydrolyze *0*-glycosyl compounds, whereas EC 3.2.2 hydrolyze *N*-glycosyl compounds. Finally, these subclasses are subdivided in four numbers indicating the specific substrate of each enzyme.

The principal exogenous enzymes used in animal nutrition belong to EC 3 and are mainly phytases (EC 3.1) and carbohydrate active enzymes (Cazy) (EC 3.2). The Cazy category includes the glycoside hydrolases (GH), the glycosyltransferases (GT), carbohydrate esterases (CE) and polysaccharide lyases (PL) (www.cazy.org).

The phytases referred to as sugar phosphatases (EC 3.1.3) are produced by numerous sources including plants, animals and microorganisms (bacteria, yeasts and fungi). Nevertheless, phytases used at industrial scale mainly originate from microbial sources (Vohra and Satyanarayana, 2003).

Phytate (myo-inositol hexakisphosphate) is the organic form of phosphorus found in grains and oil seeds. Phytases catalyze the hydrolysis of the indigestible phytate, and release a digestible form of inorganic phosphorus thus eliminating its anti-nutritional properties (Mullaney et al., 2000; Vohra and Satyanarayana, 2003; Singh et al., 2011; Singh and Satyanarayana, 2011). Phytases are classified into two categories differing on the site where the hydrolysis of the phytate molecule is induced. The 3-phytase (EC 3.1.3.8) preferentially liberates the phosphate (P) moiety at position *C-3*, whereas 6-phytase (EC 3.1.3.26) launches at position *C-6* of the myo-inositol hexaphosphate ring (Selle and Ravindran, 2007). The first phytases commercialized as feed additive in 1991 were the 3-phytase from *Aspergillus niger* and the 6-phytase from *Peniophora lycii* and *Escherichia coli* (Selle and Ravindran, 2007). Enzymes in granulate form are added to feed in pre-pelleting process. The liquid form is sprayed post-pelleting to avoid thermostability issues in case of high pelleting temperature (Selle and Ravindran, 2007). Along the digestive tract, the crop is probably the first place of action of the phytases. However in poultry the hydrolysis takes place mainly in the proventriculus and in the gizzard where the pH (3.4-4.6) is favorable (Mullaney et al., 2000; Vats and Banerjee, 2004). Other *in vivo* studies revealed that the phytase activities may occur in the ileum (Camden et al., 2001; Tamim and Angel, 2003) and that endogenous phytases produced by the cecal microbiota may influence the phytate degradation (Liebert et al. 2005; Khodambashi Emami et al. 2013).

I.4.2- CAZy

The carbohydrate active enzymes (CAZy) are a very broad category of enzymes that includes enzymes with different activities. The CAZy classification is a sequence-based classification of enzymes that synthesize or break-down carbohydrates, which originated from the seminal grouping of glycoside hydrolases (Henrissat et al. 1989; Henrissat 1991; Henrissat and Bairoch 1996; Henrissat and Coutinho 2001; Lairson et al. 2008; Cantarel et al. 2009). Each created family requires at least one biochemically characterized member, and is based on the protein sequence that defines its function (Cantarel et al., 2009). The CAZy database identifies 135 GH, 24 PL 16 CE and 98 GT families on the last checked update (02-06-2016). Updated information about the characteristics and the classification of enzymes are found in the Carbohydrate-Active Enzyme (CAZy) database (www.cazy.org). This database contains the sequence annotations, family classifications and known functional information.

The glycoside hydrolases (GH) are enzymes that catalyze the hydrolysis of the glycosidic linkage of glycosides and belong to EC 3.2.1. Depending on the anomery of the hydrolysis product, they follow a retaining (α→α or β → β) or inverting (α→ β or α → β) mechanism. Inverting enzymes utilize two amino acids, typically carboxylate residues that act as acid and base, respectively. Retaining enzymes follow the Koshland double-displacement mechanism. Generally, it is achieved in 2-steps, involving a covalent glycosyl-enzyme intermediate (Koshland, 1953). The first step is the glycosylation through a nucleophilic attack of the anomeric center to displace the aglycon and form a glycosyl enzyme intermediate. The second step is the deglycosylation where the glycosyl enzyme is hydrolyzed by water (Davies and Henrissat, 1995; Lairson et al., 2008).

Depending on their recognition site on the substrate, endo- and exo-enzymes can be distinguished. The endo-enzyme refers to the ability of a glycoside hydrolase to cleave a substrate within the middle of a chain. The exo-enzyme cleaves its substrate at the end (most frequently, but not always the non-reducing end) (Vernon, 1908; Davies and Henrissat, 1995).

Polysaccharide lyases (PL) cleave uronic acid-containing polysaccharides via a β-elimination mechanism to generate an unsaturated hexenuronic acid residue and a new reducing end at the point of cleavage (Garron and Cygler, 2010; Lombard et al., 2010). The mechanism of PL action can be described as consisting of three events:

- abstraction of the *C-5* proton on the sugar ring of an uronic acid or ester by a charge stabilizing cation such as Ca^{2+} or a basic amino acid side chain

- stabilization of the resulting anion by charge delocalization onto the *C-6* carbonyl group
- lytic cleavage of the *O-4:C-4* bonding that is facilitated by proton donation from a catalytic acid (Yip and Withers, 2006; Lombard et al., 2010).

Carbohydrate esterases (CE) are enzymes catalyzing the *O*- or *N*- de-acylation of substituted saccharides (Cantarel et al., 2009). They remove ester-linked groups from mono-, oligo- and polysaccharides and thereby facilitate the action of GH on complex polysaccharides. CE comprise 16 families (www.cazy.org). The sequence based classification incorporates some enzymes that may act on non-carbohydrate esters since the specificity barrier between carbohydrate esterases and other esterase activities is low (Cantarel et al., 2009).

The glycosyltransferases (GT) (EC 2.4) are enzymes that catalyze the formation of the glycosidic linkage to form a glycoside. These enzymes utilize activated sugar phosphates as glycosyl donors, and catalyze glycosyl transfer to a nucleophilic group, usually an alcohol. Depending on their mechanism, GT can be qualified as retaining or inverting enzymes, according to whether the stereochemistry of the donor's anomeric bond is retained or inverted during the transfer (Lairson et al., 2008).

Some of the CAZy are multimodular and contain a carbohydrate-binding module (CBM). A CBM is defined as a contiguous amino acid sequence within a carbohydrate-active enzyme, able to bind to carbohydrate ligand and direct the catalytic machinery onto its substrate, thus enhancing the catalytic efficiency of the carbohydrate-active enzyme (Boraston et al., 2004; Shoseyov et al., 2006; Hashimoto, 2006). CBM are themselves devoid of any catalytic activity. CBM are most commonly associated with GH but have also been identified in PL, CE, polysaccharide oxidases, GT and plant cell wall-binding expansins (Gilbert et al., 2013). Based on their protein sequence, CBM are classified into 74 families (www.cazy.org), the majority of which contains members that target components of plant cell walls (Gilbert et al. 2013).

In the next section, enzymes used in animal feed will be detailed, based on the substrate they target, which are the main cell wall polysaccharides of wheat endosperm, arabinoxylans and mixed-linked-β-glucans.

I.4.2.1- Arabinoxylan-degrading enzymes

As described in section I.3.2.4.1, AX are linear backbone of (1,4)-β-D-xylopyranosyl residues substituted with individual α-L-arabinofuranose residues (Izydorczyk and Biliaderis, 1995). To degrade AX different enzymes with different activities are needed (Figure I.15).

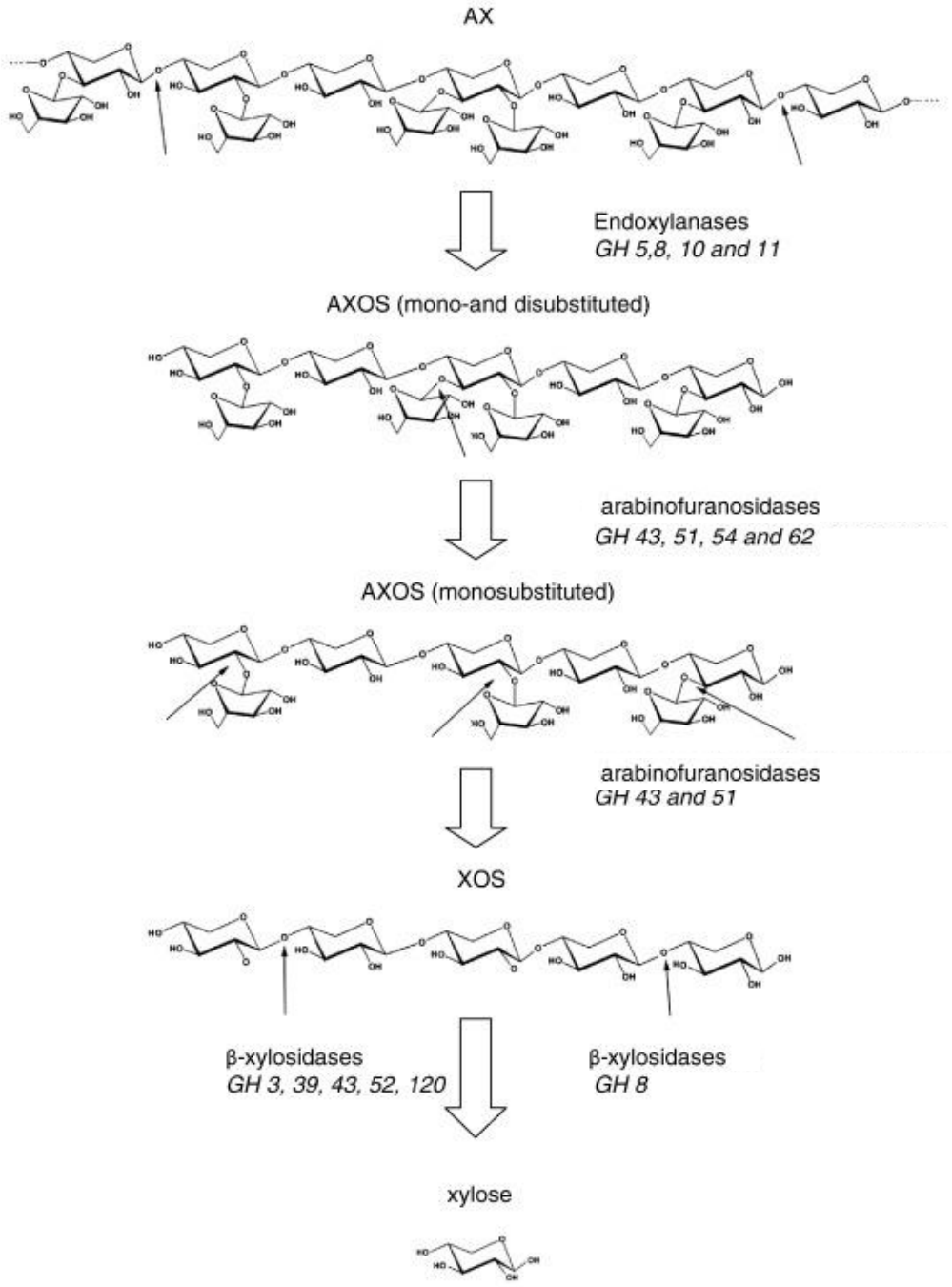

Figure I.15: Schematic overview of arabinoxylan (AX) degradation (Lagaert et al., 2014). Arrows indicate cleavage sites. Endo-xylanases cleave the xylan backbone randomly, endo-xylanase families are taken from Collins et al. (2005).

I.4.2.1.1- Xylanases

The xylanases are able to catalyze the hydrolysis of the glycosidic (β-1,4) linkage between two xylose residues (Jeffries, 1996). They have been isolated mainly from bacteria and fungi but also from algae, protozoa, gastropods and arthropods (Prade, 1996).

The endo-xylanases (EC 3.2.1.8) are clustered in CAZy families 5, 8, 10, 11, 30, 43 and 51, according to their amino acids sequences. Enzymes within the same family have the same three-dimensional structure (Henrissat and Coutinho, 2001) and the same molecular hydrolytic mechanism (Gebler et al., 1992). Only the xylanases from families 10 and 11 are used as feed additives.

The most numerous enzymes in GH10 are endo-β-xylanases (E.C 3.2.1.8), although GH10 includes endo-β-1,3-xylanases (E.C 3.2.1.32) (Jeffries, 1996; Biely et al., 1997). The GH10 enzymes are characterized by a high Mw (>30 kDa) and an acidic isoelectric point (pI) (Collins et al., 2005a; Lairson et al., 2008).

Similarly to GH10, GH11 includes mainly endo-β-1,4-xylanases. Unlike GH10 xylanases, their action is limited by the substitution on the xylan backbone by 4-*O*-methyl-D glucuronate, acetate and α-L-arabinofuranose, since they do not tolerate such backbone decoration in their active site (Biely et al., 1997; Beaugrand et al., 2004). The GH11 enzymes are characterized by a low Mw (<30 kDa) and an alkaline pI (Collins et al., 2005).

I.4.2.1.2- Arabinofuranosidases

α–L-Arabinofuranosidases (EC 3.2.1.55) are classified in families GH 10, 43, 51, 54 and 62. They remove arabinofuranose side chains that are linked either α-1,2 or α-1,3 to the xylose backbone residues. The GH43 includes also endo-1,5-α-L-arabinanase (EC 3.2.1.99) which endo-hydrolyses the (1,5)-α-arabinofuranosidic linkages in (1,5)-α-L-arabinans. The GH62 enzymes are described as specific of the AX. They release arabinose linked on both *O-2* and *O-3* of the xylose moiety. Several of these enzymes contain cellulose- or xylan-binding modules (Kellett et al., 1990), Dupont et al., 1998).

I.4.2.1.3- Carbohydrate Esterases

Carbohydrate esterases (CE) catalyze the *O*- or *N*- de-acylation of substituted saccharides. The AX can be *O*-esterified by acetic or ferulic acids. In both cases, the polysaccharide behaves as the alcohol to built the ester linkage. Consequently, the esterases involved in the degradation of AX are

- feruloyl esterase (EC 3.1.1.73) classified in CE 1 family and catalyzing the following reaction: feruloyl-polysaccharide + H_2O = ferulate + polysaccharide
- acetyl xylan esterase (EC 3.1.1.72) belonging to the CE 1 to 7 and CE12 families and catalyzing the deacetylation of xylans and xylo-oligosaccharides.

I.4.2.2- β-glucan-degrading enzymes: Glucanases

As described in section I.3.2.4.2, mixed linked β-glucans are linear homoglucans of D-glucopyranose arranged as blocks of consecutive 1,4-linked β-D-glucose residues separated by single 1,3-linkages. The β-glucan-degrading enzymes are called glucanases and are GH belonging to different families (5, 6, 7, 8, 9, 12, 16, 19, 44, 45, 48, 51, 74, 124 and 131). Their activity consists on endo-hydrolysis of (1-4)-β-D-glucosidic linkages (Biely et al., 1985) of the glucan chain (Figure I.16).

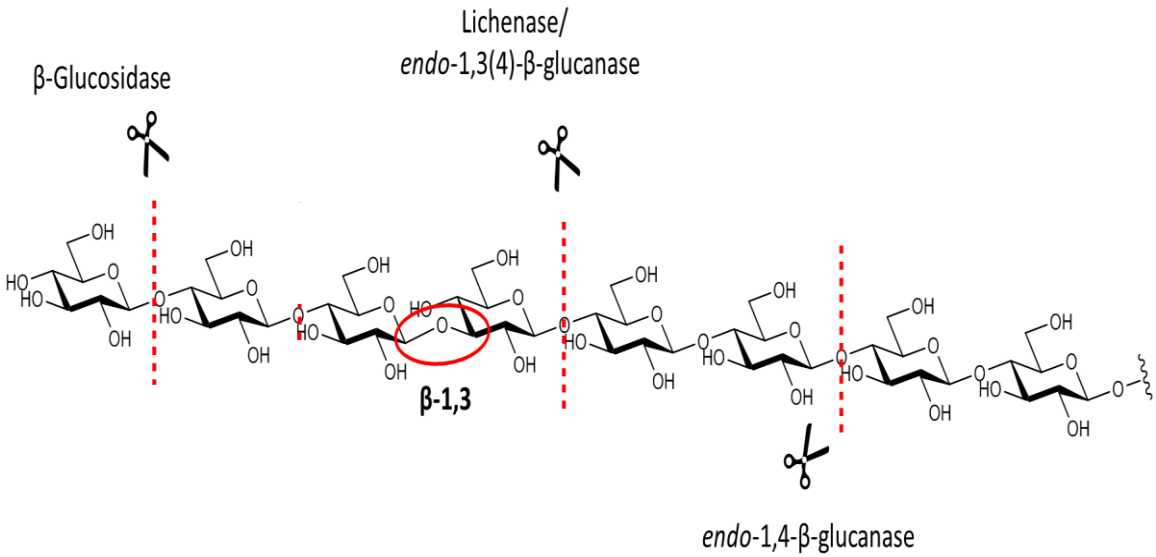

Figure I.16: Schematic overview of mixed linked β-glucans (MLG) degradation (www.megazyme.com).

Glucanases have been purified from a variety of fungi, plant and bacteria (Gilkes et al., 1991). This category includes:

- 1,3(4)-β-D-glucanases also named 1,3;1,4-β-D-glucan-4- glucanohydrolase or lichenase (EC 3.2.1.73), belonging to 9 different GH families (GH5, 7, 8, 9, 11, 12 16, 17, 26) that strictly cleave β-1,4-glycosidic linkage adjacent to a β-1,3 linked glucose residue but are inactive against β-1,3 or β-1,4-glucans;

- endo-1,4-β-D-glucanases also called endo-cellulase (EC 3.2.1.4), belonging to 15 different GH families (mainly GH5) and which hydrolyze glucosidic bonds in a β-1,4 backbone;
- β-1,3(4)-glucanases (EC 3.2.1.6, GH9 and GH16) active on mixed-linked (1,3-1,4) β-glucans as well as β-1,3 glucans, able to cleave β-1,3 as well as β-1,4 linkages;
- 1,3-β-D-glucanases also named 1,3-glucan-3-glucanohydrolases (EC 3.2.1.39, GH16, 17, 55, 64, 81, 128) that cleave exclusively β-1,3 linkages between two glucose residues, mainly in β-1,3-glucans and have a very limited activity on (1,3-1,4) β-glucans.

All these glucanases are endo-enzymes, thus decreasing rapidly the molecular weight of their substrate.

I.4.3- Effects of the exogenous enzymes in animal nutrition

As reviewed by Choct (2006), the first studies on exogenous enzymes in animal nutrition used "crude amylases" and reported significant improvement of animal performance (Hastings, 1946; Fry et al., 1958). Since this date, it was established that cereal starch is well digested by broiler endogenous enzymes. This ascertainment suggested that the improvements recorded are due to the presence of NSP-degrading enzymes that improve nutrient utilization, increase metabolizable energy, reduce digesta viscosity and indirectly improve nitrogen and amino acids utilization by increasing access to protein for digestive proteases (Adeola and Cowieson, 2011). Moreover, it has been confirmed that enzymes were more efficient when the feedstuffs were poorly digestible (Classen and Bedford, 1991; Scott et al., 1998b; a).

The strategy of using enzymes as feed additives is based on the fact that those enhance nutrient digestibility. The NSP-degrading enzymes extenuate the anti-nutritive effects of the NSP especially of viscous and moderately viscous grains such as rye, barley, oat, wheat and triticale. Therefore, most of the GH used in animal nutrition target cereal soluble NSP (*i.e* 1,4-β-D-glucan and 1,4-β-D-xylan) and specifically their endo-linkages allowing reducing Mw without releasing monomeric sugars (Bhat and Hazlewood, 2001).

The NSP found in other diet compounds such as protein sources, are mainly pectic substances and cellulose. Soybean meal contains 21.7-30.3 % total NSP, including 6.3-13.9 % water-soluble NSP (WS-NSP) (Smits and Annison, 1996; Knudsen, 1997, 2014). Rapeseed meal contains 22.0-46.1 % total NSP including 5.5-34.8 % WS-NSP (Smits and

Annison, 1996; Knudsen, 1997, 2014). In these diets, NSP-degrading enzymes degrade the cell-wall polysaccharides that possibly improve the release of encapsulated nutrients from inside the cell. Secondly, they reduce the digesta viscosity induced by the WS-NSP. Moreover, NSP-degrading enzymes affect the digesta transit time as well as gut motility (Potkins et al. 1991). To be efficient, the enzymes should match with the specific structure of NSP present in the diet, but due to the large use of cereals endo-1,4-β-xylanase and endo-1,3(4)-β-glucanase activities, represent 80 % of the global market of animal feeding, (Adeola and Cowieson, 2011).

The efficiency of the enzymes used as feed additives depends on many factors. The optimal temperature for their activity is around 40 °C, and they exhibit different thermostability (Ravindran, 2013). The enzymes have an optimum activity at a pH between 4 and 6 (Ravindran, 2013). The moisture content is essential for the solubility and solubilization of substrate and the mobility of the enzyme. The concentration/activity of the enzyme as well as the feedstuff used are crucial to improve enzyme efficiency and consequently, animal performances.

The efficiency of the enzyme depends on the enzyme source as well as the substrate. Choct et al. (2004) compared 3 xylanases belonging to the same family GH11 but from different sources (xylanase A from *Thermomyces lanuginosus*, xylanase B from *Humicola insolens* and xylanase C from *Aspergillus aculeatus*) and showed that they differ on their effect on soluble and insoluble NSP degradation and on digesta viscosity. Many factors influence the wheat apparent metabolizable energy (AME) such as starch, protein and lipid content as well as the NSP content. Wheat varieties have different AX content and structure that might impact its AME and by consequence the animal performance (Annison and Choct, 1991; Choct et al., 1995, 1999). Different studies demonstrated that the response of the xylanases *in vivo* is positively correlated with the NSP content (Campbell and Bedford, 1992; Bedford, 1996; Bedford and Schulze, 1998). Recently, it was reported that the A/X ratio better predicts the xylanase efficiency through a negative correlation with the response of the xylanases *in vivo* (Smeets et al., 2014).

The enzyme accessibility to the substrate depends on the complexity of the structure and the relationship between the different polymers as well as the crystallinity (in case of cellulose) and the DP of the substrate (Agbor et al., 2011). The enzyme activity can be improved by the pretreatment of the biomass. Physical pretreatment such as grinding aims at increasing the specific surface area and reduce the DP (Sun and Cheng, 2002). Chemical

pretreatments (acids, alkali, organic solvents, and ionic liquids) have been reported to significantly affect the polymers structure and improve enzyme action (Chandra et al., 2007), but are not compatible with animal feeding.

The efficiency of the exogenous enzymes depends not only on the physical form of the diet (mash, pellet and whole grains) but also on the amount of antinutritional factors (trypsin inhibitor, polyphenols, phytate,…) (Walsh et al., 1993). In this respect, cereals contain xylanase inhibitors that can obstruct the activities of exogenous enzymes produced by microorganisms (Simpson et al., 2003; Juge and Delcour, 2006). Two types of structurally different xylanase inhibitors have been reported in the literature, namely, *Triticum aestivum* xylanase inhibitor (TAXI) (Furniss et al., 2002; Brutus et al., 2004) and xylanase inhibitor protein (XIP) (Sørensen et al., 2004; Bedford, 2006). The level of these inhibitors in wheat differs according to cultivar (Bonnin et al., 2005; Dornez et al., 2006; Gebruers et al., 2010). Bonnin et al. (2005) quantified the apparent XIP and TAXI amounts in 20 wheat cultivars TAXI ranged from 0.05 to 0.19 mg/g in flour and from 0.07 to 0.2 mg/g in grain, whereas XIP amount was higher with 0.12 to 0.6 mg/g in flour and 0.21 to 0.56 mg/g in grain. TAXI inhibition activities vary between 17 and 137 InhU, while XIP inhibition activities vary between 234 and 355 InhU. InhU is the amount of inhibitor per gram sample that inactivates 50 % of a given activity of xylanase under the conditions described by Gebruers et al. (2002). Nevertheless, not all xylanases are inhibited by these XIP and TAXI (Berrin and Juge, 2008).

The effects of exogenous enzymes on animal performance depend on animal species and age (Classen and Bedford, 1991). The effect of feeding β-glucan-rich diet is negatively correlated with age (Salih et al., 1991). Adeola and Bedford (2004) recorded 13 % improvement on duck body weight gain (BWG) and 12 % on feed conversion ratio (FCR) using a xylanase preparation derived from *Trichoderma longibrachiatum* on wheat-based diet. Olukosi et al. (2007) showed that using 3200 U of GH11 xylanase from *Bacillus circulans* per kg of rye, wheat and soybean meal diet increased broiler BWG by 18 %. In the other hand, Olukosi et al. (2008) showed that using a cocktail containing xylanases, amylases and proteases had no effect on broiler BWG fed a maize-soybean-based diet. Same outcomes were observed by Troche et al. (2007) using similar diet and the same enzyme cocktail on turkeys during the first 56 days of age. Meng et al. (2005) showed that a combination of cellulases, pectinases, xylanases, glucanases, galactanases, and mannanases induced a greater effect on broiler performance and nutrient utilization than the single enzymes. Using a

mixture of different enzymes targeting different linkages in different substrates maximize the beneficial effect on animal performance.

Recent researches investigated the effect of enzymes on gut health. The fact that high digesta viscosity decreases its flow rate and supports the proliferation of pathogens is generally accepted. Wheat-based diet is also responsible of morphological degradation of the intestinal tissues, increase inflammation and T-lymphocytes infiltration in the mucosa (Teirlynck et al., 2009b) and a higher *Salmonella enteritidis* colonization of the gut (Teirlynck et al., 2009a). These effects are diminished by enzyme supplementations. Hübener et al. (2002) observed an increase of microbiota growth and SCFA production on wheat-fed broilers when supplemented with xylanases. The GH supplementation allowed also increasing villi length, reduced digesta transit time and relative weight of the digestive tract (Choct et al., 2004; Sieo et al., 2005).

Most recent researches on exogenous enzymes explore new pathways to explain their beneficial effects. The undigested NSP are fermented in the ceca to produce SCFA, which provides energy for the host. Bedford and Cowieson (2012) claimed that the GH influence the intestinal health through two mechanisms. At first, they provide fermentable oligosaccharides resulting from depolymerization of NSP (Courtin et al., 2008). Secondly, they ensure the removal of fermentable starch and protein through accelerated digestion and promote the proliferation of NSP-degrading microbial populations (Choct et al., 1996; Bedford, 1996, 2000). These two pathways decrease the completion between the host and its intestinal microbiota for the nutrients and allow a more efficient symbiosis. Nevertheless, these effects depend on enzyme dose, class, and substrate/ enzymes combination.

I.4.4- Rovabio® Excel

Rovabio ® Excel is an enzyme cocktail commercialized by the company Adisseo. It is used as feed additive and exists in liquid and solid forms. It has been approved by the European Commission based on the scientific opinion of European Food Safety Authority (EFSA) as a feed additive for chickens and turkeys for fattening, laying hens, piglets (weaned) and pigs for fattening, ducks, guinea fowls, quails, geese, pheasants and pigeons (European Food Safety Authority 2013).

It is produced by the non-genetically modified strain of the filamentous fungus *Talaromyces versatilis sp.nov* (previously named *Penicillium funiculosum)* deposited at CABI Bioscience United Kingdom with the IMI CC number 378536 (former deposit number

IMI SD 101).

The liquid form of Rovabio® Excel LC contains 4-8 % enzyme concentrate, 10-30 % sorbitol, 0.1-0.4 % potassium sorbate and water up to 100 %. The ingredients used to formulate the product are food grade (European Food Safety Authority, 2013). The liquid form ensures a minimum of 7500 viscosimetric U/mL or 1075 DNS U/mL for the glucanase and 5500 viscosimetric U/mL or 800 DNS U/mL for the xylanases (European Food Safety Authority, 2013).

The stability of the liquid formulation, Rovabio® Excel LC, was investigated for up to six months at three different temperatures: 20, 30 and 40 °C (Table I-1). The stability of xylanases contained in Rovabio® Excel LC showed that the enzyme retained its activity over a period of more than one year (European Food Safety Authority, 2013).

Table I-1: Rovabio® Excel LC enzymes recovery results measured, after 3 months, as viscosimetric units (European Food Safety Authority, 2013).

	20 °C	30 °C	40 °C
Xylanase	88 %	61 %	41 %
Glucanase	-	90 %	-

The secretome of *Talaromyces versatilis sp.nov* analyzed by Guais et al. (2008) allowed identifying a wide range of enzymes (Table I-2). Nevertheless, only 19 enzymatic activities were detected and quantified (Table I-3).

Table I-2: Putative enzymatic activities present in Rovabio® Excel and identified by 2-dimensional gel electrophoresis (2-DE), single SDS-PAGE electrophoresis (1-DE) and peptide shotgun methods (Guais et al., 2008)

Protein name /enzyme name	**Family**
Acetyl xylan esterase	CE 1,2,3,4,5,6,7 and 12
α-1,2-mannosyltransferase	GT 1
α-mannosidase	GH 47
α-galactosidase	GH 27
Endopygalacturonase	GH 55
Endo-1,4- β -galactanase	GH 53
Neutral endopolygalacturonase	GH 28
Polygalacturonase	GH 28
Rhamnogalacturonase	GH 28
α-glucuronidase	GH 67

α-L-rhamnosidase	GH 78
α-xylosidase	GH 31
Arabinofuranosidase	GH 62
B-xylosidase	GH 3
Endo-1,4-xylanase	GH 11
Endo-1,4-xylanase B	GH 11
Endo-1,4-xylanase D	GH 11
Xylanase/cellobiohydrolase	GH 7
β-1,3-glucanosyltransferase	GT 3
β-1,4-xylosidase	GH 3
β-1,6-glucanase	GH 5
β-galactosidase	GH 35
β-glucosidase	GH 3
Endo-glucanase	GH 5
Cellulase	GH 5
Cellobiohydrolase I	GH 7
Cellobiohydrolase II	GH 6
Dextranase	GH 49
Exo-β-D-glucosaminidase	CE 1
Ferulic acid esterase A	CE 1
Feruloyl esterase B	GH 15
Glucoamylase	
Avicelase III	
Carboxypeptidase	
Catalase	
FAD binding monooxygenase	
GDP-mannose 4,6-dehydratase	
Glutaminase A	
Laccase	
Lipase	
Manganese peroxydase precursor	
Oxidoreductase	
Polyketide synthase	
Steroid monooxygenase	
Swollenin	
Type I phosphodiesterase / Nucleotide pyrophosphatase	
Ubiquitin-conjugating enzyme	
Alkaline protease	
Aspartic protease	

Table I-3: The main enzymatic activities of Rovabio® Excel LC (batch RXL LC 4862469236) (Adisseo/Cinabio-Internal data)

Enzyme name	Activity	Substrate
endo-1,5-α-L-arabinanase	567 UAZCL/mL	AZCL-Polysaccharides[1]
feruloyl esterase	0.391 U/mL	methyl ferulate[2]
β-D-glucosidase	42 U/g	p-nitrophenyl-β-D-glucoside[3]
endo1,4-1,3-β-glucanase	1249 U/g	β-glucan[4]
endo1,4-β-D-glucanase	1703 U/g	carboxymethyl cellulose[4]
endo-1,3-β-D-glucanase	117U/g	laminarin[4]
Exoglucanase	32U/g	p-nitrophenyl-β-D-lactoside[3]
β-D-mannosidase	0.3 U/g	p-nitrophenyl-β-D-mannoside[3]
endo1,4-β-D-mannanase	497 UAZCL/mL	AZCL-Polysaccharide[1]
β-D-xylosidase	7 U/g	p-nitrophenyl-β-D-xyloside[3]
endo1,4- β-D-xylanase	910 U/g	birchwood xylan[4]
endo1,4- β-D-xylanase	5589 Uvisco/g	wheat arabinoxylan[5]
rhamnogalacturonase	1092 UAZCL/mL	AZCL-Polysaccharide[1]
α-D-galactosidase	7 U/g	p-nitrophenyl-β-D-glucoside[3]
polygalacturonase	288 U/g	polygalacturonic acid[4]
Pectinase	25 U/g	Citrus pectin[4]
dextranase	11 UAZCL/mL	AZCL-polysaccharide[1]
protease (casein pH7)	0.020 UAZCL/mL	AZCL-casein
protease (collagen pH7)	0.043 UAZCL/mL	AZCL-collagen
protease (collagen pH 4,5)	0.304 UAZCL/mL	AZCL-collagen

[1] AZCL: Azurine-Crosslinked Polysaccharides are dyed and crosslinked highly purified polysaccharides. They are supplied as a fine powder (milled to pass a 0.5 mm screen). These substrates are insoluble in buffered solutions, but rapidly hydrate to form gel particles, which are readily and rapidly hydrolyzed by specific endo-hydrolases releasing soluble dye-labelled fragments. One unit of the enzyme activity is defined as the amount of enzyme that gives rise to 1 absorbance unit.

[2] Method described by Kermasha et al. (1995)

[3] The specific activity was expressed as nmole of *p*-nitrophenol released per min per mg of protein

[4] Dinitrosalicylic acid (DNS) colorimetric method (Miller, 1959)

[5] Viscosimetric method: One viscosimetric unit (U) is the amount of enzyme which hydrolyses the substrate (barley β-glucan for the endo-1,3(4)-β-glucanase activities and wheat arabinoxylans for the endo-1,4-β–xylanase activities), reducing the viscosity of the solution, to give a change in relative fluidity of 1 dimensionless unit/min at 30 °C and pH 5.5.

Talaromyces versatilis sp.nov genome was sequenced in 2013 and since allowed identifying new genes encoding for new GH from different families. Genomic analysis identified 200 genes encoding putative GH (Adisseo, internal data). It contains a broad range of GH such as xylanases and glucanases.

To date, 5 endo-xylanases were identified, one GH10 (XynD) and 4 GH11 (XynB, C, E, F). Recombinant xylanases were produced to study their biochemical properties and specificities (Lafond et al., 2011; 2014). They differed in their thermostability and in the production of xylose, xylobiose and some AXOS as end products of the degradation of low viscosity AX (Lafond et al., 2014). The active site of these xylanases accommodated at least 6 xyloses residues, since their catalytic activity increased when xylan DP increased. The secretome also contained arabinofuranosidases, 4 belonging to GH54 (Guais et al., 2008) and 3 belonging to GH62 (De La Mare et al., 2013). The GH62 enzymes were shown to be able to remove arabinosyl residues from positions *O-2* and *O-3* of substituted xylose in AX.

Maisonnier-Grenier et al. (2006) investigated the effects of Rovabio® Excel on several wheat cultivars and wheat extracts. The *in vitro* experiment showed an important solubilization of water-insoluble AX in wheat flour ranging from 65 to 85 % depending on wheat cultivars. Using the TNO gastrointestinal model-1 (TIM-1) which is an *in vitro* model system mimicking the digestive tract, Lafond et al. (2011) showed that Rovabio® Excel improved feed digestibility and suggested that the efficiency of the enzyme cocktail depended on the wheat cultivar especially its AX content and structure. *In vitro* studies using xylanases produced by *Talaromyces versatilis* increased AX degradation mainly at the jejunal level. It also increased the amount of reducing end sugars into the jejuno-ileal dialysates (Lafond et al., 2015).

The *in vitro* studies do not always show the same improvement and effects on all the cereals (corn, wheat, barley, rye…) neither on apparent digestibility and apparent metabolizable energy (AME). They bring essential elements to understand the mechanisms of action of the enzyme and to pretend their effect *in vivo*. The enzymes produced by *Talaromyces versatilis* increased AME of broilers fed wheat-based diet (Maisonnier-Grenier et al., 2006). However, the results showed an important variation on AME depending on the wheat cultivars.

In vivo trials using Rovabio® Excel showed an improvement of the BWG, the final body weight, the FCR and mortality of broilers fed wheat-based diet (West et al., 2007; European Food Safety Authority, 2013). It also increased AME content of the diet. When combined

with phytase (such as in Rovabio® MAX), it increases significantly the average body weight, the BWG, and the FCR of broilers fed a corn-wheat-soybean (50-10-28) diet (Lee et al., 2010).

On barley-based diet, the efficacy of the exogenous enzymes produced by *Talaromyces versatilis* depends on the level of the endogenous β-glucanases (Ribeiro et al., 2011). Though, it is not the only parameters that influence its efficacy. Mushtaq et al. (2009) showed that it depends on the digestible lysine levels in the diet and that the enzyme supplementation can compensate the lysine deficiency. They also showed a positive response of enzyme addition in low nutrient density and high sunflower meal (300 g/kg) diets.

I.5- Aim of the study

The preceding chapter stressed on the importance of the avian intestinal tract for animal performance and described the most important features of its anatomy, physiology and the associated immune system. It is followed by a review of wheat grain structure and chemical composition and their influence on animal performance as a main source of energy in animal nutrition. Finally, exogenous enzymes used as feed additives and their effects on wheat structure and composition as well as on animal performances were described. Though, the mechanisms of action of these enzyme preparations are not fully comprehended and seem to be more complex than exposed in the literature. The aim of this study is to investigate the effects of the enzymes on wheat cell wall structure and compositions and the effects of these degradation products on animal performance.

To fulfill the research aim the following strategy was adopted.

First, we studied the effects of the enzymes on wheat. Water-soluble fractions from wheat treated or not with MEP were isolated at a laboratory scale. The fractions were characterized and their effect on cecal microbiota was tested in order to identify the fraction(s) of interest (Chapter II).

Second, the fraction(s) of interest identified were then produced at a pilot scale. The fractions produced at different levels (laboratory and pilot scale) were compared *in vitro* in order to confirm the effects observed previously (Chapter III).

Third, the fraction(s) of interest produced at pilot scale were added to a wheat-based diet to feed broilers during the starter period (2 first weeks post-hatch) and their effects on animal performance and intestinal health were investigated. In order to establish a connection between the different effects on animal performance and the use of MEP, a special attention was paid to the interaction between the diet and the host especially the intestinal health and microbiota (Chapter IV).

Finally, based on the different results a model of the mechanisms of action of the MEP is proposed.

Chapter II

Water-soluble fractions obtained by enzymatic treatment of wheat grains promote short chain fatty acids production by broiler cecal microbiota

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II.1- Abstract

The use of exogenous cell wall degrading enzymes to improve the nutritional values of cereal grains has increased during the last decades. In this study, products from enzymatic hydrolysis of wheat grain were isolated and their ability to induce short-chain fatty acids was investigated using *in vitro* fermentation with cecal microbiota. Water-soluble fractions were obtained following incubation without and with a multi-enzyme preparation (MEP) (2 mL/kg) containing essentially endo-xylanase and endo-glucanase activities. These were further fractionated by size exclusion chromatography into high molecular weight (HMw) and low molecular weight (LMw) sub-fractions. The MEP-treatment increased the content in water-soluble arabinoxylans **(AX)** and decreased the degree of polymerization (DP) of the xylan-backbone. *In vitro* fermentation assays using broiler cecal content as inoculum demonstrated that low substituted AX with reduced molecular weight isolated after MEP-treatment yielded higher concentrations of acetate and butyrate. This result indicates that cecal fermentation of enzymatic degradation products into short-chain fatty acids, particularly butyrate, is one of the important mechanisms of MEP action on wheat-based diets that can contribute to the improvement of intestinal health and animal performance.

Key words: Cereals, xylanase, non-starch polysaccharides, arabinoxylans, short chain fatty acids

II.2- Introduction

Wheat is the most commonly used cereal in poultry diets in Europe. However differences in metabolizable energy are observed amongst wheat based diets, partly due to the presence of non-starch polysaccharides (NSP) (Gutierrez del Alamo et al., 2008). In wheat, the NSP content ranges from 12 to 16 % of dry matter. Starchy endosperm NSP consist mainly of arabinoxylans (AX), that represent 70 % of the cell walls (Saulnier et al., 2012). Arabinoxylans are composed of a linear backbone of β-(1,4)-linked D-xylose residues that is partially substituted with individual α-L-arabinose residues attached through *O-2* and/or *O-3* (Izydorczyk and Biliaderis, 1995). The second most important wheat NSP are mixed-linked β-glucans, which are linear homoglucans arranged as blocks of consecutive (1,4)-linked β-D-glucose residues separated by single (1,3)-linkages (Li et al., 2006). It is well demonstrated that these two major components of cereal grain endosperm exert anti-nutritional effects through the viscosity-inducing property of their water-soluble fraction. In the case of wheat, the water-soluble NSP are almost exclusively composed of AX.

Non-starch polysaccharides degrading enzymes (NSP-enzymes) constitute a category of safe additives that improve body weight gain (BWG) and feed conversion ratio (FCR) in broilers (Mathlouthi et al., 2002a; b; Choct et al., 2004; Wang et al., 2005; Walk et al., 2011). The NSP-enzymes like endo-1,4-β–xylanases and endo-1,3(4)-β-glucanases are widely used separately, in combination or in a more complex mixture to decrease the viscosity of the intestinal content and enhance the nutritional value of cereal diets (Mathlouthi et al., 2002b; Choct et al., 2004). However, the improvement of nutritional performance of wheat-based diets by NSP-enzymes is not only linked to viscosity effect. NSP-enzymes also increase short-chain fatty acids concentration (SCFA) in the ceca (Choct et al., 1999) and alter the microbiota (Van der Wielen et al., 2000).

However, it is not fully explained in the literature what substrates are released by the NSP-enzymes which contribute favorably to the SCFA production in the ceca. The aim of this paper is to contribute to the understanding of the beneficial effects of NSP-enzymes supplementation to wheat-based diets for broilers. Therefore, water-soluble NSP were isolated from wheat grain treated or not with the multi-enzyme preparation (MEP) Rovabio® Excel. Water-soluble NSP were further separated by size exclusion chromatography into high molecular weight (HMw) and low molecular weight (LMw) sub-fractions. Fractions were characterized for their chemical composition and physico-chemical features, and their SCFA production pattern was determined *in vitro* using broiler cecal microbiota.

II.3- Materials and methods

II.3.1- Materials

The multi-enzyme preparation (MEP) Rovabio® Excel was provided in liquid form by Adisseo SAS (Commentry, France) (Table II-1). The MEP contains glycosyl hydrolases, mainly endo-1,3(4)-β-glucanase (1200 U/g) and endo-1,4-β–xylanase (800 U/g), produced from the fermentation of *Talaromyces versatilis sp.nov.* (previously named *Penicillium funiculosum).*

The wheat cultivar Barok, harvested in 2012, was purchased from Euronutrition (St Symphorien, France). Xylo-oligosaccharides (XOS), XOS95P, were purchased from Shandong Longlive Bio-technology CO.LTD, Yucheng, China. Fructo-oligosaccharides (FOS), Fibrulose® F97, were purchased from Cosucra, Warcoing, Belgium.

Table II-1: Characterization of Rovabio Excel*

Designation	Activity**
endo-1,4-β-xylanase	811 U/g
endo-1,3(4)-β-glucanase	1215 U/g

***Major enzyme activities of Batch Rovabio Excel LC 13.M.002152

****Activities as measured by DNS colorimetric method. For endo-1,3(4)-β-glucanase, one unit corresponds to the amount of enzyme which produces 1 µmole of glucose per minute from barley β-glucan at pH 5.0 and 50 °C. For xylanase, one unit corresponds to the amount of enzyme which produces 1 µmole of xylose per minute from birchwood xylan at pH 4.0 and 50 °C.

II.3.2- Isolation of the wheat fractions

The flowchart of the isolation of the wheat fractions is described in figure II-1. Wheat grains were decontaminated with successive washing in water, 70 % ethanol, sodium hypochlorite (NaClO) and water. After drying in a stove at 40 °C for 24 h, grains were ground using a 0.5 mm grid (Retsch, Haan, Germany). Ground grains (300 g) were suspended in Milli-Q water (1/3, w/w) leading to pH around 6.0. The incubation lasted 6 h at 40°C under gentle agitation in a water-bath orbital shaker at 60 rpm, with or without MEP addition (2 mL/kg). After 6 h, the water-soluble fraction (WSF) was recovered by centrifugation at 11800 g for 20 min in a Sigma 6K10 centrifuge (Osterode am Harz, Germany). The WSF was placed for 10 min in a boiling water bath to deactivate enzymes, and then centrifuged (11800 g, 20 min). The supernatant was then precipitated at 4 °C

overnight with 5 volumes of 96 % ethanol to get a final concentration of 80 % ethanol. Ethanol-insoluble fraction (EIF) was recovered by centrifugation and successively washed with 80 % ethanol, 96 % ethanol, acetone and finally dried overnight in a stove at 40 °C. An EIF sample (3 g) was dissolved in Milli-Q water (150 mL) and 50 mL aliquots were further fractionated into 2 sub-fractions using size exclusion chromatography on Sephacryl^{TM} S300 (GE Healthcare, Uppsala, Sweden). The column (90 cm x 5 cm) was eluted with water at 100 mL/h. Ten mL fractions were collected and analysed for neutral sugars content using an automated orcinol procedure (Tollier and Robin, 1979). Carbohydrates eluting between 700 and 1100 mL were pooled as high molecular weight fraction (HMw), whereas those eluting between 1300 and 1700 mL were pooled as low molecular weight fraction (LMw).

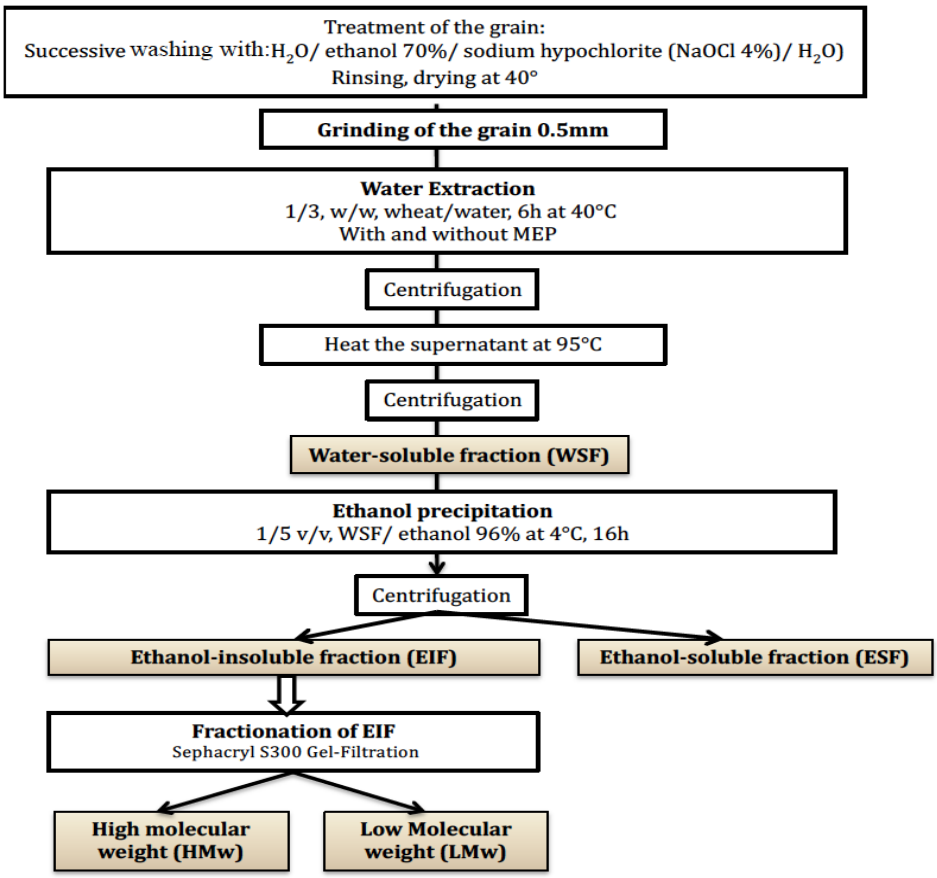

Figure II-1: Flow chart of extraction procedure and fractionation of the water-soluble fraction (WSF), ethanol-soluble fraction (ESF), ethanol-insoluble fraction (EIF), ethanol-insoluble fraction with high molecular weight (HMw) and ethanol-insoluble fraction of low molecular weight (LMw). MEP: multi-enzyme preparation

II.3.3- Characterization of the wheat fractions

II.3.3.1- Neutral sugars content

Neutral sugar composition of starting material and isolated fractions was determined by Gas Liquid Chromatography (GLC) based on the Englyst and Cummings (1988) method. Samples were hydrolysed using 1 M sulfuric acid at 100 °C for 2 h. Released monosaccharides were reduced to alditol in presence of sodium borohydrure ($NaBH_4$), and then acetylated with acetic anhydride and N-methylimidazole as catalyst. The alditol acetates were extracted by dichloromethane and analysed by GLC as previously described (Saulnier et al., 1995).

Starch content was analysed using thermostable □-amylase and amyloglucosidase (McCleary et al., 1997). The method is adapted from the AOAC method 996.11 and AACC 76-31.01.

II.3.3.2- Protein quantification and amino acids analysis

The protein content was measured by the Kjeldahl method (N x 6.25). Amino acids composition was investigated as described previously by Rezig et al. (2013). The method consists in a hydrolysis with hydrogen chloride (HCl) 6 N for 24 h at 110 °C, followed by drying-step using ethanol/water/Triethylamine (TEA): 2/2/1. Then a derivatization using ethanol/water/TEA/Phenyl isothiocyanate (PITC): 7/1/1/1. Calibration was done from 250 to 1500 pmol of amino acids in the injected sample. The analysis was done with reverse phase high-performance liquid chromatography HPLC (Alliance HT system, module 2795, Waters, Milford, MA, USA) on a Picotag column C_{18} 3µ, 15cm x 4.6mm (Waters, Milford, MA, USA). Signal was detected at 254 nm with an absorbance detector (Waters model 2487).

II.3.3.3- Degree of polymerization by gas–liquid chromatography (GLC)

Reducing end sugars in HMw and LMw fractions were determined as alditol acetates by GLC as described by Courtin et al. (2000) with minor modifications. The method consists of a reduction, acid hydrolysis and acetylation of the samples. The reducing end sugars are separated as alditol acetates from their acetylated aldose counterparts. In parallel, neutral sugars were determined in the sample as described above.

The ratio of the reducing end xylose to total xylose measured in the samples allowed calculating an average degree of polymerization (DP) of the xylan backbone.

II.3.3.4- Analytical high performance size-exclusion chromatography (HPSEC)

Approximately 5 mg of sample was solubilised in 1 mL of MilliQ water and filtered through a 0.45 µm filter (Millex-HV, PVDF) prior to injection. The HPSEC was performed at room temperature on a system consisting of a Shodex OH SB-G guard column (Shodex, Tokyo, Japan) and two Shodex OH-Pak columns SB-805HQ and SB-804HQ. Injection volume was 50µL and columns were eluted at 0.7 mL/min with 50 mM sodium nitrate. A Viscotek tri-SEC model 270 was used for light scattering and differential pressure detection, and a Viscotek VE 3580 refractive index (RI) detector was used for the determination of polymer concentration. A RI increment per unit concentration increment (dn/dc) value of 0.146 mL/g was used for concentration determination. Data were collected with Omnisec 4.7 software (Viscotek), and all calculations on polymer peaks (concentration, average molecular weights, intrinsic viscosity) were carried out using Omnisec software. .

II.3.4- In Vitro Fermentation of Wheat Fractions

Cecal contents of 10 4-week-old male broilers fed a wheat-based diet without any additives were pooled, diluted 10 times with anaerobic Phosphate-buffered saline PBS buffer (1 mg cysteine-HCl/mL HBSS, pH= 6) and used as inoculum. Fifty mL stock solutions of each wheat fraction at 1 mg/mL concentration were prepared. XOS (1 mg/mL) and FOS (1 mg/mL) were used as controls. Two biological replicates were performed in batch under anaerobic conditions at 38 °C in minimal medium and in triplicate (Williams et al., 2005). One hundred µL of the fraction stock solutions and 100 µL of the inoculum solutions were mixed in 10 mL medium and incubated for 24 h. After incubation, the supernatants and the pellets were separated by centrifugation for 20 min at 5200 g. The concentration of SCFA in the supernatants was determined by gas chromatography as described by Schäfer (1994) and Zhao et al. (2006). The method consists of an extraction in oxalic acid 0.5 M followed by direct injection on the gas chromatograph. Lactic acid concentrations were determined using the D/L-Lactic acid kit (ref 023, Biosentec, Toulouse, France).

II.3.5- Statistical Analyses

One-way ANOVA was employed to analyse the data using IBM® SPSS® Statistics 22.0 software. Results are given as means. Statements of statistical significance were based on $P \leq 0.05$.

II.4- Results

II.4.1- Characterization of water-soluble NSP Fractions

Water-soluble NSP fractions were isolated from Barok wheat with or without MEP-treatment and were characterized (Table II-2). The enzymatic treatment increased the total amount of water-soluble polysaccharides that were recovered by ethanol precipitation (Ethanol-Insoluble Fraction, EIF). The MEP-untreated EIF represented 2.84 g/100 g of wheat *vs*. 3.75 g/100 g of wheat after MEP-treatment, corresponding to an increase of 32 % of water-soluble polysaccharides amount. The EIF isolated after enzymatic treatment contained a larger amount of arabinose (13.8 g/100 g with MEP-treatment *vs*. 9.0 g/100 g without MEP-treatment) and xylose (22.3 g/100 g *vs*. 12.5 g/100 g) indicating that the enzyme preparation mainly solubilised AX.

The MEP-treated EIF and MEP-untreated EIF were further separated by size exclusion chromatography into LMw and HMw sub-fractions that were collected as indicated on figure II-2. The MEP-treatment increased LMw fraction yield that represents almost 50 g/100 g of the EIF with MEP-treatment and only 41 g/100 g of the EIF without MEP-treatment. Conversely the yield of the HMw fraction decreased (16.7 g/100 g with MEP-treatment *vs*. 22.8 g/100 g without MEP-treatment).

The chemical composition and the physicochemical properties of the different sub-fractions are reported in table II-2. A corrected A/X ratio was calculated for all the fractions taking into account the contribution of arabinogalactan-proteins (AGP) to the arabinose content and assuming an average A/G ratio of 0.7 (Loosveld, 1998). Minor amounts of glucose arising from starch were also detected. The average molecular weight (247 kDa) and the intrinsic viscosity (492 mL/g) and in good agreement with the average degree of polymerization (DP 470) of the xylan backbone calculated from the chemical determination of xylose reducing ends and total xylose contents in the HMw sub-fraction. The HPSEC chromatography (Figure II-3) showed that the collected HMw sub-fraction was heterogeneous with different populations of polymers eluting in the 12-17 mL range. Contrary to the HMw fraction, the MEP-untreated LMw sub-fraction was almost devoid of arabinose and xylose and contained large amounts of glucose originating from starch.

The MEP-treated HMw sub-fraction contained less xylose (21.1 g/100 g *vs*. 33.2 g/100 g) but the same amount of arabinose when compared to MEP-untreated HMw. On figure II-3, population corresponding to AX eluted between 12-18 mL was shifted to higher elution volume, while AGP elution profile remained unchanged (18-20 mL elution volume).

Table II-2: Yield, sugar composition, molecular weight (Mw) average, intrinsic viscosity and degree of polymerization (DP) of xylan backbone of the ethanol-insoluble fractions (EIF), ethanol-insoluble fraction of high molecular weight (HMw), ethanol-insoluble fraction of low molecular weight (LMw) prepared with and without the multi-enzyme preparation (MEP).[1]

		MEP-untreated			MEP-treated		
	Wheat	**EIF**	**HMw**	**LMw**	**EIF**	**HMw**	**LMw**
Yield* (g/100 g)		2.84	22.82	41.38	3.75	16.66	49.66
Sugars (g/100 g**)**	75.0						
Arabinose	2.3	9.0	23.7	1.3	13.8	24.5	8.0
Xylose	2.3	12.5	33.2	1.0	22.3	21.1	19.4
Mannose	0.5	0.9	0.2	1.4	0.7	0.1	1.0
Galactose	0.6	4.5	9.7	1.6	3.7	11.2	1.0
Glucose	69.3	29.9	4.3	36.1	21.0	3.2	28.3
Starch		28.4	3.9	32.3	21.0	3.1	28.2
Total sugars		56.8	71.2	41.4	61.4	60.0	57.7
Ratio A/X**	0.82	0.47	0.51	nd	0.5	0.79	0.38
Sum A+X**	4.21	18.4	50.1	1.2	33.5	37.7	26.7
DP (xylan)		263	470	nd	47	227	32
Mw (kD)* **		nd	247	nd	nd	161	nd
Intrinsic viscosity (mL/g)		nd	492	nd	nd	303	nd

*The yield is expressed as g/100 g grain for EIF and g/100 g EIF for HMw and LMw

**Corrected from arabinogalactan contribution

*** Population eluted from 12 to 18 mL

Rhamnose was detected as traces (≤ 0.1 %)

[1] Results given are mean of duplicates and coefficient of variation was less than 5 %

The AX recovered in the LMw fraction, that exhibited a lower branching degree (A/X: 0.38) and a drastically reduced DP (32) compared to AX recovered in the HMw fraction. However the measured DP indicated that oligosaccharides (DP< 10) were not formed upon MEP-treatment. This was confirmed by high-performance anion exchange chromatography analysis (data not shown). In addition MEP-treated LMw still contained glucose (28.3 g/100 g) totally originating from starch.

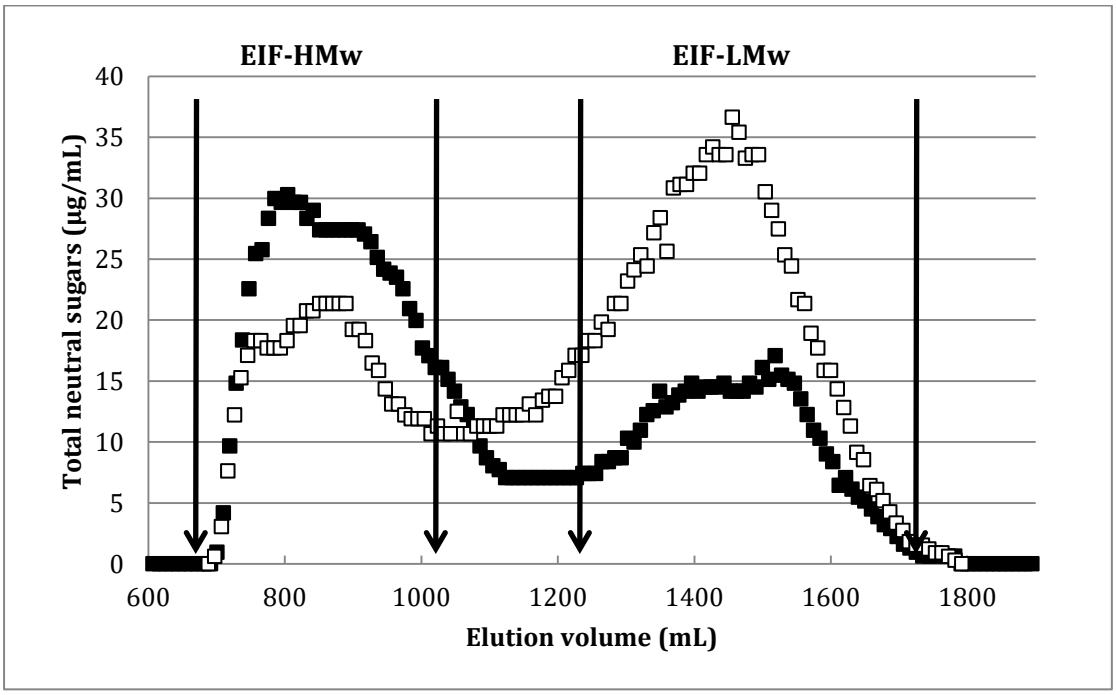

Figure II-2: Fractionation of the multi-enzyme preparation (MEP) untreated (filled square) and treated wheat (empty square) ethanol-insoluble fractions (EIF) by size exclusion chromatography (Sephacryl^{TM} S300).

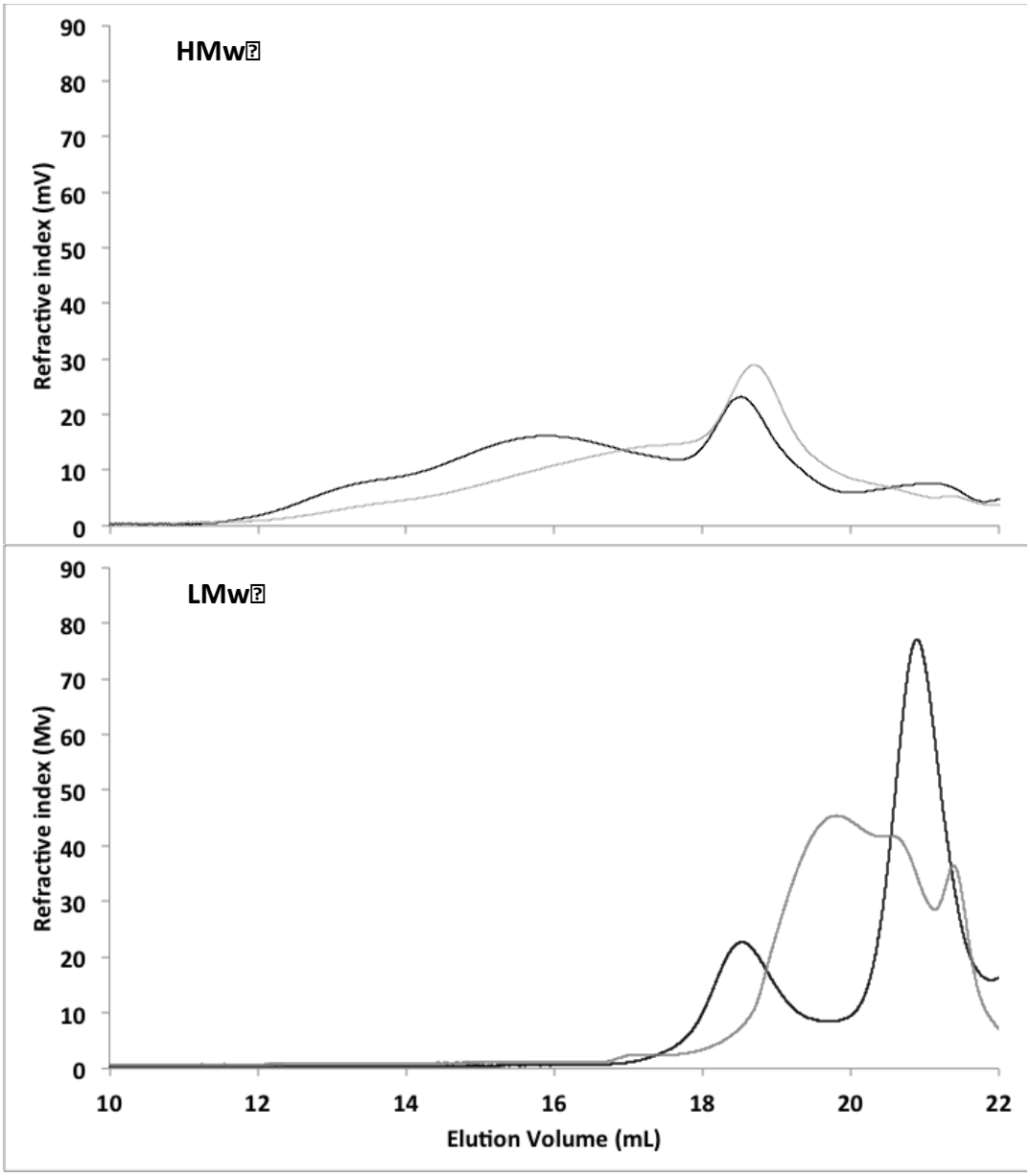

Figure II-3: HPSEC elution profile of the high molecular weight (HMw) sub-fractions and low molecular weight (LMw) sub-fractions prepared with (grey line) and without (black line) the multi-enzyme preparation (MEP).

The protein content of the MEP-treated EIF and MEP-untreated EIF fractions was similar (Table II-3). Nevertheless, LMw sub-fractions contained less protein than HMw sub-fractions. The amino acids composition of the fractions was close except that LMw sub-fractions were enriched in aspartic acid and cysteine, while HMw sub-fractions were enriched in glutamic acid and phenylalanine.

Table II-3: Protein and amino acids composition of the ethanol-insoluble fraction (EIF), ethanol-insoluble fraction with high molecular weight (HMw) and ethanol-insoluble fraction of low molecular weight (LMw) prepared with and without the multi-enzyme preparation (MEP).[1]

Fraction		**MEP-untreated**			**MEP-treated**	
	EIF	**HMw**	**LMw**	**EIF**	**HMw**	**LMw**
Protein (g/100 g)	23.9	22.2	17.3	21.6	28.2	15.9
Amino acids (mol/100 mol AA)						
Asparagine/aspartic acid	5.8	3.6	6.4	5.7	4.5	7.61
glutamine/glutamic acid	15.1	14.9	12.5	17.6	17.1	15.2
Serine	6.3	6.4	6.8	6.9	6.3	7.4
Glycine	10.2	10.9	10	11.5	10.4	11.9
Histidine	2.2	2.6	1.9	2.9	2.3	1.4
Arginine	6.8	5.6	6.9	6.5	5.6	5.7
Threonine	4.7	5.3	4.2	5	5.5	4.9
Alanine	8.1	9.3	8.1	8.2	9	8.7
Proline	7.5	7.7	7.2	7.7	7.6	8
Tryptophan	1.1	0.9	1	0.2	1.1	0.5
Valine	5.9	6.2	6.8	5.5	5.9	6.2
Methionine	0.8	1.1	0.7	0.2	1.1	0.3
Cysteine	3	1.9	4.1	1.5	1.9	3.6
Isoleucine	3.5	3.3	4.1	2.9	3.1	3.1
Leucine	7.2	7.9	8.2	6.5	7.3	7.2
Phenylalanine	3.3	4.4	2.4	3.2	4.2	2.1
Lysine	8.6	8.1	8.5	8.1	6.9	7.6

[1] Results given are mean of duplicates and coefficient of variation was less than 5 %.

II.4.2- Fermentation of the wheat fractions by cecal microbiota

The wheat fractions produced with and without MEP-treatment were tested in *in vitro* fermentation assays using pooled cecum content of 10 4-week old broilers as inocula. The production of SCFA and lactic acid was quantified (Table II-4) and analysed statistically. The results were compared with non-supplemented culture medium (NC) and with FOS- and XOS-supplemented media both as positive controls. All the wheat fractions increased significantly ($P \leq 0.05$) the SCFA production and the lactate concentration when compared to the non-supplemented medium. The MEP-treated fractions increased significantly ($P \leq 0.05$) SCFA production when compared to MEP-untreated fractions. Branched SCFA (isobutyrate and isovalerate) were present in all samples but only in traces amounts (< 0.1 mM).

The MEP-treated LMw fraction showed the most important increase of total SCFA (+ 12.74 mM, corresponding to 42.4 %). It multiplied by 6.62 and 6.75 the butyrate and acetate concentrations, respectively, when compared to the non-supplemented medium, and by 2.8 and 1.3 respectively, when compared to MEP-untreated LMw. The MEP-treated LMw fraction allowed also increasing the propionate concentration to the same level as the positive controls FOS and XOS, and reaching higher level than MEP-untreated LMw (+17 %). For all MEP-treated fractions butyrate proportion represents up to 30 % of SCFA while it was only 15-18 % for MEP-untreated fractions.

Table II-4: *In vitro* short chain fatty acids (SCFA) and lactic acid production (mM) after fermentation of different wheat fractions at 38 °C for 24 h, under anaerobic conditions, using broiler cecal content as bacterial inoculum. Non-supplemented medium (NC), xylo-oligosaccharides (XOS) and fructo-oligosaccharides (FOS) supplemented media were used as controls. HMw: Ethanol-insoluble fraction of high molecular weight; LMw: ethanol-insoluble fraction of low molecular weight.

	NC	FOS	XOS	MEP-untreated			MEP-treated			SEM	P-value
				EIF	HMw	LMw	EIF	HMw	LMw		
Lactic acid	0.39^{e}	3.43^{bc}	3.37^{bc}	3.35^{c}	4.57^{a}	4.66^{a}	3.38^{bc}	2.63^{d}	3.76^{b}	0.23	0.000
Acetic acid	3.10^{h}	20.14^{b}	18.49^{c}	10.94^{f}	10.59^{f}	16.38^{d}	10.11^{g}	12.35^{e}	20.93^{a}	1.07	0.000
Propionic acid	1.15^{d}	5.56^{a}	5.25^{a}	0.94^{d}	1.36^{d}	4.45^{b}	2.09^{c}	2.12^{c}	5.22^{a}	0.36	0.000
Butyric acid	1.95^{h}	9.08^{b}	9.15^{b}	2.92^{g}	3.75^{f}	4.58^{e}	5.95^{d}	7.56^{c}	12.90^{a}	0.65	0.000
SCFA* Sum	6.59^{i}	38.21^{b}	36.26^{c}	18.14^{h}	20.27^{g}	30.07^{d}	21.53^{f}	24.66^{e}	42.81^{a}	2.11	0.000

$^{a\text{-}i}$ Values with different superscripts are significantly ($P \leq 0.05$) different from each other.

*Branched SCFA were detected in traces (<0.1 mM).

II.5 - Discussion

The enzymatic treatment allowed, not only solubilising more polysaccharides, but also decreasing their molecular weight. Before MEP-treatment, the HMw sub-fraction was essentially composed of arabinose and xylose arising from AX, and also contained a substantial amount of galactose coming from AGP. The average molecular weight and the intrinsic viscosity of the MEP-untreated HMw fraction were in the range typically observed for water-soluble wheat AX (Faurot et al., 1995; Dervilly et al., 2000) and in good agreement with the average degree of polymerization of the xylan backbone.

After MEP-treatment, the distribution of AX between HMw and LMw sub-fractions was shifted, with a much higher AX content of the MEP-treated LMw sub-fraction.

The MEP-treated HMw AX content indicated that some highly branched AX resisted the enzymatic attack as indicated by the higher A/X ratio determined for this fraction. Comparing the galactose content in the different fractions suggested that the MEP-treatment did not modify the AGp distribution. Molecular weight and DP determinations indicated a substantial depolymerization of AX. Quite large amounts of AX were recovered in the LMw fraction that exhibited a lower branching degree and reduced DP as a result of endo-xylanase action. Grain treatment with MEP increased the solubilisation of AX and reduced its Mw. Beyond the depolymerization effect; MEP-treated HMw sub-fraction exhibited a higher A/X ratio and MEP- treated LMw a lower one compared to MEP-untreated HMw AX.

To recapitulate, four fractions exhibiting large differences in composition, structure and Mw of AX were obtained and their effect on cecal microbiota fermentation was investigated *in vitro.*

The FOS and XOS used as positive controls in the *in vitro* fermentation are prebiotic oligosaccharides commercialised not only for animals but also for humans because of their ability to stimulate the growth of beneficial microbiota (Laparra and Sanz, 2010). Interestingly MEP-treated fractions and especially LMw fraction seem to have similar impact on the growth of cecal microbiota with even a greater impact on butyrate proportion. Both LMw fractions contained a high amount of glucose originating from starch (36 g/100 g of the MEP-untreated and 28 g/100 g of the MEP-treated fractions) that explains the increase of SCFA concentration between the HMw fractions and the LMw ones, as described before by Persia et al. (2002) and Wang et al. (2004). However, MEP-treated LMw produced significantly more SCFA, in particular butyrate, than the MEP-untreated one, which is probably due to its AX content (26.7 %) (Damen et al., 2011).

Butyrate has a known role on digestive health (Guilloteau et al., 2010) and is an important source of energy for the gastrointestinal epithelial cells that increases their proliferation and differentiation (Mariadason et al., 1999; Manzanilla et al., 2006). It also decreases lymphocyte proliferation in monogastrics *in vitro* (Kyner et al., 1976; Cavaglieri et al., 2003). The proliferation of butyrogenic bacteria in the ceca is known to protect against pathogenic microbiota (Andoh et al., 1999; Fernández-Rubio et al., 2009; Guilloteau et al., 2010).

The AX solubilisation efficiency of MEP depends on the initial quantity, structure and molecular weight of AX in wheat. It also depends on the enzymatic composition of the MEP, and Rovabio® Excel was already shown to be particularly efficient on wheat (Lafond et al., 2011, 2015; De La Mare et al., 2013). The MEP-treatment solubilised higher amounts of less branched AX that stimulated the cecal microbiota. Several *in vitro* and *in vivo* studies showed that polymeric AX are fermented 3 times slower than starch and produce mainly acetate, propionate and butyrate (Rycroft et al., 2001; Yu Lan et al., 2005; Lin et al., 2011). Depending on the polymerization and the substitution degrees of AX the proportion as well as the quantity of the SCFA produced vary (Broekaert et al., 2011). Many authors studied the effects of adding AX or arabinoxylo-oligosaccharides (AXOS) to monogastric diets and found that oligosaccharides with a degree of polymerization lower than 5 contribute to the proliferation of beneficial bacteria and improve microbial diversity (Van Craeyveld et al., 2008; Sanchez et al., 2009; Damen et al., 2011). The addition of AXOS derived from wheat AX selectively stimulates the presence of *bifidobacteria* in the ceca of chickens without affecting BWG, while AX polymers although promoting *bifidobacteria* exert negative impact on BWG due to their viscous properties (Courtin et al., 2008). Adding AXOS modifies the microbiota and promotes beneficial bacteria and also provides protection against infection (Eeckhaut et al., 2008) and thus improves digestive health. The degree of substitution of the oligosaccharides influences the fermentation speed, the lower the degree of substitution the faster the fermentation (Bedford and Cowieson, 2012). Linear unsubstituted xylo-oligosaccharides are rapidly fermented, whereas arabino-oligosaccharides are fermented more slowly (Kabel, 2002).

In this study AX fractions produced after enzymatic degradation of wheat clearly have a higher DP than reported in the literature for AXOS active on rat or human microbiota. However, MEP-treatment clearly increases the proportion of AX fraction with reduced Mw that have beneficial effect on the cecal microbiota by stimulating the production of butyrate.

The reduction of Mw compared to MEP-untreated AX has an impact on the fermentation and its products, but also eliminate the possible negative viscous properties of AX. MEP-treated LMw seems to have the greater impact either due to its lower DP and possibly to a lower degree of substitution by arabinose of the xylan backbone.

II.6 - Conclusion

The MEP-treatment of wheat increases the proportion of water-soluble AX with reduced molecular weight and did not affect AGP. The MEP-treated HMw and LMw fractions rich in AX stimulate the production of SCFA to same level as recognized prebiotics (FOS an XOS) when incubated *in vitro* with cecal microbiota. Interestingly, they promoted the production of SCFA, particularly acetate and butyrate. These results suggest that the beneficial action of MEP on broiler performance is notably mediated by the solubilizing and depolymerizing effect of endo-xylanase on wheat cell wall AX. This work also indicates that an extensive depolymerization of AX is not required to favour butyrate production by cecal microbiota. *In vivo* experiments using AX with intermediate chain length inducing no viscosity will allow establishing the microbiota shift in broilers. This should contribute to understand the mechanisms of the beneficial effects of in-feed enzymes on host digestive health and performance.

Chapter III

Production of wheat water-soluble fractions at pilot scale and their *in vitro* fermentation by broiler cecal microbiota.

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III.1- Abstract

During the last decades, there is an increasing interest in plant cell wall polysaccharides as sources of prebiotics or prebiotic-like substances in animal nutrition. In this study, an enzymatic degradation protocol was setup to produce wheat fractions at a pilot scale in order to use them *in vivo* to feed broilers. The products from enzymatic hydrolysis of wheat grain at a pilot scale were isolated and their ability to induce short-chain fatty acids (SCFA) was investigated using *in vitro* fermentation with cecal microbiota.

Water-soluble fractions were obtained following incubation without and with a multi-enzyme preparation (MEP) (2 mL/kg) containing essentially endo-xylanase and endo-glucanase activities. Sub-fractions were obtained by graded precipitation of the water-soluble extracts first with 65 % ethanol and then with 80 % ethanol. The MEP-treatment increased the amount of water-soluble arabinoxylan (AX), but decreased their molecular weight (Mw) and their degree of polymerization (DP). *In vitro* fermentation assays using broiler cecal content as inoculum demonstrated that low substituted AX with reduced molecular weight isolated after MEP-treatment yielded higher concentrations of SCFA, especially acetate and butyrate. The enhancement of butyrate production due to the fermentation of AX degradation products by cecal microbiota might be the main mechanism contributing to the beneficial effect of MEP on wheat-based diets.

Key words: Cereals, xylanase, non-starch polysaccharides, arabinoxylans, butyrate.

III.2- Introduction

There is an increasing interest of cell-wall polysaccharides as prebiotics in human and animal nutrition in the last decades. Wheat grain contains a large amount of non-starch polysaccharides (NSP) (12-16 % dry matter) mainly composed of arabinoxylans (AX). Their AX are constituted of (1,4)-β-linked-D-xylopyranosyl units partly substituted by α-L-arabinofuranosyl units at position *O-2* and/or *O-3* (Gruppen et al., 1992). Part of AX are water-soluble and when enzymatically degraded can act as prebiotic-like substances. In the previous study (Chapter II), water-soluble fractions produced by enzymatic treatment of wheat grain were sub-fractionated by size exclusion chromatography. Results showed that these fractions exert a prebiotic effect and increased short chain fatty acids (SCFA) production *in vitro* using cecal content as inoculum. In this section we setup an enzymatic degradation protocol at a pilot scale and a fractionation of the products to produce a large amount of the wheat-fractions. First, the sugar composition and some physico-chemical features of the pilot scale fractions were analysed. Second, the effects of these fractions were tested *in vitro* and compared to those produced at the lab scale in the previous study.

III.3- Materials and methods

III.3.1- Materials

The multi-enzyme preparation **(MEP)** Rovabio® Excel, produced by the fermentation of *Talaromyces versatilis* sp.nov. (previously named *Penicillium funiculosum)*, was provided in liquid form by Adisseo SAS (Commentry, France). MEP contains mainly endo-1,4-β–xylanase (5500 U/mL) and endo-1,3(4)-β-glucanase (7500 U/mL).

The wheat grain (cultivar Barok) harvested in 2013 was purchased from Euronutrition (St Symphorien, France).

MEP-treated HMw and LMw fractions were isolated by size exclusion chromatography of water extracts from whole grain wheat flour treated with a multi-enzyme preparation. MEP-untreated HMw and LMw are the fractions obtained without MEP-treatment (Chapter II).

Xylo-oligosaccharides (XOS), XOS95P, were purchased from Shandong Longlive Bio-technology CO.LTD (Yucheng, China). Fructo-oligosaccharides (FOS), Fibrulose® F97, were purchased from Cosucra (Warcoing, Belgium).

III.3.2- Preparation of the wheat fractions at pilot scale

III.3.2.1- Wheat pre-treatment

Wheat grains (50 kg) were decontaminated with successive baths of water, ethanol 70 % and sodium hypochlorite (NaOCl 0.4 %). They were then rinsed with water. Grains were dried in a stove at 40 °C for 24 h and finally ground in a grinder, equipped with a 2 mm grid (Forplex, Les Ateliers Réunis, Boulogne-Billancourt, France).

III.3.2.2- Setup of the enzymatic treatment of the wheat

In order to produce water-soluble fractions at a pilot scale, different incubation times were tested beforehand at the lab scale. Wheat whole grain flour (10 g) was incubated with Milli-Q water (30 mL) with (2 mL/kg) or without MEP for 6 h at 40 °C under gentle agitation in a water-bath orbital shaker at 60 rpm. Two mL samples were withdrawn from each treatment at 1 h interval. Neutral sugars composition and molecular weight of the samples were determined as described below.

III.3.2.3- Isolation of water-soluble extracts at a pilot scale

The whole grain flour (20 kg) was incubated with deionized water (60 L) with (40 mL) or without MEP for 3 h at 40 °C under agitation in a double-walled tank (Cartier S.A, Surgères, France) equipped with a helical stirrer. The slurry was decanted for 2 h at room temperature. One g/L of Celite® (Roth Sochiel E.U.R.L, Lauterbourg, France) was added to the supernatant to help the filtration, and the mixture was filtered with a hydraulic food-grade filter press (Seitz Orion, Bad Kreuznach, Germany). The filtrate was heated for 10 min at 95 °C in a 50 L homogenizer (COSIMIC- MP315, Carcassonne, France) to deactivate endo- and exogenous enzymes. The mixture was then cooled to 60 °C, mixed with 1 g/L of Celite® and filtered again to remove flocculated proteins. The clear supernatant was finally concentrated using the 50 L homogenizer connected to a vacuum generator unit (UT404P, Deltalab SMT, Carcassonne, France). The concentrated supernatants, referred to as WE-WSF for wheat+enzyme water-soluble fraction and WC-WSF for wheat control water-soluble fraction, were frozen (-20 °C) until further use.

III.3.2.4- Graded ethanol precipitation of WSF

Graded ethanol precipitation was used to separate polymeric fractions from the oligomers present in the soluble fractions resulting from water extraction in the presence or not of enzyme. Different ethanol ratios were tested as indicated in table III-1 to reach in a first step a final concentration in ethanol of 50 %, 60 %, 65 % and 70 %. After liquid/solid separation by centrifugation, the supernatant recovered from the first step of ethanol precipitation was mixed with ethanol 96 % to reach a final concentration of 80 % ethanol. Precipitates formed in ethanol 80 % were recovered by centrifugation and dried in a stove for 24 h at 40 °C. The amounts and neutral sugar compositions of the different fractions were determined.

Table III-1: Ethanol concentrations and the different ethanol ratios used in the 2 steps of the graded ethanol precipitation.

Step 1		Step 2 (Final concentration ethanol 80 %)
Ethanol concentration	Ethanol 96 % / WSF	Ethanol 96 % / step1 supernatant
50 %	1/1	2/1
60 %	1.5/1	1.5/2
65 %	2/1	1/1
70 %	3/1	1/2

The WSF obtained at pilot scale from treated and untreated grains were finally fractionated by adding 2 volumes of ethanol 96 % to 1 volume of the concentrated WSF to reach a final ethanol concentration of 65 %. The mixture was left overnight at 4 °C. The pellet was recovered by centrifugation at 3000 g for 30 min on a Sigma 6K10 centrifuge (Osterode am Harz, Germany), then washed twice with ethanol 80 % and with acetone, and finally dried for 24 h in stove at 40 °C. The fractions recovered from treated and untreated grains were named WE-1 (Wheat+enzyme) and WC-1 (wheat control) respectively.

The supernatant recovered after the ethanol 65 % precipitation was then mixed with ethanol 96 % (1/1 V/V ratio) to get a final ethanol concentration of 80 %. The mixture was left to precipitate overnight at 4 °C. The pellet was then collected by centrifugation at 11000 g for 20 min on a Jouan SR 12-22 centrifuge (Jouan industries S.A.S, Château-Gontier, France), dissolved in Milli-Q water and then freeze-dried. The fractions recovered from treated and untreated grains were named WE-2 and WC-2, respectively.

III.3.3- Characterization of isolated fractions

Molecular weight of the fractions produced during the different steps of the preparation setup was estimated by size exclusion chromatography. A Shodex OHpak SB-804 HQ column (8 mm × 300 mm, Shodex, Tokyo, Japan, fractionation domain for pullulan 5-400 kDa) eluted at 0.8 mL/min with a 50 mM sodium nitrate solution was used to analyse the samples from solubilization kinetic. A Shodex OHpak SB-802.5HQ column (8 mm × 300 mm, Shodex, Tokyo, Japan, fractionation domain for pullulan 0.5-10 kDa) eluted at 1 mL/min with a 50 mM sodium nitrate solution was used to analyse the samples from graded ethanol precipitation.

Fractions produced at the pilot scale were analysed for their neutral sugar composition, molecular weight using HPSEC, protein content and degree of polymerization of the xylan backbone as described in chapter II, section II.2.3.

Proton ^{1}H NMR spectra (400 MHz) were recorded at 60 °C on a Brücker AdvanceIII 400NB ARX spectrometer. The fractions were dissolved in deuterium oxide (D_2O; 10 mg/mL). Approximately 128 pulses were collected, the pulse repetition time was 4 s, and the pulse angle was 6 µs. The percentage of mono- and di-substitution was calculated from the signal of anomeric protons of arabinose and xylose residues according to Petersen et al. (2014).

III.3.4- *In vitro* fermentations of the wheat fractions

In vitro fermentations were conducted as described in chapter II, section II.2.4, using 4-weeks old broilers cecal content as inoculum. The SCFA concentration was measured as described in the same section.

III.3.5- Statistical analyses

One-way ANOVA followed by a post hoc Tukey's multiple comparison test was employed to analyse the data using IBM® SPSS® Statistics 22.0 software. Results are given as means. Statements of statistical significance were based on $P \leq 0.05$.

III.4- Results

III.4.1- Setup of the enzymatic treatment

Time course of degradation/solubilization was performed on Barok wheat. Samples were collected each hour and characterized for neutral sugar composition and Mw distribution. Figure III-1 shows the evolution of neutral sugar composition whereas figure III-2 shows the HPSEC profiles during the 6 h of incubation.

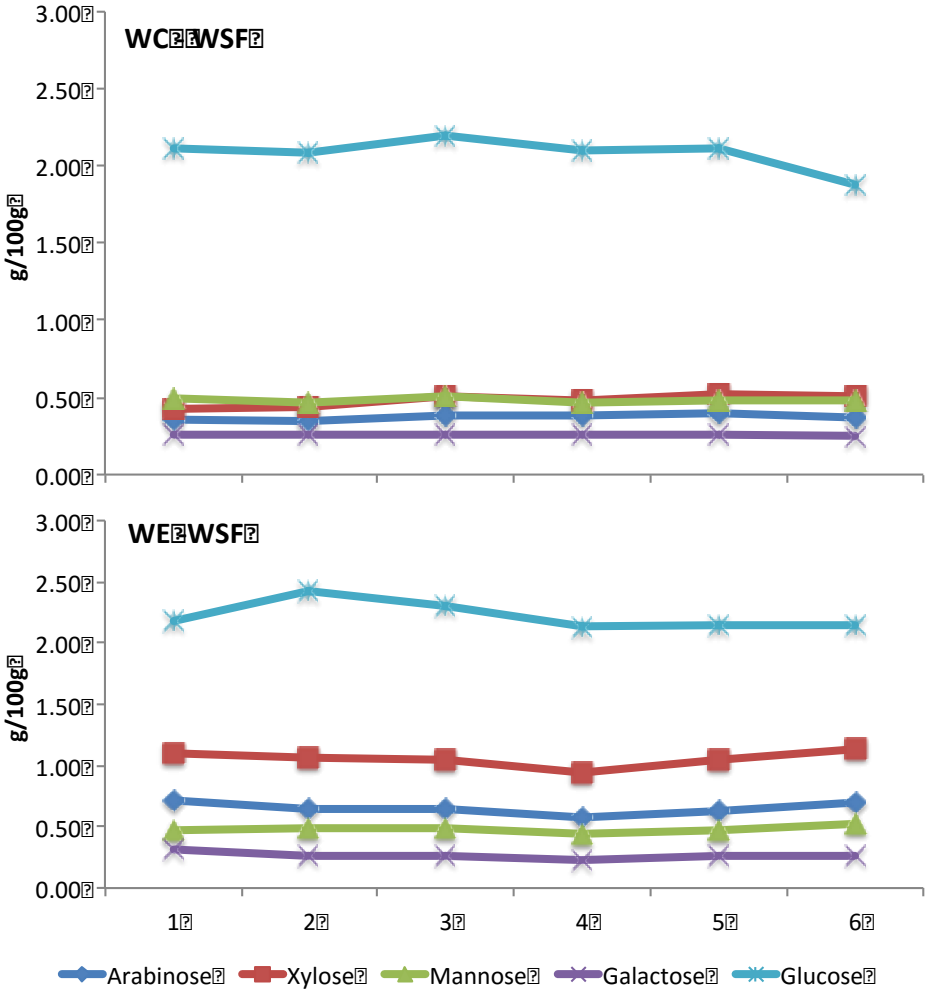

Figure III-1: Time course of the solubilizationof the neutral sugars of wheat+enzyme water-soluble fraction (WE-WSF) and the wheat control water-soluble fraction (WC-WSF).

The neutral sugar composition of the different samples each hour showed that glucose is the most solubilized sugar in all fractions with and without enzymatic treatment. The enzymatic degradation solubilized more sugars, mainly xylose and arabinose (x 2) already

after one hour of incubation. However, the amount of the different sugars did not evolve during the time course between 2 h and 6 h incubation.

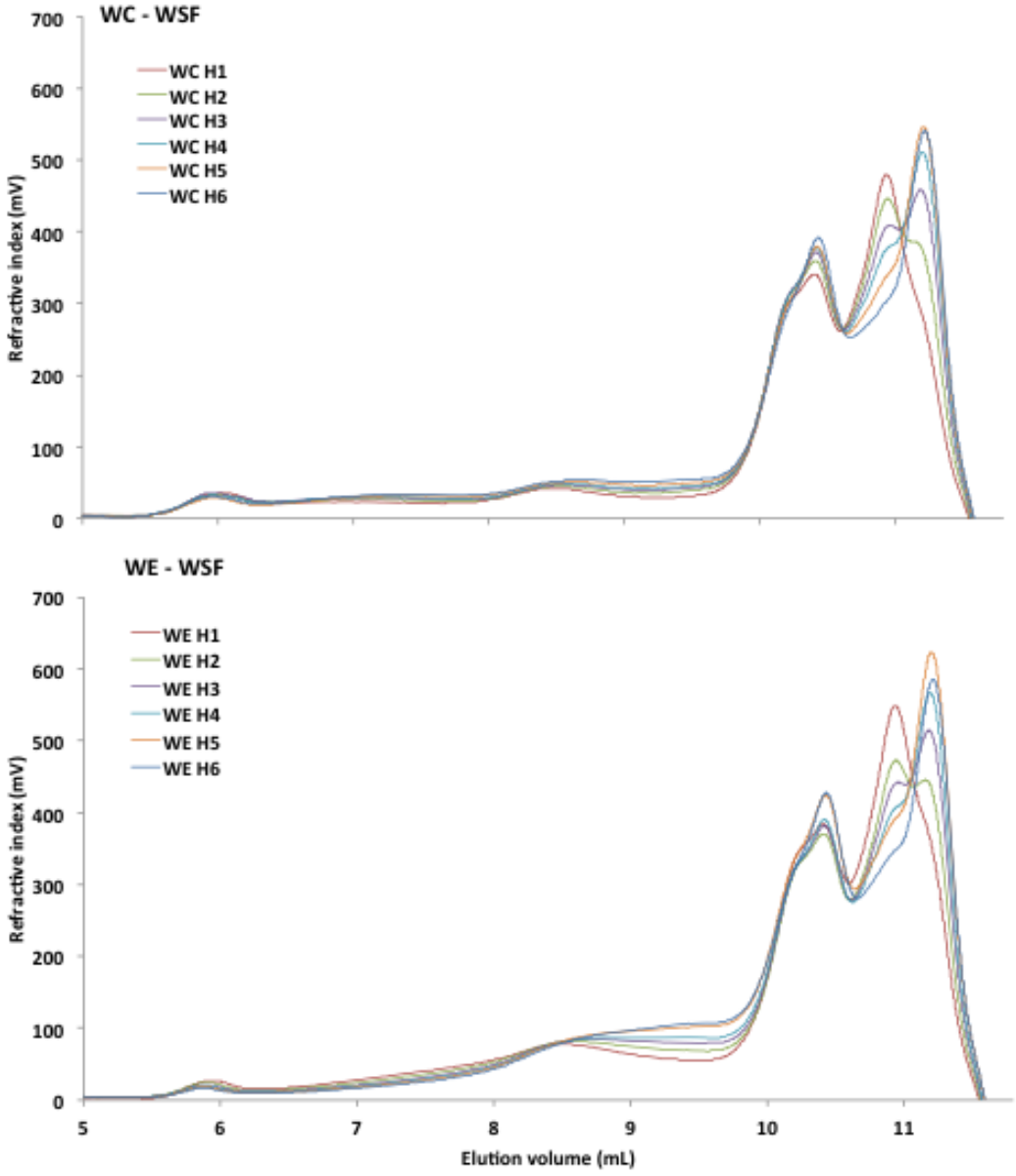

Figure III-2: Time course of HPSEC elution profiles of wheat+enzyme water-soluble fraction (WE-WSF) and wheat control water-soluble fraction (WC-WSF) using a Shodex, OHpak SB-804 HQ column.

The HPSEC elution profiles of the WC-WSF along incubation showed that AX eluting between 5.5 and 10 mL are divided into two sub-populations. The first one eluted between elution volume 5.5 and 6.5 mL and corresponded to AX with highest Mw. The

second population eluted between 8 and 10 mL and corresponded to AX of lower Mw. Other constituents with the lowest Mw eluted between 10.5 and 11.5 mL.

The elution profiles of WE-WSF showed similar sub-populations of AX. However, the AX shifted toward lower Mw since the proportion of AX eluting in the 8-10 mL range was higher than for WC-WSF and clearly increased with incubation time. However, the Mw as well as the amount of the different sugars remained stable from 3 to 6 h of incubation. According to these results the incubation time was fixed at 3h (Figure III-4).

Finally, the water extraction was carried out at pilot scale during 3 h at 40 °C with or without MEP. The fractions WC-WSF and WE-WSF were concentrated and frozen.

III.4.2- Setup of the ethanol fractionation

Water-soluble fractions produced at the pilot scale with MEP (WE-WSF) and without MEP (WC-WSF) were used to perform preliminary test of fractionation using ethanol precipitation. In the first step, various ethanol concentrations (50 %, 60 %, 65 % and 70 %) were used. In the second step, the ethanol concentration of the 1st step supernatant was brought to 80 % and ethanol-insoluble solids (EIS) were recovered.

The analysis of the different fractions (Table III-2) showed important differences in the amount and composition of the recovered fractions at each condition of ethanol precipitation. The amounts of the complementary fractions (with or without MEP-treatment) 0-50 % / 50-80 % ethanol and 0-70 % / 70-80 % ethanol were unbalanced. Without MEP-treatment 39.8 % of EIS (% dry matter) were recovered in 50 % ethanol and 77.5 % in 70 % ethanol. In these conditions 65 % ethanol precipitation allowed the highest recovery 55.53 g/100 g.

With MEP-treatment, only 12.3 % of the EIS were recovered in 50 % ethanol while up to 72 % were recovered in 70 % ethanol. The composition of the different fractions showed that AX amount recovered during the first ethanol precipitation increased according to ethanol concentration till 65 %.

Without enzymatic degradation, the A/X ratio was low after the 1st step of ethanol precipitation and high after the 2nd step. With enzymatic treatment, the A/X ratio was rather higher in the 1st fractions than in the 2nd ones. However, the difference between the 2 steps fractions was smaller. The A/X did not vary when ethanol ratio increased with and without MEP-treatment.

Table III-2: The amount and neutral sugar composition of the different control water-soluble fractions (WC-WSF) and MEP-treated water-soluble fractions (WE-WSF) insoluble at the different ethanol concentrations.

	WC-WSF							
	0-50	**50-80**	**0-60**	**60-80**	**0-65**	**65-80**	**0-70**	**70-80**
Amount[1]	131.1	198.3	183.7	230.5	191.7	196.7	278.1	80.9
Arabinose[2]	12.91	9.55	14.27	6.45	17.98	6.18	11.46	1.23
Xylose	30.3	7.02	29.66	2.28	35.66	1.73	17.45	0.78
Mannose	0.51	3.06	0.4	3.11	0.49	5.79	0.53	6.66
Galactose	0.42	5.58	0.57	6.36	1.98	6.62	4.31	1.05
Glucose	2.66	11.63	4.58	9.86	6.3	13.99	5.01	14.58
Total NS	**46.8**	**36.83**	**49.47**	**28.05**	**62.4**	**34.3**	**38.75**	**24.3**
Sum A+X[3]	42.92	12.66	43.53	4.28	52.25	3.28	25.89	1.28
Ratio A/X[4]	0.42	0.80	0.47	0.88	0.47	0.89	0.48	0.63
	WE-WSF							
	0-50	**50-80**	**0-60**	**60-80**	**0-65**	**65-80**	**0-70**	**70-80**
Amount[1]	34.9	248.6	77.5	207.6	133.2	193.4	233.2	90.6
Arabinose[2]	nd	14.44	12.61	12.27	16.52	8.48	15.06	3.3
Xylose	nd	24.97	21.5	21.3	30.96	14.4	24.8	8.67
Mannose	nd	1.27	0.57	1.72	0.35	2.44	0.58	2.82
Galactose	nd	4.23	0.93	4.94	1.77	4.81	4.55	0.77
Glucose	nd	7.7	6.63	8.25	7.64	8.34	6.41	9.38
Total NS		**52.61**	**42.24**	**48.47**	**57.22**	**38.46**	**51.39**	**24.93**
Sum A+X[3]	-	36.45	33.46	30.11	46.24	19.51	36.68	11.43
Ratio A/X[4]	-	0.46	0.56	0.41	0.49	0.36	0.48	0.32

[1] The amount of the insoluble fraction recovered from 10 mL of the corresponding WSF expressed in mg of dry matter

[2] g/100 g

[3] A+X: sum of arabinose and xylose corrected from arabinogalactan contribution

[4] A/X: arabinose/xylose ratio corrected from arabinogalactan contribution

nd: not determined

To investigate the molecular masses, a column with a smaller fractionation range allowing the separation of oligosaccharides (DP 1-10) in the 10-13 mL elution range was used. The HPSEC profiles of all the fractions (with and without MEP-treatment) indicated that no oligosaccharides were present, except for WE 0-50 %. For WC most of EIS isolated from 50 % up to 70 % of ethanol eluted in the 6-8 mL elution volume, indicating high Mw polymers. The elution profile of the fraction insoluble in ethanol 70-80 % was characterized by the absence of peak in the 6-8 mL region, attesting that AX were precipitated in 70 % ethanol as indicated by neutral sugar composition. The elution profiles of the fractions produced with MEP-treatment (WE) were similar but with a shift of 6-8 mL towards 8-9 mL indicating lower Mw of the polymers.

According to the different amounts of fractions recovered in the 2 steps of ethanol precipitation, the ethanol concentrations 0-65 % and 65-80 % were chosen for both treatments (with and without MEP). They allowed isolating large quantities of well-purified AX in the 0-65 % ethanol fractions. However, these fractions exhibited large differences in Mw as indicated by HPSEC chromatography profiles (Figure III-3). The EIS isolated in 65-80 % ethanol showed important differences in the composition of the different sugars between treatments, mainly arabinose and xylose.

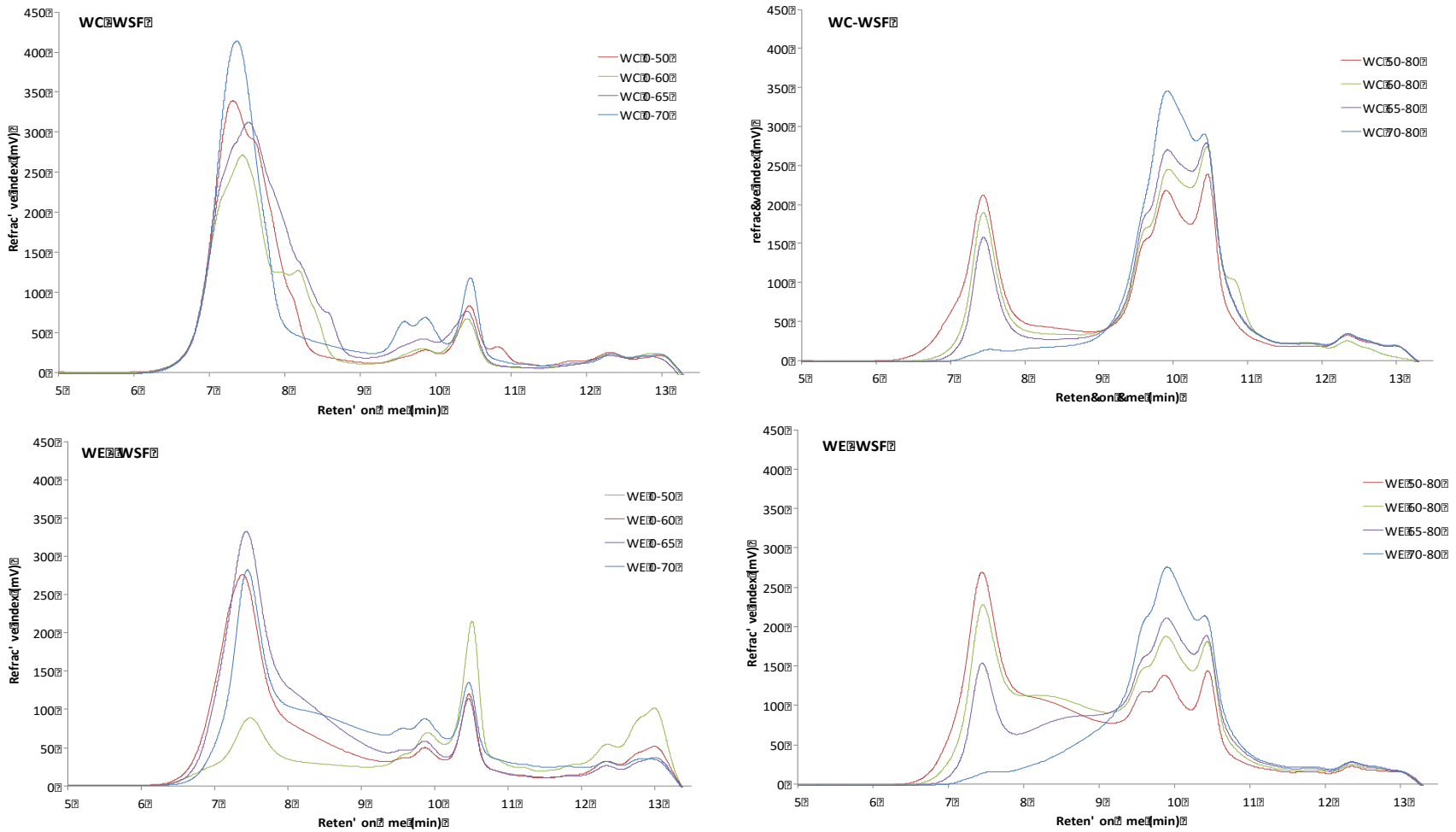

Figure III-3: HPSEC elution profiles of the wheat+enzyme water-soluble fractions (WE-WSF) and the wheat control water-soluble fractions (WC-WSF) precipitated at different ethanol concentrations, analysed on a Shodex, OHpak SB-804 HQ column.

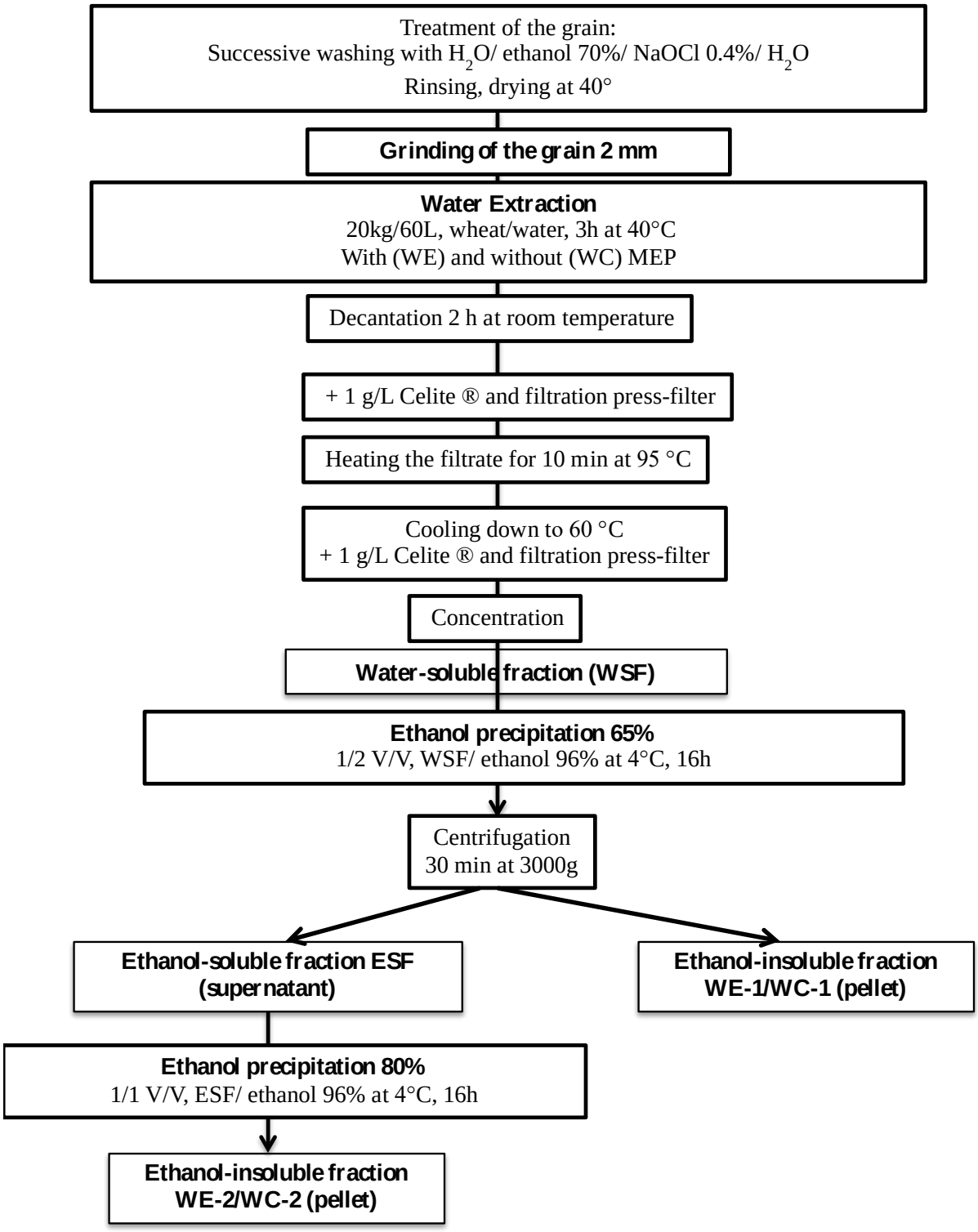

Figure III-4: Flow chart of extraction and fractionation procedure of the water-soluble fraction (WSF) with and without multi-enzyme preparation (MEP) to produce wheat+enzyme 1 and 2 (WE-1 and WE-2) and wheat control 1 and 2 (WC-1 and WC-2).

III.4.3- Characterization of the wheat fractions

Finally, the water extraction was carried out at pilot scale during 3 h at 40 °C with or without MEP as shown on figure III-4. The sub-fractions produced in the first step of ethanol precipitation were referred to as WE-1 (wheat+enzyme -1) and WC -1 (wheat control-1). The fractions recovered in the second step are referred to as WE-2 and WC-2 (Figure III-4). Sub-fractions WE-1 and WC-1 were mainly constituted of xylose (34.5 % and 32.2 %, respectively) and arabinose (19.3 % and 19.1 %, respectively) (Table III-3), in relation with the presence of AX. However, both fractions contained galactose, indicating the presence of arabinogalactan-protein (AGP), which contributed also to the arabinose content. A corrected A/X ratio and A+X sum were calculated taking into account the contribution of AGP to the arabinose content and assuming an average A/G ratio of 0.7 (Loosveld et al., 1998). The AX contents of WE-1 and WC-1 were similar (A+X= 50.8 % *vs.* 49.0 %) while the A/X was slightly lower for WE-1 (0.47 *vs.* 0.52). The glucose amounts in both fractions were also similar and originated from starch. The WE-1 fraction also contained 21 % proteins while WC-1 contained 14 %.

Table III-3*:* Characterization of sub-fractions isolated from WE-WSF and WC-WSF by ethanol precipitation (WC-1 and WE-1: ethanol 65 % insoluble fraction; WC-2 and WE-2: ethanol 65-80 % insoluble fraction)[1].

Fractions	**WC-1**	**WC-2**	**WE-1**	**WE-2**
Yield (g/kg of wheat)	5.8	5.2	6.25	5.15
Protein (g/100g)	14.0±0.2	30.0±0.1	21.0±0.1	33.0±0.1
Sugars (g/100g)				
Arabinose	19.1±1.6	6.6±0.7	19.3±1.7	7.0±1.1
Xylose	32.2±1.7	0.8±0.1	34.5±2.1	10.6±1.2
Mannose	0.2±0.1	3.3±0.1	0.5±0.1	2.5±0.1
Galactose	3.2±0.4	8.8±0.3	4.4±0.3	4.1±0.6
Glucose	5.2±0.5	12.1±0.5	5.4±0.6	7.6±0.4
Starch	4.6±0.4	7.5±0.4	5.3±0.3	3.5±0.2
β -glucan	0.3±0.1	4.4±0.1	0±0.1	3.5±0.1
Total	59.9±4.8	31.7±2.2	64.2±5.2	31.8±3.7
A+X[2]	49	1.2	50.8	14.8
A/X[3]	0.52±0.13	nd	0.47±0.12	0.39±0.11
DP	270±13	nd	54±7	29±5
Mw average (kDa)	176.8	nd	49.6	nd
Intrinsic viscosity (mL/g)	215.7	nd	54.1	nd

[1] Values are expressed as mean±sd. Four determinations were carried out/treatment. Rhamnose and fucose were detected as traces (<0.01 %). [2]A+X: sum of arabinose and xylose corrected from arabinogalactan contribution [3]A/X: arabinose/xylose ratio corrected from arabinogalactan contribution. nd: not determined

The WE-1 and WC-1 fractions had different Mw distributions (III-Figure 5-A). WC-1 eluted in the 12-20 mL elution range with a main population in the 12-17.5 mL range, corresponding to polymers of high hydrodynamic volume. A second population of lower hydrodynamic volume eluted in the 17.5-19.5 mL range, whereas WE-1 eluted mainly in the 16-20 mL range.

The macromolecular features determined for WE-1 and WC-1 confirmed the HPSEC profiles (Table III-2). The WC-1 fraction had a higher average Mw (176.8 kDa), intrinsic viscosity (215.7 mL/g) and degree of polymerization (270) (calculated for the xylan backbone) than the WE-1 fraction (49.6 kDa, 54.1 mL/g and 54, respectively).

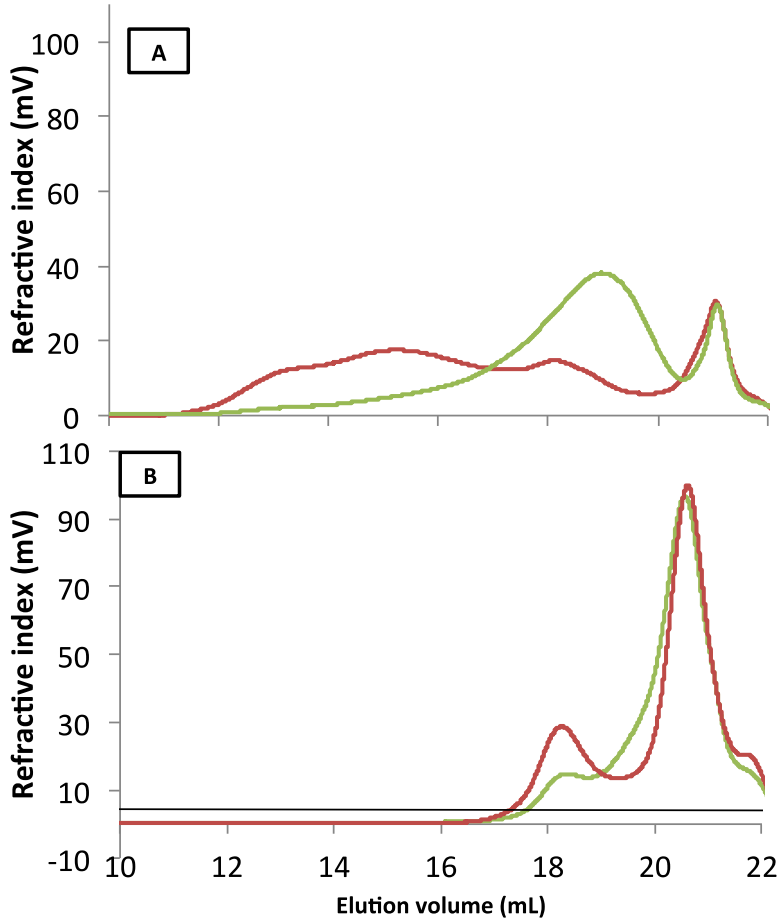

Figure III-5: HPSEC elution profiles of the WE-1 sub-fractions (green line) and WC-1 sub-fractions (red line) recovered in ethanol 65 % (A), and WE-2 sub-fractions (green line) and WC-2 sub-fractions (red line) recovered in ethanol 65-80 % (B).

NMR was used to investigate the AX structure of the WE-1 and WC-1 fractions (Figure III-6). The anomeric ^{1}H NMR signals of AX were found in spectral region of 4.30-5.40 ppm. The signal in the spectral region of 5.0-5.40 ppm is originated from the α-L-

arabinofuranosyl residues. The signal at 4.30–4.60 ppm is originated from β-D-xylopyranose residues. The identification and assignments of the individual structural motifs were made as described previously (Petersen et al., 2014). Both fractions had a very similar substitution pattern of the xylan backbone by arabinose residues, as already suggested by their similar A/X ratio. WE-1 contained 73 % of un-substituted, 13 % of mono-substituted and 14 % of di-substituted xylose, while WC-1 contained 72 %, 13 % and 15 %, respectively.

The characterization of sub-fractions WC-2 and WE-2 isolated in ethanol 65-80 % (Table III-3) showed that both fractions contained an important amount of galactose coming from AGP, glucose originating from mixed-linked β-glucan and starch, and a high level of proteins (>30 %). The WC-2 contained a very low amount of AX (1.3 %). In contrast, the WE-2 contained 14.8 % of AX. About 20 % of the total AX were recovered in the WE-2 fraction, that exhibited a lower degree of branching (A/X: 0.39) and a reduced DP (29) compared to AX recovered in the WE-1 fraction. However the measured DP indicated that oligosaccharides (DP< 10) were not formed upon MEP-treatment.

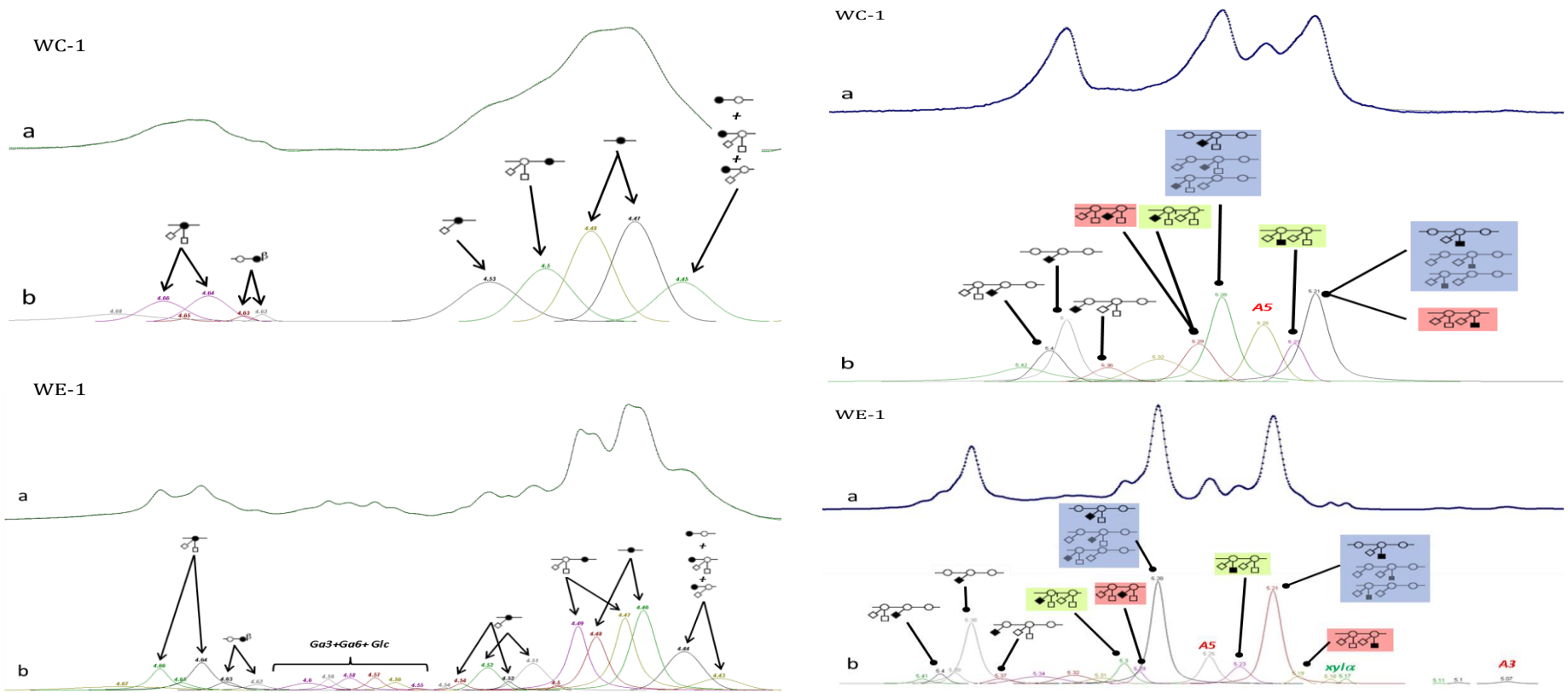

Figure III-6: ^{1}H NMR spectrum of anomeric ^{1}H signals of xylose zone (left) and the arabinose zone (right) of the AX of the fractions wheat+enzyme-1 (WE-1) and wheat control-1 (WC-1). (a) are the raw spectrums and (b) are the deconvoluted spectrum allocations for the different xylose or arabinose motifs. Schematic depiction of arabinoxylans: horizontal lines indicate β-1,4- glycosidic bonds between D-xylopyranosyl units (circles), diagonal lines indicate β–1,3- glycosidic bonds of arabinofuranosyl units (squares) to the xylan backbone and vertical lines indicate α-1,3-glycosidic bonds of arabinofuranosyl units to the xylan backbone**.** The black motif is the one detected. A5 is α-1,5 arabinofuranosyl from arabinogalactan (AGP). A3 is α-1,3 arabinofuranosyl from AGP.

III.4.4- *In vitro* fermentation of the wheat fractions by cecal microbiota

The effects of the wheat fractions produced with and without MEP-treatment at the pilot scale on broiler microbiota were investigated during *in vitro* fermentation assays. These have been carried out to compare the pilot fractions with the fractions produced at the lab scale in the previous study (Chapter II). The fractions were added to pooled cecum content of 10 4-weeks old broilers as inoculum and the concentration of SCFA and lactic acid (LA) was quantified (Table III-4) and analysed statistically. The results were compared with non-supplemented culture medium (negative control, NC) and with FOS- and XOS-supplemented media both as positive controls. All the wheat fractions increased significantly ($P \leq 0.05$) the SCFA and the LA concentrations when compared to the non-supplemented medium. Branched SCFA (isobutyrate and isovalerate) were present in all samples but only in trace amounts (< 0.1 mM).

In more details, the WE-1 increased SCFA and the LA significantly when compared to all treatments including FOS and XOS. It multiplied the total SCFA by 5.95, butyrate by 9.66, lactate by 8.96 and acetate by 4.66 when compared to the non-supplemented medium, and by 1.5, 1.78, 1.75 and 1.34 respectively, when compared to WC-1. It also induced the production of more SCFA and LA than lab scale MEP-treated LMw fraction. Both fractions increased the butyrate proportion to 20 % of the total SCFA while it represented only 16 % for the corresponding fractions without enzymatic treatment.

The WE-2 sub-fraction produced with enzymatic treatment at the pilot scale induced significantly higher concentration of total SCFA (x 1.12), butyrate (x 1.24), propionate (x 1.85) and acetate (x 1.10) when compared to WC-2.

Finally, the wheat fractions produced by enzymatic treatment either at the laboratory scale or at pilot scale increased significantly ($P \leq 0.05$) SCFA and LA production when compared to MEP-untreated fractions.

Table III-4: *In vitro* short chain fatty acids (SCFA) and lactate production (mM) after fermentation of different wheat fractions at 38 °C for 24 h, under anaerobic conditions, using 4-weeks old broilers cecal content as bacterial inoculum. Non-supplemented medium (negative control, NC), xylo-oligosaccharides (XOS) and fructo-oligosaccharides (FOS) supplemented media were used as controls. MEP-treated and MEP-untreated HMw: high molecular weight; LMw: low molecular weight; wheat fractions insoluble in ethanol 65 %: wheat+ enzyme (WE-1), wheat control (WC-1); and wheat fractions insoluble in ethanol 65-80 %: wheat+ enzyme (WE-2), wheat control (WC-2).

	Lactate	**Acetate**	**Propionate**	**Butyrate**	**SCFA Sum***
NC	0.55^{g}	5.48^{g}	0.12^{d}	0.87^{f}	7.01^{f}
FOS	2.94^{de}	24.67^{ab}	2.63^{ab}	7.58^{b}	37.82^{b}
XOS	3.27^{cd}	23.69^{b}	2.30^{b}	7.28^{b}	36.54^{b}
WC-1	2.81^{de}	18.96^{ef}	1.32^{c}	4.71^{d}	27.80^{d}
WC-2	3.93^{b}	20.08^{de}	1.34^{c}	3.53^{e}	28.88^{d}
WE-1	4.93^{a}	25.54^{a}	2.83^{a}	8.41^{a}	41.71^{a}
WE-2	3.59^{bc}	21.99^{c}	2.49^{ab}	4.40^{d}	32.47^{c}
MEP-untreated HMw	1.99^{f}	18.03^{f}	1.25^{c}	3.48^{d}	24.74^{e}
MEP-untreated LMw	2.13^{f}	19.35^{ef}	1.37^{c}	4.30^{e}	27.15^{e}
MEP-treated HMw	2.47^{ef}	20.79^{cd}	1.50^{c}	6.45^{c}	31.20^{c}
MEP-treated LMw	3.18^{cd}	23.82^{b}	2.23^{b}	7.22^{b}	36.45^{b}
SEM**	0.565	0.394	0.116	0.196	0.154
P-value	0.000	0.000	0.000	0.000	0.000

[a-g] Values within a column with different superscripts are significantly ($P \leq 0.05$) different from each other.

*Branched SCFA were detected as traces (< 0.1 mM).

**SEM: Standard error of the mean

III.5- Discussion

Graded ethanol precipitation allowed precipitating polysaccharides according to their DP and structure. In the case of AX and as shown by Dervilly et al. (2000), the arabinose/xylose (A/X) ratio and the amount of disubstituted xylose residues are positively correlated with the ethanol concentration. However, DP is negatively correlated with ethanol concentration (Swennen et al., 2005). In the present work, the 0-65 % and 65 %-80 % ethanol allowed producing balanced quantities of the fractions with fundamental differences on their structure and composition.

Water-soluble AX from wheat grain were fully recovered in WC-1 (untreated fraction). The average Mw (176.8 kDa) and the intrinsic viscosity (215.7 mL/g) of WC-1 were in the range observed for water-soluble wheat AX (Dervilly et al., 2000; Van Craeyveld et al., 2008). The MEP efficiency to solubilise AX depends on the initial quantity, structure and molecular weight of AX in wheat. It also depends on the enzymatic composition of the MEP, and Rovabio® Excel was already shown to be particularly efficient on wheat, and particularly on wheat AX (De La Mare et al., 2013; Lafond et al., 2011; 2015). The enzymatic treatment in the conditions applied to wheat grain (3 h and 40 °C) allowed solubilizing more proteins (+23 %) and sugars (+16 %, mainly originating from cell wall insoluble AX). This resulted in an increased amount of water-soluble AX with a concomitant reduction of their Mw. The evaluation of DP of the xylan backbone (270 and 54 for WC-1 and WE-1, respectively) confirmed this evolution. However, approximately 80 % of the water-soluble AX in the presence of enzymes precipitated in 65 % ethanol, indicating their polymer behaviour. The enzymes partially degraded the xylan backbone, resulting in AX with shortened chains in WE-1 compared with WC-1. Consequently, AX in WC-1 can be considered as long-chain AX whereas those in WE-1 were short-chain AX, all being polymers, as the absence of oligosaccharides in these fractions was demonstrated.

To recapitulate, four fractions exhibiting large differences in composition, structure and Mw of AX were obtained. Next, their effects on cecal microbiota fermentation were investigated *in vitro.*

The FOS and XOS were used as positive controls in the *in vitro* fermentation since their prebiotic effects have been demonstrated not only for animals (Swanson et al., 2002; De Maesschalck et al., 2015) but also for humans (Laparra and Sanz, 2010). The fractions produced at lab scale (Chapter II) resulted in different SCFA productions when fermented *in*

vitro, with the highest SCFA amount and increased butyrate proportion produced in the presence of the MEP-treated LMw fraction.

MEP-treated fractions prepared at pilot scale and especially the WE-1 seem to have similar impact on the growth of cecal microbiota with even a greater impact on butyrate proportion. The WE-1 was mainly composed of AX and increased significantly the SCFA concentration, which suggested that AX were responsible for this effect. The MEP-treatment solubilised higher amounts of less branched short-chain AX that stimulated the cecal microbiota. Several *in vitro* and *in vivo* studies showed that polymeric AX are fermented 3 times slower than starch and produced mainly acetate, propionate and butyrate (Rycroft et al., 2001; Lan et al., 2005b; Lin et al., 2011). As reviewed by Broekaert et al. (2011), the SCFA production increases when polymerization and substitution degrees of AX decrease.

Though, the MEP-treated LMw fraction contained an important amount of starch that partially explain the significant SCFA production as described before by Persia et al. (2002) and Wang et al. (2004). Still, this fraction comprised an important content of AX (26.7 %) that supposes a complementary effect between starch and AX to stimulate cecal microbiota (Damen et al., 2011). Nevertheless, both MEP-treated LMw and WE-1 increased significantly the butyrate proportion.

As reviewed by Guilloteau et al. (2010) butyrate has a key role on digestive health and is an important source of energy for the gastrointestinal epithelial cells as it increases their proliferation and differentiation (Mariadason et al., 1999; Manzanilla et al., 2006). The proliferation of butyrogenic bacteria in the cecum is known to protect against pathogenic microbiota (Andoh et al., 1999; Fernández-Rubio et al., 2009; Guilloteau et al., 2010). The DP of the oligosaccharides influences the fermentation speed, the lower the DP the faster the fermentation (Bedford and Cowieson, 2012). Accordingly, XOS are rapidly fermented, whereas arabino-xylo-oligosaccharides (AXOS) are fermented more slowly (Kabel, 2002).

In this study AX fractions produced by enzymatic degradation of wheat clearly have a higher DP than reported in the literature for AXOS active on rat or human microbiota (Courtin et al., 2008; Van Craeyveld et al., 2008). Besides, MEP-treatment clearly increases the proportion of AX with reduced Mw that have beneficial effects on the cecal microbiota by stimulating the production of butyrate.

III.6- Conclusion

The MEP-treatment of wheat increases the proportion of water-soluble AX with reduced molecular weight and degree of polymerization. The fractions produced at the pilot scale by graded ethanol precipitation revealed structural and chemical differences when compared to those fractionated by size exclusion chromatography. However, WE-1 stimulates the production of SCFA to a higher level than MEP-treated LMw fraction, FOS and XOS. It promoted particularly the production of acetate and butyrate. In this study we were able to produce sufficient quantities of water-soluble AX with and without MEP-treatment having a prebiotic-like behaviour *in vitro.* The next step of the study will be to investigate, *in vivo,* the effect of these AX with intermediate chain length on animal performance and intestinal health. This should contribute to understand the mechanisms of the beneficial effects of in-feed enzymes on host digestive health and performance.

Chapter IV

Short-chain arabinoxylans prepared from enzymatically treated wheat grain exert prebiotic effects during the broiler starter period

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Key words: T-lymphocyte, L-cell, butyrate, *Lachnospiraceae, Enterococcaceae*

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IV.1- Abstract

Carbohydrate-degrading multi-enzyme preparations (MEP) are used to improve broiler performances. Their mode of action is complex and not fully understood. In this study we compared the effect of polysaccharide fractions isolated at the pilot scale from wheat grain incubated with (WE) and without (WC) MEP. The fractions were incorporated in a wheat-based diet (0.1% w/w) to feed Ross PM3 broilers and compared with a non-supplemented control group (NC). The body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) until day 14 were determined. At day 14, ileal and cecal contents and tissue samples were collected from euthanized animals. The MEP treatment increased the amount of water-soluble arabinoxylans (AX) and reduced their molecular weight while retaining their polymer behavior. The WE fraction significantly ($P<0.05$) increased feed intake by 13.8% and BWG by 14.7% during the first week post-hatch when compared to NC. No significant effect on FCR was recorded during the 2 weeks trial. The WE increased the abundance of *Enterococcus durans* and *Candidatus arthromitus* in the ileum and of bacteria within the *Lachnospiraceae* and *Ruminococcaceae* families, containing abundant butyrate-producing bacteria, in the ceca. It also increased the concentration of short-chain fatty acids (SCFA) in the ceca (butyrate and acetate), decreased the T-lymphocyte infiltration in the cecal and ileal mucosa and increased the glucagon-like-peptide-2 (GLP-2)-producing L-cell density in the ileal epithelium compared with WC and NC. No significant effects were observed on villi length.

These results showed that AX present in the WE fraction altered the microbiota composition towards butyrate producers in the ceca. Butyrate may be responsible for the reduction of inflammation as suggested by the decrease in T-lymphocyte infiltration, which may explain the higher feed intake leading to improve animal growth.

IV.2- Introduction

Wheat is commonly used as the major cereal in poultry diets, especially in Europe. The metabolisable energy content of cereal grain is known to vary according to its non-starch polysaccharide (NSP) content and structure (Annison, 1991; Choct and Annison, 1992; Svihus and Gullord, 2002). Wheat contains up to 16% NSP, mainly arabinoxylans (AX) (Saulnier et al., 2012). In the starchy endosperm, AX constitute up to 70% of the cell walls (Saulnier et al., 2007). The water-extractable fraction of AX has recognized anti-nutritional effects, inducing high viscosity of the intestinal content and increasing intestinal inflammation (Austin et al., 1999; Bao and Choct, 2010). NSP-degrading enzymes, especially endo-1,4-β–xylanases (further referred to as xylanases) and endo-1,3(4)-β-glucanases (further referred to as glucanases), are used in poultry diets to reduce these effects and thereby improve animal performance. It is commonly accepted that these enzymes exert their beneficial effects by reducing the viscosity of the intestinal content and by releasing oligosaccharides from plant cell wall polysaccharides in the digestive tract of the animals (Mathlouthi et al., 2002a; b; Choct et al., 2004; Mushtaq et al., 2006; Bozkurt et al., 2010; Lee et al., 2010; Masey O'Neill et al., 2014). The prebiotic effects of arabino-xylo-oligosaccharides (AXOS) from wheat bran (accounting for 0.25 % of the dry matter) and their impact on broiler digestive health have been established (Courtin et al., 2008). Prebiotics are selectively fermented and allow specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confer benefits upon host well-being and health (Wang, 2009). Dietary fibers such as AX, AXOS and XOS showed prebiotic effects when used as feed additives, though they are not yet recognized as "prebiotics". In the literature, these are referred to as prebiotic-like compounds. However, to the best of our knowledge, the effect of pretreating wheat grain with NSP-degrading enzymes to produce prebiotic-like compounds have not been studied *in vivo* in broilers.

The first two weeks post-hatch are critical in the life of the broilers. During this starter period, the birds are particularly susceptible to the harmful effects of NSP (Uni et al., 1999; Sklan, 2001). This can be explained partly by the fact that digestive functions are not fully developed in young chickens and that the microbiota is not settled at hatch and thus does not aid in degrading the soluble NSPs (Jin et al., 1998; Maiorka et al., 2006). According to some authors, the broiler microbiota could reach a stable dynamic state after 2 weeks post-hatch (Barnes, 1979; Lumpkins, 2007; Wei et al., 2013). Environment and nutrition are among the major factors influencing the broiler development (Noy et al., 1998; Uni, 2006; Maiorka et

al., 2006) and microbiota (Koutsos and Arias, 2006; Torok et al., 2009). An additional factor influencing performance is the immune response, which can confer protection against infections but also decrease performance when the gut wall is heavily inflamed (Teirlynck et al., 2009b; Bao and Choct, 2010; Van De Wiele et al., 2011; Lawley and Walker, 2013). Finally, performance also depends on the absorption capacity of the intestinal mucosa and thus on the maturation and development of the small intestinal villi. Intestinal enterocyte proliferation and maturation are influenced by the hormone glucagon-like-peptide 2 (GLP-2) produced by enteroendocrine L-cells in the intestinal epithelium (Shousha et al., 2007).

We previously showed that AX polysaccharides enhanced short chain fatty acids (SCFA) production, and especially butyrate, during *in vitro* fermentation with a broiler microbiota compared with the oligosaccharides xylo-oligosaccharides (XOS) and fructo-oligosaccharides (FOS) (Yacoubi et al., 2016). The effect was markedly impacted by the chain length of AX. Therefore, we now investigate the effect of AX with different chain lengths *in vivo*. We treated wheat grain with enzymes and incorporated the degradation products to the diet at a level compatible with the extent of solubilisation that could occur *in vivo*. For this, water-soluble fractions were produced by the treatment of wheat grain with or without a multi-enzyme preparation (MEP) containing xylanases and glucanases and further purified by ethanol precipitation. The chemical composition and physicochemical features of these fractions were determined. They were further included in a wheat-based broiler starter feed at 0.1 %, corresponding to a 20-25 % increase of the amount of water-soluble AX intrinsically provided by the wheat in the feed. The effects on animal performance, T-lymphocyte infiltration in the gut wall, L-cell density, intestinal microbiota composition and SCFA concentration were investigated during the starter period.

IV.3- Materials and Methods

IV.3.1- Materials

The multi-enzyme preparation **(MEP)** Rovabio® Excel, produced by the fermentation of *Talaromyces versatilis* sp.nov. (previously named *Penicillium funiculosum)*, was provided in liquid form by Adisseo SAS (Commentry, France). MEP contains mainly xylanase (5500 U/mL) and glucanase (7500 U/mL).

The wheat grain (cultivar Barok) was harvested in 2013 and purchased from Euronutrition (St Symphorien, France). This cultivar was used to isolate fractions and to prepare the basal diet of the broiler trial.

The WE (wheat+enzyme) fraction was isolated by ethanol precipitation in ethanol 65 % of water extracts from whole grain wheat flour treated with a multi-enzyme preparation. The WC fraction obtained without MEP-treatment (Chapter III).

IV.3.2- Animals and housing

The *in vivo* trial was approved by the regional ethic committee of Auvergne (France) approval number F 03 159-4. A total of 288 1-day-old Ross PM3 male broilers were divided into 3 groups of 96 animals. Each treatment group comprised 8 pens of 12 animals. The broilers were reared in floor pens on wood shavings at a density of 8 birds/m^2. The lighting programme was as follows: from day 1 to 7, 23 hours of light and 1 hour of darkness and from day 7 to day 14, 18 hours of light and 6 hours of darkness. The temperature was maintained at 32 °C for the first 5 days and then gradually reduced until 22 °C according to EU husbandry management practices.

IV.3.3- Experimental design and feed composition

A starter feed was given during the entire trial (14 days). The same basal experimental wheat-based diet was given to all the birds. Its composition is shown in table IV-1. The diet was free of antibiotics and anticoccidials. The birds were separated in three groups, i.e., one non-supplemented control (NC) and 2 treatments comprising the 2 fractions (WE and WC) incorporated into the basal diet at 0.1%. At day 7 and 14, the animals were weighed individually and the feed intake was measured per pen to calculate the feed conversion ratio (FCR) and the body weight gain (BWG). At day 14, 3 animals/pen with a live body weight in the range of the group average body weight were euthanized using CO_2. This led to 24 animals of each treatment group. Tissue samples of approximately 1 cm from the distal end of the caecum and at the level of Meckel's diverticulum of the ileum were taken immediately post-euthanasia, placed in RNALater (Sigma-Aldrich, St, Louis, MO, USA) and stored at -20 °C. The caecum and ileum content were collected in two Eppendorf tubes (2 mL). One tube was weighed, immediately mixed with distilled water at a ratio of 2 mL/g and stored at -20 °C for SCFA and lactic acid analyses. The second tube was stored at -80 °C for bacterial DNA extraction.

Table IV-1: Feed composition of the basal experimental diets used in the *in vivo* trial.

Ingredient (%)	**Starter diet (Day 1-14)**
Wheat	44.000
Rye	5.000
Soybean meal 48[1]	23.300
Soybeans	7.500
Sunflower meal 27[1]	2.500
Rapeseed meal 36[1]	7.500
Animal fat	3.900
Soy oil	2.800
Mineral-vitamin premix[2]	1.000
$CaCO_3$	0.550
Dicalcium phosphate	0.900
NaCl	0.210
Na-bicarbonate	0.100
L-Lys-HCl	0.139
DL-Methionine	0.500
L-threonine	0.037
Phytase[3]	0.020
Nutrient composition[4]	
Crude protein (%)	23.00
Crude fat (%)	10.23
Non-soluble polysaccharides (%)	13.87
Metabolisable energy (Kcal/kg)	2800
Dig. Lysine (%)	1.12
Dig. Methionine (%)	0.59
Dig. Sulfur amino acids (TSAA) (%)	1.1
Dig. Threonine (%)	0.73
Dig. Valine (%)	0.84
Ca (%)	0.85
Available Phosphorus (%)	0.40
Total Phosphorus (%)	0.55

[1] % crude protein content

[2]Vitamin–mineral premix provided per kg of complete diet: Cu, 15 mg; Fe, 70 mg; Mn, 62mg; Zn, 80 mg; I, 0.6 mg; Se, 0.2 mg; vitamin A, 10 000 IU; vitamin D3, 1000 IU; vitamin E, 100 IU; vitamin K, 2 mg; vitamin B12, 15 mg; riboflavin, 5 mg; nicotinic acid, 12 mg; pantothenic acid, 10 mg; choline chloride, 500 mg; biotin, 200 mg; folic acid, 5 mg; vitamin B1, 2 mg; vitamin B6, 3 mg.

[3] Phytase (500 FTU units/kg) produced by fermentation of *Schizosaccharomyces pombe.*

[4] Determined values using the Precise Nutritional Evaluation (PNE) service of Adisseo based on Near Infra Red spectroscopy (NIR) technology.

IV.3.4- Quantification of SCFA

The concentration of SCFA in the cecal and ileal content samples was determined by gas chromatography as previously described (Schäfer, 1994; Zhao et al., 2006). The method consists of sample extraction in oxalic acid 0.5 M followed by direct injection on the gas chromatograph. The LA concentration was determined using a D/L-Lactic acid kit (ref 023, Biosentec, Toulouse, France).

IV.3.5- Immunohistochemical examinations

RNALater-fixed tissues of the ileum and caecum of 24 birds per group were embedded in paraffin, and 5 µm sections were cut and stained with haematoxylin and eosin. The villus length was measured in the ileum by random measurement of 10 villi per section and per animal using image analysis software (Leica Application Suite LAS V3, Wetzlar, Germany) as described previously (Teirlynck et al., 2009b). Only intact villi were measured, *i.e.*, villi for which the tip as well as the base were in the plane of the section.

To quantify the infiltration of T-lymphocytes in the caecum and ileum, immunohistochemistry was performed as described previously (Van Immerseel et al., 2002) using a monoclonal antibody targeting CD3 (Dako, Glostrup, Denmark). The number of T-lymphocytes in the different sections was evaluated using image analysis software (Leica Application Suite LAS V3, Wetzlar, Germany) measuring the average area percentage occupied by labelled cells from 10 microscopic fields per tissue and per animal at a magnification of 400x. The results were expressed as the percent area occupied by labelled cells.

To detect L-cells, we used a polyclonal rabbit anti-human GLP-2 antibody (Phoenix Pharmaceuticals Inc., Burlingame, USA) for immunohistochemical staining. The number of labelled cells and the area of 10 villi and 30 crypts per sample and per animal were measured in the ileum using the image analysis software LAS V3 (Leica Application Suite, Wetzlar, Germany). The L-cells were expressed as the number of cells/ mm^2 of mucosal tissue.

IV.3.6- Microbiota characterization

IV.3.6.1- DNA extraction

The DNA was extracted from cecal and ileal content using the CTAB method as described previously (Griffiths et al., 2000). The samples (100 mg) were mixed with 0.5 g of unwashed glass beads (Sigma-Aldrich, 117 St. Louis, United States), 0.5 mL of CTAB buffer (hexadecyltrimethylammonium bromide, 5 % w/v, 0.35 M NaCl, and 120 mM K_2HPO_4) and

0.5 of mL phenol-chloroform-isoamyl alcohol mixture (25:24:1) (Sigma-Aldrich, St. Louis, United States) in a 2 mL destruction tube. The samples were shaken 3 times for 1 min using a bead-beater (FastPrep 24^{TM} 5G, MP Biomedicals, Solon, Ohio, USA) at 5000 rpm with 30 seconds of rest between shakings. After centrifugation (10 min, 6000 g), 0.3 mL of the supernatant was transferred to a new tube. The rest of the tube content was re-extracted with 0.25 mL CTAB buffer and homogenized with a bead-beater. The samples were centrifuged for 10 min at 6000 g, and 0.3 mL of supernatant was added to the first 0.3 mL of supernatant. The phenol was removed by adding an equal volume of chloroform-isoamyl alcohol (24:1) (Sigma-Aldrich, St. Louis, United States) and a short spin. The aqueous phase was transferred to a new tube. The nucleic acids were precipitated with 1.2 mL of PEG-6000 solution (poly-ethylene-glycol 30 % w/v, 1.6 M NaCl) for 2 h at room temperature. After centrifugation (20 min, 13000 g), the pellet was rinsed with 1 mL of ice-cold ethanol 70%. The pellet was dried and dissolved in 0.1 mL of RNA-free water (VWR, Leuven, Belgium).

IV.3.6.2- 16S rRNA sequencing

The DNA quantity and quality were assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and an Agilent 2100 Bioanalyzer according to the method described previously (Patterson et al., 2006). According to the DNA quantity and quality 10 samples per group were chosen to perform the 16S sequencing. The 16S rRNA sequencing using MiSeq 250-pb technology from Illumina was performed at the GenoToul Genomics and Transcriptomics facility (Auzeville, France).

The primers used in PCR 1 were 515F (5'CTTTCCCTACACGACGCTCTTCCGATCTGTGYCAGCMGCCGCGGTA) and 928R (5'GGAGTTCAGACGTGTGCTCTTCCGATCTCCCCGYCAATTCMTTTRAGT) targeting the hypervariable 16S rRNA V4-V5 region. The amplification mix contained 5U of FastStart high fidelity polymerase (Roche Diagnostics, Vilvoorde, Belgium), 8µL of dNTP mix 250µM (Eurogentec, Liège, Belgium), 2 µL of each primer (20µM) and 100 ng of genomic DNA in a volume of 100 µl. The thermocycling conditions consisted of a denaturation at 94 °C for 2 min followed by 30 cycles at 94 °C for 60 s, 65 °C for 40 s, and 72 °C for 30 s and a final elongation step of 10 min at 72 °C. These amplifications were performed on an Ep Master System gradient apparatus (Eppendorf, Hamburg, Germany). Subsequently, the DNA was purified using HighPrep™ PCR (MagBio Genomics Inc, Gaithersburg, USA) following the manufacturer's protocol. Single multiplexing was

performed using a 6-bp index, which was added during a second PCR with 12 cycles using the forward primer P5 (5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC) and the reverse primer P7 (5'CAAGCAGAAGACGGCATACGAGAT-index-GTGACTGGAGTTCAGACGTGT). The PCR 2 products were purified, and the quality and the fragment length were controlled using an Agilent DNA 7500 DNA chip (Agilent Technologies, Santa Clara, USA) following the manufacturer's protocol. The resulting products were purified and loaded onto an Illumina MiSeq cartridge according to the manufacturer's instructions (Illumina Inc., San Diego, CA). The quality of the run was internally checked using control libraries generated from the PhiX virus (Illumina PhiX control; Illumina Inc., San Diego, CA) as previously described (Drouilhet et al., 2016).

The resulting sequences were pre-clustered and then independently divided into operational taxonomic units (OTU). Singletons were eliminated, and the non-redundancy of the OTU was verified. The phylogenetic affiliation of one representative sequence per OTU was performed using the LTP database (www.arb-silva.de/projects/living-tree) using the pipeline FROGS of the platform Galaxy (www.sigenae-workbench.toulouse.inra.fr).

IV.3.7- Statistical analyses

The data were analysed with IBM® SPSS® Statistics 22.0 software. The Shapiro–Wilks test was used to test the normal distribution of the data. The results are given as the means ± SD. The sugar composition, AX content, A/X and DP were analysed using one-way ANOVA. The BWG, FCR, feed intake (FI), villus length, L-cell count and T-lymphocyte infiltration were subjected to repeated measure analysis of variance with the general linear model of SPSS followed by a post hoc Tukey's multiple comparison test. The statistical power was ≥ 80 %. Statements of statistical significance were based on $P \leq 0.05$.

The pyrosequencing data were analysed using the Phyloseq package (McMurdie and Holmes, 2013) in R software (Version 3.2.3). The α-diversity of the cecal and ileal microbiota, defined as the species richness within each sample (broiler), was measured using the Shannon index. The Shannon index (H') was calculated as $H' = -\sum pi \text{ Ln } pi$, where pi is the proportion of individuals found in the i^{th} OTU. Higher values indicate greater diversity, with 0=complete homogeneity.

4- Results

IV.4.1- Broiler performances: body weight gain, feed intake and feed conversion ratio

Three groups of 96 animals were included in the trial: 2 treatment groups (WE and WC) and a negative control (NC). The fractions were incorporated at 0.1 % in a wheat-based diet during the two-week *in vivo* trial.

Adding WE to the diet significantly increased the feed intake (FI) during the entire trial ($P \leq 0.05$) compared with WC and NC (Table IV-2). In-feed administration of WE also significantly ($P \leq 0.05$) increased BWG by 14.7 % after the first week post-hatch and by 7.8 % throughout the two weeks compared with NC.

Table IV-2: Effect of dietary supplementation of 0.1 % WC (MEP-untreated) and WE (MEP-treated) fractions to a wheat-based diet on feed intake (FI), body weight gain (BWG) and feed conversion ratio (FCR). MEP: multi-enzyme preparation.

Parameters[1]	NC	WC	WE	P-values
FI (g/bird)				
0-7 d	130^{b} ±10	130^{b} ±18	148^{a} ±12	0.000
8-14 d	417^{b} ±20	418^{b} ±27	443^{a} ±21	0.000
0-14 d	547^{b} ±29	559^{b} ±33	591^{a} ±31	0.000
BWG (g/bird)				
0-7 d	109^{b} ±8	110^{b}±8	125^{a} ±8	0.000
8-14 d	316^{b} ±20	319^{b}±20	333^{a} ±21	0.001
0-14 d	425^{b} ±26	430^{b}±25	458^{a} ±26	0.000
FCR				
0-7 d	1.19^{a} ±0.20	1.19^{a} ±0.27	1.18^{a} ±0.14	0.241
8-14 d	1.32^{a} ±0.43	1.31^{a} ±0.32	1.33^{a} ±0.30	0.429
0-14 d	1.29^{a} ±0.18	1.30^{a} ±0.19	1.29^{a} ±0.18	0.397

[1] Raw data (n=96, for BWG and n=8 for FI and FCR) were analysed using one-way ANOVA. Values are expressed as mean ±SD.

[a and b] Values with different superscripts are significantly ($P<0.05$) different from each other within each row.

4.2- Gut morphology and immunohistochemistry

The density of L-cells/mm^2 was significantly higher in the ileal crypt and villus epithelium (Table IV-3) of the chickens receiving a diet supplemented with WE compared with WC and NC.

Adding WE to the diet significantly decreased the T-lymphocyte infiltration in the ileum (8.14 % of labeled area *vs*. 15.86 %) and in the cecum (6.96 % of labeled area *vs*. 15.68 %) compared with WC (Table IV-3).

Adding the wheat fractions produced with or without the MEP pretreatment had no significant effect on the villus length compared with NC (Table IV-3).

Table IV-3: Effect of dietary supplementation 0.1% of WC (wheat control) and WE (wheat+enzyme) fractions to a wheat-based diet on L-cell density in the ileum, T-cells and villus length in the ileal and cecal mucosa of 14-day-old broilers. NC: non-supplemented control.

		NC	WC	WE	P-values
L-cells [1] **(Cells/mm^2)**	In crypt	1.1^b±0.1	2.2^b±0.3	7.8^a±0.2	0.000
	In villi	15.9^c±1.1	25.2^b±2.8	79.9^a±2.6	0.000
T-cells[1] **(% labelled area)**	Ileum	16.1^b±1.1	15.9^b±0.6	8.1^a±0.6	0.000
	Caecum	16.8^b±0.9	15.7 ^{b}±0.7	7.0^a±0.6	0.000
Villus length[1] **(µm)**	Ileum	372^a ±9	389^a ±8	401^a ±7	0.196

[1] Raw data (n=24) were analysed using one-way ANOVA. Values are expressed as mean ±SD.

[a,b and c] Values with different superscripts are significantly (P<0.05) different from each other within each row.

IV.4.3- Microbiota characterization

IV.4.3.1- Microbiota composition

The α-diversity of the ileal (Figure IV-1- A) and cecal (Figure IV-1- B) microbiota of the same group (WE) increased compared with WC and NC.

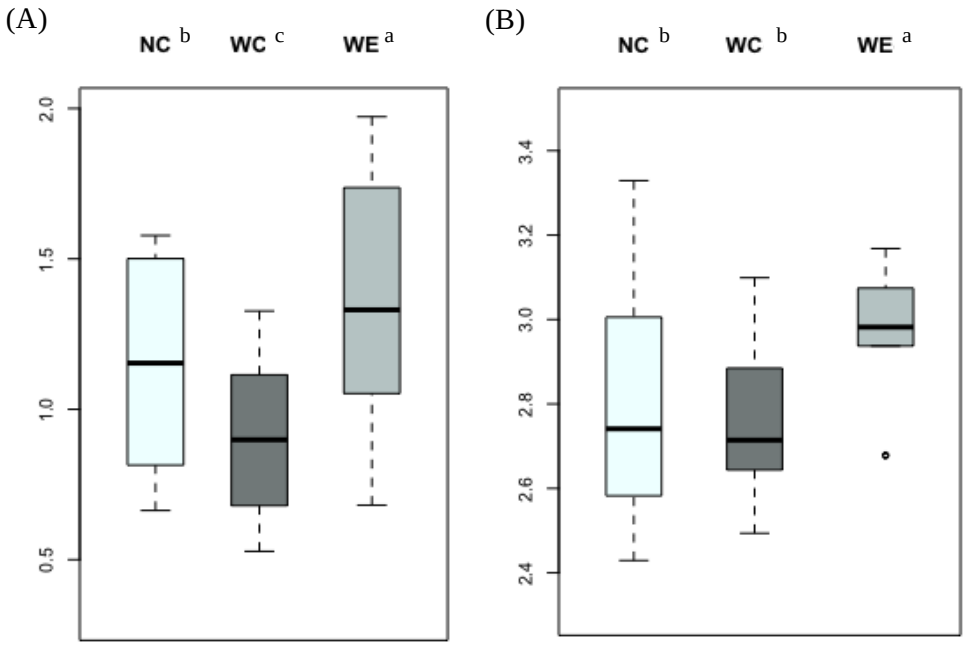

Figure IV-1: The Shannon index (H') of the α-diversity in the ileal (A) and cecal samples (B) of 14-days old broilers fed a wheat-based diet supplemented with WC (MEP-untreated) and WE (MEP-treated). NC : non-supplemented diet.

[a, b and c] boxplots with different superscripts are significantly ($P<0.05$) different from each other.

In table IV-4, we summarised, first, the main families identified in the ileal contents. Second, we reported the families and species significantly different between the 3 treatments. The ileal samples comprised 12 families, mainly *Lactobacillaceae* which represented up to 79 % of the WE, 92 % of the WC and 89 % of the NC microbiota (Table IV-4). Though no significant effect was identified on the *Lactobacillaceae* between the treatments. The WE supplementation significantly increased *Enterococcaceae* and *Clostridiaceae 1* ($P<0.05$) compared with WC and NC. Fifty-six species were identified in the ileal samples, and 8 species represented up to 99 % of the microbiota relative abundance. Only 2 species were significantly increased by WE supplementation to the diet, i.e., *Enterococcus durans* and *Candidatus arthromitus sp.*

In the cecal samples, 14 families were identified, mainly the *Lachnospiraceae* and *Ruminococcaceae* (more than 50 % of the total sequences). One hundred sixteen species were identified, and 18 species represented up to 85 % of the microbiota relative abundance.

Adding WE to the diet significantly increased the abundance of bacteria from these two main families compared with NC and WC (Table IV-4). *Lachnospiraceae* represented 52 % of the cecal microbiota of animals supplemented with WE versus 46 % for the animals supplemented with WC and 43 % for the NC group. Adding WE to the diet significantly increased *Ruminococcaceae* (12 %) compared with WC (9 %) and NC (8 %). Only 9 species were significantly increased by WE supplementation ($P<0.05$) compared to WC and 11 compared to NC (Table IV-4). No significant differences were observed between NC and WC.

Table IV-4: Effect of dietary supplementation of 0.1% of the WC (wheat control) and WE (wheat+enzyme) fractions to a wheat-based diet on the abundance (%) of the taxonomic groups (main families and families and species significantly different for the three treatments) within the ileal samples and the cecal samples of 14-day-old broilers[1]. NC: non-supplemented control.

Cluster	NC	WC	WE	P-Value	Accession n°	Highest 16S rRNA gene sequence similarity	
Ileum							
Lactobacillaceae	89.24^{a}± 3.21	92.13^{a}±4.16	79.13^{a}±2.38	0.754			
Enterococcaceae	0.65^{b}±0.12	1.28^{b}±0.19	6.08^{a}±0.54	0.005			
Enterococcus durans	0.64^{b}±0.24	1.26^{b}±0.17	5.06^{a}±0.38	0.000	AHYU01000027		
Clostridiaceae 1	0.36^{b}±0.01	0.41^{b}±0.02	0.54^{a}±0.01	0.004			
Candidatus arthromitus sp	0.35^{b}±0.03	0.40^{b}±0.02	0.53^{a}±0.02	0.010	DQ342328		
Caecum							
Lachnospiraceae	42.87^{b} ±0.23	46.30^{b}±0.31	51.63^{a}±0.36	0.001			
Ruminococcus gauvreauii group bacterium ic1296	0.08^{b} ±0.001	0.12^{b}±0.001	0.41^{a}±0.002	0.000	DQ057459	*Blautia producta*	(93%)
Blautia unknown sp	0.76^{b}±0.02	0.94^{b}±0.03	1.06^{a}±0.01	0.000	DQ456377	*Blautia hansenii*	(96%)
Coprococcus 1 unknown sp	2.25^{b} ±0.08	3.35^{b}±0.07	8.21^{a}±0.12	0.000	DQ800811	*Coprococcus catus*	(95%)
Eisenbergiella unknown sp	0.06^{b}±0.001	0.13^{b}±0.001	0.43^{a}±0.002	0.005	HQ807825	*Eisenbergiella tayi*	(95%)
Fusicatenibacter unknown sp	0.17^{b}±0.001	0.44^{b}±0.002	1.42^{a}±0.005	0.005	GQ175482	*Fusicatenibacter saccharivorans*	(96%)
Lachnoclostridium bacterium ic1294	2.18^{b}±0.04	3.91^{ab}±0.05	4.72^{a}±0.03	0.010	DQ057458	*Ruminococcus torques*	(96%)
Lachnoclostridium unknown sp	0.39^{b}±0.03	0.42^{b}±0.02	1.11^{a}±0.03	0.004	FJ440057	*Ruminococcus torques*	(96%)
Lachnospiraceae FE2018 group unknown sp	5.11^{b}±0.05	7.13^{b}±0.08	9.45^{a}±0.10	0.000	DQ326866	*Blautia faecis*	(93%)
Tyzzerella unknown sp	0.14^{b} ±0.006	0.28^{a}±0.004	0.36^{a}±0.002	0.004	HQ796125	*Clostridium lactatifermentans*	(99%)
Ruminococcaceae	7.85^{b}±0.38	9.46^{b}±0.42	11.91^{a}±0.19	0.000			
Ruminiclostridium unknown sp	0.57^{b}±0.03	1.08^{b}±0.08	3.14^{a}±0.12	0.004	EF025214	*Eubacterium desmolans*	(97%)
Ruminococcaceae UCG 013 unknown sp	0.06^{b}±0.001	0.08^{b}±0.001	0.17^{a}±0.001	0.000	AF371825	*Clostridium straminisolvens*	(87%)

[1] Raw data (n=10) were analysed using one-way ANOVA. Values are expressed as mean ±SD. [a and b] Values with different superscripts are significantly (P<0.05) different from each other within each row.

IV.4.3.2-Quantification of SCFA and lactic acid in cecal and ileal content

Adding WE to the diet had no significant effect on the SCFA concentration in the ileum when compared to NC and WC (Table IV-5). The WE increased the total SCFA concentration in the ceca and favoured the acetate and butyrate concentrations compared with WC and NC (Table 6). The propionate concentration in the presence of WE was significantly increased compared to NC but not compared to WC.

Table IV-5: Effect of dietary supplementation of 0.1 % WC (MEP-untreated) and WE (MEP-treated) fractions to a wheat-based diet on the short chain fatty acid and lactic acid concentration in the cecal and ileal content. MEP: multi-enzyme preparation.

	Parameters[1]	**NC**	**WC**	**WE**	**P-values**
Ileum	**Total SCFAs** (µM)	27.1^{a}±3.0	21.1^{a}±2.3	21.4^{a}±1.9	0.109
	Acetate (µM)	1.9^{a}±1.0	1.6 ^{a}±0.4	1.8 ^{a}±0.3	0.617
	Propionate[2] (µM)	-	-	-	-
	Butyrate[2] (µM)	-	-	-	-
	Lactate (µM)	25.2^{a}±3.0	19.5^{a}±2.2	19.7^{a}±2.0	0.105
Caecum	**Total SCFAs** (µM)	43.6^{b}±3.4	39.4^{b}±2.5	65.6^{a}±5.6	0.000
	Acetate (µM)	33.4^{b}±2.8	30.6^{b}±2.1	49.7^{a}±4.9	0.000
	Propionate (µM)	0.8^{b}±0.1	1.1^{ab}±0.2	1.9^{a}±0.3	0.014
	Butyrate (µM)	6.6^{b}±0.6	5.3^{b}±0.6	10.2^{a}±1.0	0.000
	Lactate (µM)	2.9^{b}±0.3	2.5^{b}±0.3	3.7^{a}±0.5	0.000

[1] Raw data (n=24) were analysed using one-way ANOVA. Values are expressed as mean ±SD.

[2] Butyrate and propionate concentrations were <0.1 µM in ileum

[a and b] Values with different superscripts are significantly (P<0.05) different from each other within each row.

IV.5- Discussion

In the present study, 0.1 % of AX containing fractions was added to diet. In previous studies, purified AXOS (Eeckhaut et al., 2008), AX or XOS (Courtin et al., 2008) were used at higher level (0.2-2.5 %). On average water-soluble AX represent 0.5% of wheat. Taking into account the proportion of wheat in the feed (44 %), adding 0.1 % of WE or WC corresponded to a 20-25 % increase of the amount of water-soluble AX. Under the action of MEP part of the water-insoluble AX were solubilized. The amount of WE fraction added was compatible with the extent of AX solubilisation likely occurring *in vivo*. The supplementation of water-soluble short-chain AX obtained by the enzymatic treatment of wheat improved the feed intake and daily weight gain with no effect on FCR in the starter period of broilers on a wheat-based diet. These beneficial effects were clearly attributable to the effect of MEP on wheat AX.

Water-extractable AX from wheat grain was fully recovered in WC (98% yield), and their molecular features were lower than previously observed (Faurot et al., 1995; Dervilly et al., 2000) (the Mw of purified AX was 300 kDa) due to prolonged time and higher temperature used for extraction. The enzymatic treatment and the conditions applied to wheat grain (3 h and 40°C) resulted in the solubilisation of cell-wall-insoluble AX, increasing the amount of water-extractable AX (125 g of WE *vs.* 103 g of WC from 20 kg of whole wheat flour). It allowed reducing the average Mw (176.8 kDa for WC to 49.6 kDa for WE). The evaluation of DP of the xylan backbone by measuring reducing ends (270 and 54 for WC and WE, respectively) confirmed this evolution. However, approximately 80% of the water-extractable AX in the wheat treated with enzyme precipitated in 65% ethanol, indicating polymer behaviour. The enzymes partially degraded the xylan backbone, resulting in AX with shortened chains in WE compared with WC. In addition NMR indicated a close structure of AX in both fractions. Finally, we considered the AX in WE as short-chain AX and that in WC as long-chain AX, all being polymers.

The *in vivo* results showed that only the fraction containing the shortest AX chains (WE) had a significant beneficial effect on animal performance. It increased FI and by consequence BWG. The WC supplementation had no negative effects on animal performance. It could be hypothesized that the quantity (0.1%) and the Mw of added SC-AX (49.6 kDa) were probably too low to increase digesta viscosity. Most previous studies (Van Craeyveld et al., 2008; Sanchez et al., 2009; Damen et al., 2011; Xu et al., 2013) showing beneficial effects of AX have used oligosaccharides of low DP (range between 5-32). Thus

far, the lower the DP of the NSP, the higher the microbial metabolite production. Nevertheless, our results showed noticeable beneficial effects despite the DP being significantly higher (DP=54) and the fraction not containing small oligomers (DP<10) (data not shown). These results suggest that AX of higher chain length than previously reported can be used beneficially by the microbiota of young chicks. In a recent study in humanized rats (Van den Abbeele et al., 2011), an increase of butyrate producers and production was recorded using AX with an average DP of 60.

The WE fraction improved BWG during the two-week trial by increasing FI. More important was the effect of this fraction during the first week post-hatch. Many authors (Jin et al., 1998; Maiorka et al., 2006; Stefanello et al., 2015) have emphasized how important the first week post-hatch is in the development of the digestive system. At hatching, the digestive system of the broilers is anatomically fully developed but physiologically immature (Nitsan et al., 1991; Hossain et al., 2014). It is important during this period that the digestive system is colonized by beneficial microbiota for the development of the gut-associated immune system and the protection against pathogens (Bao and Choct, 2010).

At the end of this study (d 14), we analysed the microbiota and observed an increase of *Enterococcus durans* and *Candidatus arthromitus sp*. in the ileum in the group that received WE. The lactate-producing bacterium *Enterococcus durans* is resistant to low pH and has been known to have probiotic effects as well as antimicrobial and antioxidant activities (Pieniz et al., 2014; Liu et al., 2016). *In vitro*, it exhibits a broad spectrum of inhibitory activity against *Listeria monocytogenes*, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* Typhimurium, *Salmonella* Enteritidis, *Pseudomonas aeruginosa*, and *Aeromonas hydrophila* (Pieniz et al., 2014). *Candidatus arthromitus sp.* is a commensal bacterium with a key role in the post-natal development of the gut immune functions by increasing the number of lymphoid cells in the lamina propria of the ileal and cecal mucosa (Klaasen et al., 1993; Bolotin et al., 2014). It is also characterized by its attachment to the intestinal epithelium, and it plays an important role in modulating host immune function (Snel et al., 1995; Thompson et al., 2013). It induces IgA secreting B-cells and steers the development of the T-cell repertoire (Maynard et al., 2012; Ericsson et al., 2014). In case of infection or pathogen challenge, it activates the interepithelial $CD8^+$ T-lymphocytes (Umesaki et al., 1995) and induces the lamina propria $CD4^+$ T-helper cells that produce interleukin IL-17 and IL-22 (Ivanov et al., 2009; Gaboriau-Routhiau et al., 2009). IL-17 and IL-22 have been associated with the induction of antimicrobial peptides (AMPs) (Zheng et al., 2008; Kolls et al., 2008;

Curtis and Way, 2009; Ivanov et al., 2009) involved in the protection of the host against pathogenic microbes by their broad-spectrum antimicrobial activities (Seo et al., 2012).

The WE fraction increased the concentration of acetate and butyrate in the ceca. As indicated previously (Miller and Wolin, 1996; Duncan et al., 2002), butyrogenic bacteria convert acetate more efficiently to produce butyrate when growing in a medium containing a carbohydrate source (glucose or xylose). In the present study, adding WE to the diet significantly increased the relative abundance of the bacteria belonging to the families of *Lachnospiraceae* and *Ruminococcaceae*, known as butyrate producers (Biddle et al., 2013; Stackebrandt, 2014). *Ruminococcaceae* are saccharolytic bacteria known to produce a range of glycosidases, enabling them to degrade resistant starch, hemicellulose and cellulose (69). Bacteria within the *Lachnospiraceae* family are known to produce butyrate by direct fermentation of polysaccharides (Meehan and Beiko, 2014) or by cross-feeding with other bacteria and converting acetate or lactate to butyrate (Duncan et al., 2004). An increase in butyrate concentration is known to protect gut against colonization with pathogenic microbiota (Andoh et al., 1999; Fernández-Rubio et al., 2009; Guilloteau et al., 2010). Butyrate also stimulates the proliferation and differentiation of intestinal epithelial cells (Mariadason et al., 1999; Manzanilla et al., 2006), and it has been shown to reduce inflammation in the intestinal mucosa of mice (Kyner, 1976) and rats (Cavaglieri et al., 2003). Moreover, butyrate plays an important role in the maintenance of intestinal mucosal integrity (Segain, 2000; Hu and Guo, 2007; Vercauteren et al., 2010; Guilloteau et al., 2010). The primary role of the gastrointestinal mucosa lies in the digestion and absorption of nutrients, but it is also a gateway for dietary antigens, pathogenic and non-pathogenic bacteria (Berg, 1999). The cellular immune response in the intestinal mucosa comprises mainly T-lymphocytes (Guy-Grand and Vassalli, 1993; Norris and Evans, 2000; Revillard, 2001). As observed previously (Teirlynck et al., 2009b; Montanhini Neto et al., 2013), diets containing a high amount of NSP increase T-lymphocyte infiltration in the intestinal mucosa. In the present study, WE in the diet decreased T-lymphocyte infiltration in the mucosa and may possibly reduce inflammation.

As recently reviewed (Candela et al., 2010), the microbiota shifts induced by prebiotic carbohydrates are associated with an increase of glucagon-like peptide-2 (GLP-2) production. The GLP-2 is a 33-amino-acid peptide produced by intestinal endocrine L-cells. The GLP-2 hormone is involved in controlling gastrointestinal epithelial integrity, motility, and secretion, local blood flow, and nutrient uptake and utilization (P. T. Schmidt, B. Hartmann, 2000;

Guan et al., 2006). AXOS can increase the number of GLP-2 producing L-cells in mice (Neyrinck et al., 2012). Our results showed an increase of FI and of the number of L-cells in the ileum when WE is included in the diet. This increase may be partially explained by the increase of butyrate observed *in vitro* (Mangian and Tappenden, 2009). The endocrine regulations underlying the increase in FI, observed with WE included in the diet, were not investigated in detail, and are unclear. Indeed, regulation of appetite and satiety is a complex and still not fully comprehended process, in which ghrelin, GLP-1 and leptin as others play a role (98). It is well documented, however, that inflammation reduces appetite (Ferket and Gernat, 2006; Todd, 2012; Montanhini Neto et al., 2013) and feed intake . Therefore, it cannot be excluded that a butyrate associated reduction in inflammation, as seen in the WE group in the present study, may have contributed to the increase in FI.

IV.6- Conclusion

The treatment of wheat with a multi-enzyme preparation containing xylanases and glucanases resulted in a short-chain arabinoxylan polysaccharide fraction that increased the BWG of newly hatched chicks and improved their microbiota diversity. These effects may be attributed to the stimulation of specific beneficial bacteria in the ileum (*Candidatus Arthromitus sp*. and *Enterococcus durans*) and in the ceca (members of the *Ruminococcaceae* and *Lachnospiraceae*). Physiologically, the ceca lie downstream of the ileum in the food passage. The lactate producers in the ileum may represent a microbial consortium that cross-feed the acetate and the butyrate producers dominant in the ceca. This could be important in the early development of the broiler to stabilize the intestinal microbiota.

Chapter V

General Discussion

Conclusion and further perspectives

V.1- General discussion

The European Union (EU) is one of the world's top producers of poultry meat and a net exporter of poultry products (1.5 Mio tons in 2014). Over the years, important legislation changes were made to ensure the development of the sector, the quality of the products and the protection of the consumers. In 2014, the EU kept the self-sufficiency level at 103 % with a real consumption of 13.1 million tons of poultry meat. The leading countries in poultry meat production in Europe are Poland (13.7 %), France (12.7 %), closely followed by UK (12.4 %), Germany (11.4 %) and Spain (11.1 %) (http://ec.europa.eu).

Broilers are fast growing animals with an average production cycle of 42 days. To ensure an average slaughter weight of 2.5 kg, they need a high amount of energy mainly obtained from wheat and corn based-diets. The price for broiler meat keeps on declining (-5.2% between 2015 and 2016 (www.ec.europa.eu)) and implies more efforts to reduce production costs. It is also important to produce homogenous products of good nutritional quality to cover the growing human population needs. To ensure good quality end products it is important to use good quality feedstuff and to maintain high health and welfare status of the animals along the production chain.

Important progress has been made during the last years to increase the poultry meat production and be cost efficient. Selective breeding and genetic selection allowed increasing the average weight at slaughter from 1.6 kg in 98 days 50 years ago to 2.5 kg in 42 days nowadays (www.animalsaustralia.org). Housing and environmental conditions were optimized and mortality reduced during the starter period. New software and effective methods to calculate and cover the growth needs of the broiler were developed. Nevertheless the efficacy of these methods depends on the quality and the nutritional value of the feed ingredients, as well as the health conditions of the animals. The nutritional value of the feedstuff varies according to the harvest year and conditions, the plant varieties and especially their non-starch polysaccharides (NSP) content. These NSP impact the digestive system and the associated immune system (Gut-associated lymphoid tissue-GALT). All these variations in the production scheme and particularly the fast growth put a lot of pressure on the heart and the fragile skeleton of the young animals and cause several health problems. They also cause a particular impact on the digestive microbiota. For decades the ultimate solution for this issue was the use of antibiotics as growth promoter. These allowed improving animal performance by limiting bacterial overgrowth and colonization by Gram-positive pathogens (Lee et al., 2012). However, due to the concerns of antibiotic resistance,

their use was banned in the EU since 2006. The post-antibiotics era was accompanied with a decrease of productivity as well as an increase of the production cost. Many bacterial disorders become frequent, such as dysbacteriosis due to general bacterial overgrowth in the small intestine, and necrotic enteritis caused by *Clostridium perfringens* (Van Immerseel et al., 2004a). Scientists, nutritionists and feed manufacturers have since been developing safe feed additives to face the production decline and to improve intestinal health. Many alternatives, such as probiotics, prebiotics, organic acids, short and medium chain fatty acids, anti-microbial peptides, exogenous enzymes, clay minerals and plant-derived phytogenic preparations were tested. Although exogenous enzymes have been used in poultry feed for decades, supplementation of the diet with new optimized GH-preparations is one of the successful alternatives.

V.1.1- Effect of MEP on cereal grains and other feed components

Exogenous enzymes as feed supplements allowed improving the nutritional value of the diet based on wheat, rye or barley (Pettersson and Åman, 1989, 1988; Annison, 1991; Bedford and Classen, 1992) and improve the animal performance, as reviewed by many authors (Bedford and Partridge, 2001; Choct, 2006; Ravindran, 2013; Masey O'Neill et al., 2014). The glycoside hydrolases (GH) increase the digestibility of organic matter, crude proteins and starch, with a significant different response according to the cereal type used (Pettersson and Åman, 1989). The first developed enzyme preparations mainly composed of β-glucanases and β-xylanases were more effective on rye and triticale than wheat (Pettersson and Åman, 1988). Different mechanisms are proposed to explain the beneficial effect of GH (Slominski, 2011). First, the effect of GH might be due to the elimination of the nutrient encapsulating effect of the cell walls improving nutriments availabilities. However, wheat endosperm cell walls are easily disrupted with the mechanical action of grinding and nutrients are available (Engberg et al., 2002; Carré et al., 2007; Amerah et al., 2008). Though, the cell walls of the aleurone layer are more resistant and could encapsulate potential nutriments (Knudsen, 2014). Indeed, the intracellular medium of aleurone cells is characterized by high amounts of protein, minerals, phytates, vitamins (Hemery et al., 2009; Brouns et al., 2012). The aleurone contains 15 % of the total wheat protein (Feillet, 2000; Brouns et al., 2012) but also up to 30 % of the total lysine, which is the first limiting essential amino acids in wheat (Pomeranz, 1988). Still, this hypothesis is very difficult to endorse. In this thesis we observed that MEP-treatment increased protein content of the water-soluble

fraction with no effect on the amino acid composition. However, it is difficult to identify their origin since the endosperm and the aleurone layer contain similar proteins (Rhodes and Stone, 2002; Brouns et al., 2012).

Second, GH solubilize the cell wall NSP and eliminate the anti-nutritive properties of certain dietary NSP. The endosperm cell walls of cereal grains are mainly composed of AX and MLG. The enzyme supplementation of cereal-based diets can significantly improve chick performance by increasing the BWG, FCR, AME and digestibility of dry matter, fat and protein (Marquardt et al., 1996). Excellent improvements are often obtained with diets containing rye, oat and barley and less with wheat. To date, little indication of success exists regarding the use of enzyme preparations on a corn-soybean meal based diets (Slominski, 2011). These differences in response are probably due to different NSP composition and nature in the different feedstuffs. Barley contains more MLG, while wheat contains more AX but AX exert lower effect on digesta viscosity than MLG. Corn contains low amount of NSP, mainly composed of highly substituted AX, that do not induce viscosity problems (Saulnier et al., 1995).

Our study showed that the enzymatic degradation of wheat, mainly by the endo-xylanase action, increased the solubilisation of AX and reduced its Mw and intrinsic viscosity. However, no oligosaccharides (DP< 10) were produced. These results are in concordance with those obtained *in vitro* under pig digestive physiological conditions (TIM-1), since just a minor amount of AX was hydrolysed to DP< 8 (Maisonnier-Grenier et al., 2006). The effects of enzyme addition to diets for poultry can be variable due to different NSP content, composition, structure and accessibility. The beneficial responses are most often seen in young birds or in birds fed diets containing poor digestible ingredients (Bedford and Cowieson, 2012). It is complicated to compare the different studies investigating the effects of GH. First, enzymes used have different activities and specificities, originate from different sources and are often used individually or in combination. Second, the enzyme efficacy depends on the inclusion rates and on the different cereals used in the diet. Third, the animals respond differently according to their species and age (Adeola and Cowieson, 2011). For example, Olukosi et al. (2007) showed that xylanase supplementation had no effect on pigs <10 kg but improved broiler performance. The literature shows that the chicks benefited more from the enzyme addition at a younger age and that the contribution of the enzymes to nutrient retention decreased with age in chickens (Olukosi et al., 2007). This is suggested to

be related to the output of pancreatic enzymes which limits digestion till 8 days post-hatch (Bedford, 2000).

V.1.2- Effects of MEP degradation products on intestinal microbiota

The enzymatic degradation of cell wall NSP allows releasing oligosaccharides (Walsh et al., 1993; Bedford and Schulze, 1998; Choct, 2006; Lee et al., 2010). The DP influences the fermentation pattern, and the lower the DP the faster the fermentation (Kabel, 2002; Bedford and Cowieson, 2012). AXOS of low DP selectively stimulate the growth of beneficial microbiota in the ceca (Courtin et al., 2008), provide protection against *Salmonella* infection (Eeckhaut et al., 2008) and improve microbial diversity (Van Craeyveld et al., 2008; Sanchez et al., 2009; Damen et al., 2011). In this study, the short-chain arabinoxylans (SC-AX) produced by enzymatic degradation of wheat clearly have a higher DP than reported in the literature for prebiotic AXOS but have similar effect and stimulate the growth of cecal microbiota with a great impact on butyrate producers *in vitro* and *in vivo*. This suggests that the optimal chain length of AX that can be fermented by the microbiota of young chicks could be higher than previously reported. Same tendency was also observed in humanized rats, using AX with an average DP of 32 that favoured butyrate producers and increased butyrate production (Van den Abbeele et al., 2011).

The chicken microbiota is very complex and is composed of bacteria, fungi, protozoa, yeasts, viruses and bacteriophages (Gong and Yang, 2012). Bacteria are the main component of gut microbiota with a high population density, high diversity, and highly complicated interactions among them and with their host and diet (Xu et al., 2007). An unbalanced gut microbiota is associated with the pathogenesis of many infectious and inflammatory diseases (Stecher and Hardt, 2008; Saleh and Elson, 2011; Siggers et al., 2011). Until recently, culture-dependent methods provided the main insights into the avian GIT microbiota. It is estimated that about 47 % of cecal bacteria can be cultured (Stanley et al., 2015). Even with new metagenomics, metatranscriptomics and pyrosequencing methods and techniques, only around 45 % of chicken intestinal bacteria can be confidently assigned to a known genus (Apajalahti et al., 2004; Apajalahti and Vienola, 2016) and around 90 % are unknown species (Bjerrum et al., 2006; Gong and Yang, 2012). Therefore, a considerable number of the GIT bacteria remains largely unidentified. These represent an important untapped biological potential in terms of newly identified bacteria, encoded enzyme activities and potentially probiotic strains of bacteria (Stanley et al., 2014).

The microbiota composition varies according to the intestinal compartments and even the host genotype and gender could play an important role (Zoetendal et al., 2004; Zhao et al., 2013). The different sections of the broiler GIT are inhabited by particular microbiota adapted to the physicochemical conditions, host physiology and available nutrients of the specific habitat (Apajalahti and Vienola, 2016). The crop and small intestine of broiler chickens are dominated by lactic acid producing bacteria, mainly *Lactobacillus spp.*, *Enterococcus spp.* and *Streptococcus spp.*. These bacteria represent up to 95 % of the total small intestinal bacteria (Barnes et al., 1972; Salanitro et al., 1974; Apajalahti et al., 2004; Bjerrum et al., 2006). The lactic acid producers are unable to synthesise amino acids for their anabolism and are therefore highly dependent on amino acid availability (Apajalahti and Vienola, 2016). The cecal microbiota is dominated by bacteria belonging to the order *Clostridiales* (families *Lachnospiraceae* and *Ruminococcaceae*, also referred to as *Clostridial* clusters XIV and IV, respectively) (Lu et al., 2003; Apajalahti et al., 2004; Bjerrum et al., 2006; Apajalahti and Vienola, 2016). These bacterial families are characterized by their ability to hydrolyse cell wall NSP into oligosaccharides and to produce butyrate. The *Ruminococcaceae* are saccharolytic bacteria known to produce a range of glycosidases, enabling them to degrade resistant starch, hemicelluloses and cellulose (Duncan et al., 2007; Torok et al., 2011). The *Lachnospiraceae* bacteria are able to degrade both NSP and starch (Biddle et al., 2013) and to produce butyrate by direct fermentation (Torok et al., 2011) or by cross-feeding with other bacteria and converting acetate or lactate to butyrate (Duncan et al., 2004). Other families composing the cecal microbiota are the *Bifidobacteriaceae* (10 %) and *Coriobacteriaceae* (7 %) families (Apajalahti and Vienola, 2016). *Bifidobacteria* have been suggested to degrade simple carbohydrates and oligosaccharides and produce lactic and acetic acids (Sanz, 2016). The role of *Coriobacteria* is poorly understood, but some reports suggested that they are connected to lipid and cholesterol metabolism (Martínez et al., 2013).

In our study, an important decrease of the α-diversity and an alteration on the ileal and cecal microbiota were observed when the wheat fraction produced with enzymes was added to the broiler diet. In the ileum, the lactate producing bacteria *Enterococcus durans* and *Candidatus arthromitus sp.* were stimulated. These species are resistant to low pH and have been shown to have probiotic effects as well as antimicrobial and antioxidant activities (Pieniz et al., 2014; Antonissen et al., 2015). In the ceca, the relative abundance of the bacteria belonging to the families of *Lachnospiraceae* and *Ruminococcaceae*, known as

butyrate producers were significantly increased. The increase of the abundance of these beneficial bacteria would improve intestinal health and animal performance.

The composition of the microbiota showed differences as compared to the studies performed previously (Choi et al., 2015; Apajalahti and Vienola, 2016) since the age of broilers was much higher (4-weeks old chickens). Our samples contained less *Ruminococcaceae* bacteria (~10 % *vs.* ~20 % in the literature) and more *Lactobacilleacea* (~ 40 % *vs.* 10 %) in the ceca. Only few reports exist that investigate the microbiota composition of young chickens and they are based on culture methods. For some authors a stable composition of the GIT microbiota could be reached from 2 weeks age (Torok et al., 2009; Pan and Yu, 2014) while others proposed the age of 4 weeks (Bedford and Cowieson, (2012). Most of the studies investigating microbiota composition endorse the fact that it evolves with the age. Multiple factors influence this composition; therefore it is difficult to compare our results with literature data.

V.1.3- Effects of fermentation products on intestinal mucosa and animal performance

The present study shows that the enzymatic degradation of NSP favours the release of prebiotic-like components. These allow a more effective hindgut fermentation resulting in an increase of SCFA production *in vivo* and *in vitro*.

The GIT bacteria exhibited an increase of the butyrate producers *Lachnospiraceae* and *Ruminococcaceae* resulting in an increase of the butyrate production. Butyrate plays a key role in the maintenance of intestinal mucosal integrity (Segain, 2000; Leeson et al., 2005; Weber and Kerr, 2006; Guilloteau et al., 2010). It is also an important source of energy for the gastrointestinal epithelial cells that increases their proliferation and differentiation (Mariadason et al., 1999; Manzanilla et al., 2006) partly by stimulating the intestinal endocrine L-cells that produce GLP-2 (Candela et al., 2010). The GLP-2 hormone is involved in controlling gastrointestinal epithelial integrity, motility, and secretion, local blood flow, and nutrient uptake and utilization (Schmidt and Hartmann, 2000; Guan et al., 2006). Different prebiotics have already shown an increase of the number of GLP-2 producing L-cells in mice (Mangian and Tappenden, 2009; Neyrinck et al., 2012). Our results showed an increase of L-cell density in the ileum when SC-AX were included in the diet.

Butyrate is also known to protect against pathogenic microbiota colonization (Andoh et al., 1999; Guilloteau et al., 2010; Mátis et al., 2015). Studies using butyrate as feed

additives showed that it is effective on intestinal pathogens by reducing *Salmonella* (Van Immerseel et al., 2004; Van Immerseel et al., 2005) and *Clostridium perfringens* (Timbermont et al., 2010) in the GIT. Butyrate has also been shown to reduce inflammation in the intestinal mucosa of mice (Kyner, 1976) and rats (Cavaglieri et al., 2003) and to activate lymphocytes in the GIT (Kyner, 1976).

The gastrointestinal mucosa has an important role in the digestion and absorption of nutrients, and is the first gateway for dietary antigens, pathogenic and non-pathogenic bacteria (Berg, 1999). The capacity of pathogens to adhere to the intestinal mucosa plays a significant role in the pathogenesis of infections, and in the induction of inflammation. Dietary NSP can also induce inflammation in the GIT. It has been shown that ~ 30 g/kg of high molecular water-soluble AX (Mw= 758 kDa) in the diet decreased severally BWG and FCR, and induced inflammation in the GIT (Annison and Choct, 1991; Choct and Annison, 1992; Teirlynck et al., 2009a; Montanhini Neto et al., 2013). The cellular immune response in the intestinal mucosa comprises mainly T-lymphocytes (Guy-Grand and Vassalli, 1993; Norris and Evans, 2000; Revillard, 2001). All lymphocytes have a CD3 receptor on their surface (Tizard, 2013) that allows detecting all lymphocytes in the GALT. By this way, the present study shows that the SC-AX decreased T-lymphocyte infiltration and reduced inflammation in the ileum and cecum indicating less inflammation in the intestinal mucosa during the starter period especially during the first week. Montanhini Neto et al. (2013) showed also that MEP is effective to reduce T-lymphocyte infiltration induced by the use of alternative feedstuffs (millet, canola and sunflower meals) in a corn-soybean based diet. Enzymes are also effective in reducing pathogens proliferation in the GIT of broilers fed a commercial diet (Singh et al., 2012).

Moreover, a balanced intestinal microbiota is crucial to the gut integrity and to the proper development of the intestinal tract but also for better performance (Torok et al., 2008). It is also important to shape the intestinal microbiota soon after hatch to favour the development of beneficial bacteria. As a consequence, the first week post-hatch is a fundamental period for broilers. As highlighted by many authors (Jin et al., 1998; Maiorka et al., 2006; Stefanello et al., 2015), the digestive system of the broilers at hatch is anatomically fully developed but physiologically immature (Nitsan et al., 1991; Hossain et al., 2014). It is essential during the starter period that the digestive system is colonized by beneficial microbiota. Once the birds have a balanced intestinal microbiota inhabiting a healthy GIT, they can be more efficient in feed conversion and grow faster.

The *in vivo* trial carried out in the present study showed that the supplementation of a wheat-based diet with only a small amount (0.1 %) of water-soluble SC-AX improved animal performance. The remarkable beneficial effects were observed during the first week post-hatch. These results support the hypothesis that the production of SC-AX exerting prebiotic effects is one of the main mechanisms explaining the beneficial action of MEP on wheat-based diet. MEP also prevents the possible negative effect of digesta viscosity of water-soluble AX present in wheat, by reducing their Mw. Our results also showed that SC-AX can induce the same effects observed with oligosaccharides (Van Craeyveld et al., 2008; Sanchez et al., 2009; Pareyt et al., 2011; Lecerf et al., 2012; Neyrinck et al., 2012). Many authors studied the effects of adding different exogenous enzymes *in vivo,* using different feedstuffs as a substrate showing a huge variability in results according to the avian species, enzymes activity and substrates (Table V-1). These results show how important is to choose the accurate enzymes for the feedstuff used in the diet in order to improve animal performance and to be cost efficient.

Table V-1 : Some studies in poultry showing effects of exogenous NSP-degrading enzymes on growth performance (Adeola and Cowieson, 2011)

Species	Ingredients	Major enzyme activity	Observations	Reference
Ducks	High- or low viscosity wheat	Xylanase	Up to 12 % improvement in BWG	Adeola and Bedford, 2004
Turkey	Wheat, barley, rye	Xylanase, β-glucanase	2 % improvement in daily BWG	Boguhn and Rodehutscord, 2010
Pullet	Corn, wheat products	Five carbohydrase activities	Greater ovary weight in enzyme-supplemented treatment	Chauynarong et al., 2007
Broiler	Corn	Xylanase, amylase, and protease combination	6 % improvement in BWG	Cowieson and Ravindran, 2008
Broiler	Wheat and wheat middlings	Xylanase	No effect on BW gain	Olukosi and Adeola, 2008
Broiler	Wheat and rye	Xylanase	18 % improvement in BWG	Olukosi et al., 2007
Broiler	Corn	Xylanase, amylase and protease	No effect on BWG	Olukosi et al., 2007
Broiler	Corn	Cellulase, hemicellulase, pectinase	Enzyme combination improved BW gain by 9 %	Tahir et al., 2008

V.1.4 - A model for the mechanisms of action of MEP

The SC-AX fraction showed prebiotic properties by improving performances partly by modifying the intestinal microbiota and increasing the SCFA production in the GIT. A prebiotic is a selectively fermented non-digestible dietary ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health (Roberfroid, 2007, 2008; Ducatelle et al., 2014). Because these are non-digestible they are not directly used by the host itself, but fulfil their beneficial effects indirectly. The advantage of NSP-degrading enzymes, however, is that they allow transforming the wheat-NSP (or more generally cereal-NSP), considered as anti-nutritional factors, to prebiotic-like components and take benefit of the associated effects.

According to the results obtained in this PhD work and based on the literature data, the mechanisms of action of NSP-degrading enzyme preparation can be resumed in the Figure V-1. The mechanisms are multiple and interconnected. They consist on direct effects (solubilizing cell wall-NSP and reducing the DP of AX and their viscous effects) and indirect effect (altering the microbiota composition, increasing the nutrient accessibility…), which results in an improved intestinal health and a better animal performance.

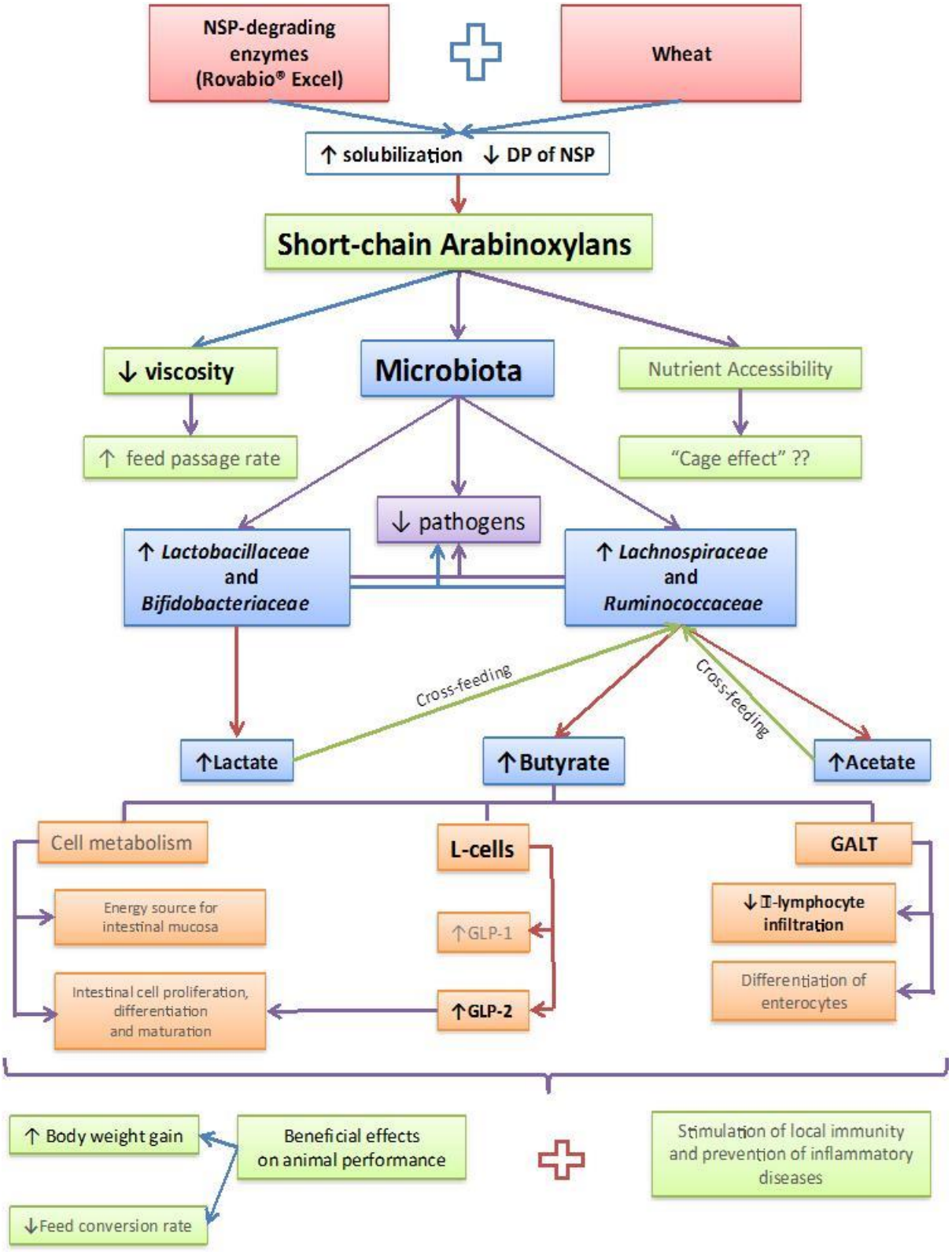

Figure V-1: Mechanisms of action of NSP-degrading enzymes

Production — Cross-feeding — Direct effects — Indirect effects

Results of the present study are in **black** and data from the literature are in grey

V.2- Conclusion and further perspectives

In-feed supplementation of enzyme preparations is an effective method to replace antibiotics to enhance animal performance. Their mechanisms of action are complex and consist on direct and indirect effects. MEP allows solubilizing wheat cell wall NSP, mainly AX, decreasing their Mw and their intrinsic viscosity and by consequence increasing the feed passage rate in the GIT. The SC-AX produced by enzymatic degradation will steer the intestinal microbiota towards one with a dominance of beneficial bacteria. In the current thesis it was shown that early post-hatch, butyrate-producing bacteria, and consequently butyrate concentrations in the ceca, were increased when SC-AX were supplemented in the feed of broilers. Elevated butyrate concentrations in the gut allow reducing inflammation and increasing the density of L-cells in the GIT. The L-cells produce GLP-2, which is recognized to have potent growth-promoting activities on intestinal epithelium.

These complementary effects are responsible for the better performance of broilers. The interaction between the microbiota and the host are suggested to be the key effect of prebiotic-like components released after enzymatic degradation *in situ*. The profiling of the microbiota along the gut showed that microbial diversity is still very low in the first days post-hatch. This period is likely the best one to target to get an influence on microbiota that could last till the end of the production cycle. One of the effects generally observed with MEP supplementation and observed in this work with MEP degradation products in-feed supplementation is a reduction of performance variations between the animals, which results in a more cost effective production. Despite all the improvements, performance within a flock varies considerably and this variation causes significant losses to the industry.

The increasing knowledge generated from the human microbiome project (Yatsunenko et al., 2012; Ravel et al., 2014), the increasing ability to sequence the metagenome as well as the advanced bioinformatic methods and databases (Yilmaz et al., 2014) are generating important data helping determining the effects of feed additives on the modulation of gut microbiota. The results of the characterization of the cecal and ileal microbiota of our samples showed important variations of the composition between the different fractions used. However, using the 16S hypervariable region V4-V5, many sequences were identical leading to genera and species multiple affiliations. In addition, different species and genera were unknown or corresponding to reference with a low quality index. Further research and characterization of the broiler microbiota are therefore necessary to allow identifying unknown genera and species and their interaction with the host. The new

methods will help provide new information about the bacterial metabolism of the different species within the intestinal microbiota that allow better understanding the interactions such as the cross-feeding between the different bacterial groups. Still, there are a lot of bacterial families belonging to the normal intestinal microbiota but with only little information about their metabolism, ecology, fermentation's end products and their role in the GIT.

Many authors adopted the hypothesis that water-insoluble cell wall NSP could impair nutrient availability for the animal by blocking the access of the endogenous enzymes digestive enzymes, the so called "cage effect" (Classen and Bedford, 1991; Bedford, 2000, 2002; Campenhout et al., 2007; Smeets et al., 2014). They suggest that NSP-degrading enzymes break down cell walls by solubilizing the NSP that allow releasing cell content and increase nutrient accessibility. However, the structure of the endosperm cell walls is disrupted by mechanical action of the grinding. In this respect, NSP-degrading enzymes have probably little effects on the accessibility of digestive enzymes to the starch and the proteins available from wheat endosperm. Generally, the tissue structure of the aleurone layer is preserved from disruption during milling. Degradation of aleurone cell walls by NSP-degrading enzymes could potentially release more nutrients, especially proteins. Further investigations are needed to verify this effect and to fix the ideal enzyme type and dose that would allow reaching it.

We have shown that SC-AX added to feed improved animal performance. SC-AX are clearly produced *in vivo* by MEP, but their amount is difficult to control due to variability in wheat grain composition, which is linked to cultivars and environmental effects, and affects enzyme action. Another possibility to improve nutritional value of feed would be to include enzymatically pre-treated wheat in the diet assuring a higher and controlled amount of SC-AX, especially during the starter period. The challenge would be to setup an efficient production protocol allowing enzymes to degrade the cell wall NSP in low-hydrated medium and to find the appropriate combination between the enzyme activity conditions and the release of SC-AX.

The dose of enzyme and its specificity for the feedstuff are the key factors of the MEP efficacy. One of the limiting factors to MEP activities is the accessibility of the substrate in feedstuffs. The biochemistry analysis provides important data about the composition and the structure of cell wall NSP. Still, more investigations are needed to identify all the variations between the structures of the different feedstuff used in animal nutrition. For example, corn AX are highly substituted by arabinose (A/X >1). Their extensive degradation needs first the

cleavage of the arabinose side chains. This increases accessibility of the xylanases to the xylan backbone. This knowledge allows selecting appropriate activities for the degradation of cell wall components. Thus, these data should support the development of new preparations containing enzymes with synergistic activities, which will efficiently degrade the corresponding substrate (feedstuff).

The sequencing of the fungi genome allowed identifying the different genes encoding the different enzymes. After a functional validation of the genes of interest, enzyme preparations can be improved based on genetic modifications of the producing organism by the over-expression of genes encoding the enzymes of interest, which will enhance the enzyme production. The introduction of new genes from other fungi will improve the preparation efficacy by introducing new activities and probably lead to adapt the secretome to be more efficient in degrading cell wall NSP. The genome of *Talaromyces versatilis* (www.ncbi.nlm.nih.gov) showed the presence of genes coding enzymes that are not currently produced in the secretome. The possibility of overexpressing certain transcription factors will increase the number of enzymes differing in their specificity. This could improve the synergistic potential between the different enzymes in the preparation. An objective for a better performance of enzyme preparation on cereals based diets is certainly to increase the expression of arabinofuranosidases. These enzymes work synergistically with xylanases to efficiently break down complex fiber structures in plant cell walls. By removing hampering arabinose branches from AX, these enzymes open the way to allow the xylanases access to the xylan backbone (De La Mare et al., 2013). The increase of arabinofuranosidase activities could improve the efficacy of MEP on corn-based diet as corn contains very highly branched AX. These newly expressed enzymes could also target other cell wall NSP of other feedstuffs. Further researches are needed to optimize the expression of new enzymes and study their effects on new substrates.

The combination of the efficient characterization of the feedstuff and the new synergistic enzymes will allow optimizing the enzyme uses in animal nutrition and reduce production costs. Further investigations are needed to optimize the use of the new improved GH preparation *in vivo* and reduce variability between animals and between the different feedstuffs used in the diets.

Abstracts

Résumé en Français

Les problèmes de santé digestive en production avicole représentent des pertes économiques très importantes, en particulier depuis l'interdiction de l'utilisation des antibiotiques comme facteurs de croissance par l'Union Européenne en 2006. Cette interdiction a engendré une augmentation de l'utilisation des antibiotiques à des fins curatives (Persoons et al., 2012; Landoni and Albarellos, 2015). Toutefois, afin d'éviter l'apparition de résistances aux antibiotiques, il est impératif de diminuer leur consommation. Différentes solutions sont possibles pour maintenir un bon niveau de production et prévenir les problèmes de santé digestive des animaux. Les enzymes dégradant les polysaccharides pariétaux des végétaux sont largement utilisées pour résoudre ces problèmes, notamment dans les régimes à base de céréales. La littérature suggère que les régimes à base de céréales, notamment le blé, induisent une inflammation du tube digestif du poulet (Teirlynck et al., 2009a). Ce phénomène inflammatoire au niveau de l'intestin est de plus en plus considéré comme étant l'élément clé de la santé digestive des animaux et aurait des conséquences importantes sur les performances animales (Montagne et al., 2003). L'action des enzymes de dégradation des polysaccharides pariétaux est vraisemblablement multifactorielle, mais elles pourraient notamment favoriser l'apparition, dans le tractus digestif, d'oligosaccharides présentant des propriétés prébiotiques et ainsi améliorer la santé digestive chez l'animal.

En effet, les prébiotiques sont des oligosaccharides résistants à l'acidité gastrique, à l'hydrolyse par les enzymes digestives de l'hôte et à l'absorption gastro-intestinale, qui sont fermentés par le microbiote intestinal et stimulent sélectivement la croissance des *lactobacilles* et *bifidobactéries*, reconnus comme des agents santé. Toutefois, les différents genres de bactéries du microbiote seraient différemment sensibles à la structure de ces oligosaccharides (Pastell et al., 2009). Parmi les produits issus de la fermentation des prébiotiques par le microbiote, le butyrate est reconnu comme agent anti-inflammatoire en santé intestinale (Guilloteau et al., 2010). Les composés prébiotiques produits par la dégradation des polysaccharides de la paroi et leur produit de fermentation pourraient donc en partie expliquer l'effet sur la santé digestive des animaux des enzymes ajoutées à l'alimentation. Cependant, à l'heure actuelle, le lien entre utilisation de glycosyde-hydrolases dans les aliments / formation de prébiotiques / effet sur la santé digestive n'est pas clairement établi.

Rovabio® est une préparation enzymatique commerciale produite par le champignon filamenteux *Talaromyces versatilis* et commercialisée par la société ADISSEO. Ce cocktail

possède un grand nombre d'activités enzymatiques de type glycosyde-hydrolases, et est particulièrement riche en activités de type xylanase et β-glucanase (Guais et al., 2008). Ces activités lui permettent de produire une large gamme d'oligosaccharides à partir de parois des grains de céréales (Maisonnier-Grenier et al., 2006; Lafond et al., 2011a). Dans cette thèse, les produits d'hydrolyse du grain de blé par la préparation enzymatique Rovabio® (nommé ci-dessous MEP pour multi-enzyme preparation) ont été isolés, et leurs effets sur les performances et la santé digestive du poulet ont été étudiés.

1- Les fractions hydrosolubles obtenues par traitement enzymatique du grain de blé favorisent la production d'acides gras à chaîne courte par le microbiote caecal des poulets.

L'objectif de **la première partie** de ce travail était d'étudier les effets des fractions produites par traitement enzymatique de grain de blé sur le microbiote caecal. D'abord le grain entier broyé en farine a été incubé à 40 °C pendant 6 h avec (MEP-treated) ou sans préparation enzymatique (MEP-untreated). Chaque fraction soluble a été précipitée avec de l'éthanol 80 %. Le culot a été récupéré puis fractionné par chromatographie d'exclusion stérique en une fraction de haute masse moléculaire (HMw) et une fraction de faible masse moléculaire (LMw). La composition en oses neutres, la teneur en protéine et la composition en acides aminés des fractions ont été déterminées. La distribution des masses moléculaires des fractions a été analysée et un degré de polymérisation moyen a été calculé. La composition en oses neutres montre que, sans traitement enzymatique, la totalité de l'arabinose et du xylose est récupérée sous forme d'arabinoxylanes (AX) dans la fraction MEP-untreated HMw. Les AX représentent 50,1 % de la masse totale et 70 % des oses totaux de cette fraction. La fraction MEP-untreated LMw contient majoritairement du glucose (32,3 %) sous forme d'amidon. Le traitement enzymatique augmente la solubilizationdes AX. Ces derniers sont répartis dans les fractions HMw et LMw : 37,7 % dans la fraction MEP-treated HMw et 26,7 % dans la fraction MEP-treated LMw. Le traitement enzymatique réduit aussi la masse moléculaire moyenne, la viscosité intrinsèque et le degré de polymérisation (DP) de la fraction HMw (RF-Tableau 1). Le DP moyen de la fraction MEP-treated LMw (32) indique l'absence d'oligosaccharides (DP< 10).

RF-Tableau 1 : Caractéristiques des fractions à haute (HMw) et à faible masse moléculaire (LMw) produites sans (MPE-untreated) et avec (MEP-treated) traitement enzymatique.

	MEP-untreated		**MEP-treated**	
	HMw	**LMw**	**HMw**	**LMw**
Total des Oses (g/100g**)**	71,2	41,4	60,0	57,7
Rapport* A/X	0,51	nd	0,79	0,38
Somme* A+X	50,1	1,2	37,7	26,7
DP (xylane)	470	nd	227	32
Mw (kD)	247	nd	161	nd
Viscosité intrinsèque (mL/g)	492	nd	303	nd

*Corrigé en tenant compte de l'arabinose issu des arabino-galactanes
nd: non déterminé

Les fractions produites sans et avec traitement enzymatique ont été fermentées *in vitro* en utilisant le contenu caecal de 10 poulets âgés de 4 semaines. Les résultats de fermentation ont été comparés à un contrôle négatif (NC, milieu non supplémenté) et à des contrôles positifs constitués de prébiotiques reconnus (fructo-oligosaccharides : FOS ; xylo-oligosaccharides : XOS). Après 24 h de fermentation en conditions d'anaérobie à 38 °C, la concentration en acides gras à chaîne courte (AGCC) a été mesurée ainsi que celle en lactate. Les résultats (RF-Tableau 2) montrent que les fractions produites par traitement enzymatique augmentent la concentration en AGCC et en lactate par rapport aux fractions produites sans traitement. La fraction MEP-treated LMw permet d'augmenter la production des AGCC au même niveau que les FOS et XOS. Elle induit la plus forte production d'AGCC et provoque une augmentation de 12,74 mM, correspondant à une augmentation de 42,4 % par rapport à la fraction MEP-untreated LMw. Elle favorise surtout la proportion de butyrate et acétate, dont la concentration est multipliée par 2,8 et 1,3 par rapport à la fraction MEP-untreated LMw.

Ainsi, la première partie de ce travail montre que le traitement enzymatique du grain de blé permet d'augmenter la solubilizationdes polysaccharides pariétaux, en particulier des AX, et de réduire la masse moléculaire ainsi que le degré de polymérisation de ces derniers sans produire d'oligosaccharides. Ces fractions, surtout MEP-treated LMw, augmentent la production des AGCC, et en particulier le butyrate, au même niveau que les prébiotiques FOS et XOS.

RF- Tableau 2 : Concentration en acides gras à chaîne courte (AGCC) et en lactate (mM) après la fermentation des différentes fractions de blé, *in vitro*, à 38 ° C pendant 24 h, en anaérobiose, en utilisant le contenu caecal des poulets de chair comme inoculum. Le milieu non supplémenté (negative control, NC), les xylo-oligosaccharides (XOS) et les fructo-oligosaccharides (FOS) ont été utilisés comme témoins. MEP-treated et MEP-untreated : fractions obtenues avec et sans enzyme ; HMw : fraction à haute masse moléculaire ; LMw : fraction à faible masse moléculaire.

		Lactate	Acétate	Propionate	Butyrate	AGCC
NC		0,39^{e}	3,10^{h}	1,15^{d}	1,95^{h}	6,59^{i}
FOS		3,43^{bc}	20,14^{b}	5,56^{a}	9,08^{b}	38,21^{b}
XOS		3,37^{bc}	18,49^{c}	5,25^{a}	9,15^{b}	36,26^{c}
MEP-untreated	**HMw**	4,57^{a}	10,59^{f}	1,36^{d}	3,75^{f}	20,27^{g}
	LMw	4,66^{a}	16,38^{d}	4,45^{b}	4,58^{e}	30,07^{d}
MEP-treated	**HMw**	2,63^{d}	12,35^{e}	2,12^{c}	7,56^{c}	24,66^{e}
	LMw	3,76^{b}	20,93^{a}	5,22^{a}	12,90^{a}	42,81^{a}

$^{a-i}$ Les valeurs de la meme colonne avec des exposants différents sont significativement (P <0,05) différentes.

2- Production pilote de fractions hydrosolubles de blé et fermentation *in vitro* par le microbiote caecal des poulets.

Suite à ces premiers résultats, nous avons souhaité étudier le potentiel de ces fractions dans l'amélioration de la santé animale chez le poulet. Cela nécessitant des quantités importantes de fractions, l'objectif de **la deuxième partie** de ce travail était de mettre au point un protocole expérimental permettant de produire 100 à 150 g des fractions de blé avec et sans traitement enzymatique dans le but de les utiliser ultérieurement lors d'un essai *in vivo*. Les fractions produites à l'échelle pilote ont été analysées et comparées aux fractions produites à l'échelle laboratoire en fermentation *in vitro*, pour étudier leur potentiel sur le microbiote caecal.

Pour la mise au point d'un protocole pilote, le temps d'incubation a été le premier paramètre ajusté. L'incubation a été réalisée avec ou sans MEP en utilisant un rapport de 1/3 p/p blé/eau à 40 °C sous agitation. Chaque heure, un échantillon a été prélevé pour établir l'évolution de la composition en oses neutres et de la distribution des masses moléculaires. La composition des fractions en oses neutres montre que le traitement enzymatique permet de solubiliser une quantité plus importante d'arabinose et de xylose sous forme d'AX, cependant, leur quantité évolue peu entre 2 h et 6 h d'incubation. Les profils HPSEC

montrent que les AX sont répartis en deux sous-populations : des AX de haute masse moléculaire et des AX de faible masse moléculaire. Avec traitement enzymatique, la proportion d'AX de faible masse moléculaire augmente. Cependant, la masse moléculaire et la quantité d'oses neutres demeurent stables entre 3 h et 6 h d'incubation. L'extraction pilote a donc été réalisée pendant 3 h à 40 °C et a permis de produire les fractions WE-WSF (avec enzyme) et WC-WSF (sans enzyme).

Une précipitation fractionnée à l'éthanol a ensuite été choisie pour fractionner à l'échelle pilote ces deux fractions. Cette technique met en jeu à la fois des critères de masse moléculaire et de structure des polysaccharides. Différentes concentrations en éthanol ont donc été testées : 50 %, 60 %, 65 % et 70 %. Les fractions précipitées par chaque concentration ont été récupérées, lyophilisées, pesées et analysées. Ensuite la concentration d'éthanol dans chaque surnageant a été amenée à 80 %, provoquant la précipitation de fractions complémentaires : 50-80 %, 60-80 %, 65-80 % et 70-80 %. Ces nouveaux culots ont été à leur tour récupérés, lyophilisés, pesés et analysés. Sans traitement enzymatique, l'augmentation de la concentration en éthanol a permis de purifier des AX qui sont récupérés en totalité dans la fraction 0-70 %. Le rapport A/X est plus faible pour les fractions de la première étape de la précipitation (0,42-0,48) en comparaison avec celles de la deuxième (0,63-0,89). Avec traitement enzymatique, la quantité d'AX augmente dans le premier précipité en fonction de la concentration de l'éthanol jusqu'à 65 %. Les AX sont répartis entre les deux fractions complémentaires et le rapport A/X n'évolue pas en fonction de la concentration en éthanol. Les profils HPSEC montrent que les fractions produites sans traitement enzymatique, précipitées en éthanol 50 % et jusqu'à 70 % correspondent à des polymères de masse moléculaire importante. Les fractions produites en présence de l'enzyme montrent des profils similaires décalés vers les faibles masses moléculaires. Ces profils montrent également l'absence d'oligosaccharides.

Les concentrations 0-65 % et 65-80 % ont été choisies pour préparer les fractions d'intérêt à l'échelle pilote. Elles permettent de récupérer des quantités équilibrées pour les deux traitements (sans et avec MEP) et des fractions présentant des différences de compositions et de structure.

L'extraction pilote a donc été réalisée pendant 3 h à 40 °C avec et sans traitement enzymatique. Les fractions précipitées en éthanol 65 % ont été nommées WE-1 (blé avec enzyme) et WC-1 (blé contrôle) et les fractions précipitées entre 65 et 80 % d'éthanol ont été nommées respectivement WE-2 et WC-2 (RF- Tableau 3). Les fractions WE-1 et WC-1 sont

essentiellement composées de xylose (34,5 % et 32,2 %, respectivement) et d'arabinose (19,3 % et 19,1 %, respectivement). Leurs contenus en AX sont similaires et le rapport A/X est légèrement plus faible pour la fraction WE-1. Les deux fractions contiennent des quantités équivalentes de glucose, essentiellement issu d'amidon. La fraction WE-1 contient plus de protéines que WC-1 (21 % *vs.* 14 %). La distribution des masses moléculaires des deux fractions est différente. WC-1 correspond à des polymères de volume hydrodynamique plus élevé que WE-1. WC-1 a une masse moléculaire moyenne (176,8 kDa *vs.* 49,6 kDa), une viscosité intrinsèque (215,7 mL /g *vs.* 54,1 mL) et un degré de polymérisation (270 *vs.* 54) plus élevés que WE-1. Ainsi, le traitement enzymatique réduit le DP moyen des chaînes de xylanes sans aboutir à la formation d'oligosaccharides de DP< 10. Les résultats de RMN montrent que les deux fractions présentent des profils similaires de substitution du squelette de xylose par l'arabinose. WE-1 contient 73 % de xylose non substitué, 13 % mono substitué et 14 % di-substitué, tandis que WC-1 en contient respectivement 72 %, 13 % et 15 %.

La caractérisation des fractions WC-2 et WE-2 montre qu'elles contiennent des quantités importantes de galactose, issu des arabino-galactanes, du glucose issu des β-glucanes et de l'amidon, ainsi que des protéines (>30 %). La fraction WC-2 contient une quantité faible d'AX (1,2 %) contrairement à WE-2 (14,8 %).

Les fermentations *in vitro* ont été réalisées comme indiqué précédemment et ont permis de comparer les fractions produites aux échelles pilote et laboratoire (RF- Tableau 4). A nouveau, les FOS et XOS ont été utilisés comme témoins positifs et un milieu non supplémenté (NC) a été utilisé comme témoin négatif. La fraction WE-1 augmente la concentration en AGCC et lactate significativement par rapport à toutes les autres fractions, y compris les FOS et XOS. Elle multiplie la quantité d'AGCC totaux par 1,5, celle de butyrate par 1,8, celle de lactate par 1,7 et celle d'acétate par 1,3 par rapport à WC-1. Elle induit également la production de quantités plus élevées d'AGCC et de lactate que la fraction MEP-treated-LMw produite à l'échelle du laboratoire. Cependant, les deux fractions (WE-1 et MEP-treated-LMw) augmentent la proportion de butyrate jusqu'à 20 % du total des AGCC, alors qu'il ne représentait que 16 % des AGCC pour les fractions obtenues sans traitement enzymatique. La fraction WE-2 produite avec un traitement enzymatique induit une concentration significativement plus élevée d'AGCC totaux (x 1,1), butyrate (x 1,2), propionate (x 1,8) et d'acétate (x 1,1) par rapport à WC-2.

RF-Tableau 3 : Caractérisation des sous-fractions isolées de WE-WSF et WC-WSF par précipitation à l'éthanol (WC-1 et WE-1: fractions insolubles en éthanol 65 %; WC-2 et WE-2: fractions insolubles en éthanol 65-80 %). Quatre répétitions / traitement, les valeurs sont exprimées en moyenne ± écart-type.

Fractions	**WC-1**	**WC-2**	**WE-1**	**WE-2**
Rendement (g/kg de blé)	5,8	5,2	6,25	5,15
Protéines (g/100g)	14,0±0,2	30,0±0,1	21,0±0,1	33,0±0,1
Oses neutres[2] (g/100g)				
Arabinose	19,1±1,6	6,6±0,7	19,3±1,7	7,0±1,1
Xylose	32,2±1,7	0,8±0,1	34,5±2,1	10,6±1,2
Mannose	0,2±0,1	3,3±0,1	0,5±0,1	2,5±0,1
Galactose	3,2±0,4	8,8±0,3	4,4±0,3	4,1±0,6
Glucose	5,2±0,5	12,1±0,5	5,4±0,6	7,6±0,4
Amidon	4,6±0,4	7,5±0,4	5,3±0,3	3,5±0,2
β-glucanes	0,3±0,1	4,4±0,1	0±0,1	3,5±0,1
Total	59,9±4,8	31,7±2,2	64,2±5,2	31,8±3,7
A+X[3]	49	1,2	50,8	14,8
A/X[4]	0,52	nd	0,47	0,39
DP	270	nd	54	29
Masse moléculaire moyenne	176,8	nd	49,6	nd
Viscosité intrinsèque (mL/g)	215,7	nd	54,1	nd

[2]Rhamnose et fucose sont presents à l'état de traces (<0,01 %)
[3]A+X: somme arabinose + xylose avec l'arabinose corrigé en tenant compte de celui issu des arabino-galactanes
[4]A/X: rapport arabinose/xylose avec l'arabinose corrigé en tenant compte de celui issu des arabino-galactanes
nd: not determined

Le traitement enzymatique du blé augmente la proportion des AX solubles dans l'eau et réduit leurs masses moléculaires et leurs degrés de polymérisation. Les fractions produites à l'échelle pilote par précipitation à l'éthanol présentent des différences structurales et chimiques par rapport à celles isolées par chromatographie d'exclusion stérique à l'échelle laboratoire. Dans cette étude, nous avons été en mesure de produire des quantités suffisantes d'AX solubles avec et sans traitement enzymatique ayant un comportement prébiotique *in vitro*. Pour la suite du travail, les fractions WC-1 et WE-1, riches en AX, ont été utilisées dans un essai *in vivo*.

RF-Tableau 4 : Concentration en acides gras à chaîne courte (AGCC) et en lactate (mM) après fermentation des différentes fractions de blé *in vitro* à 38 ° C pendant 24 h, en anaérobiose, en utilisant le contenu caecal de poulets de chair comme inoculum. Le milieu non supplémenté (negative control, NC), les fructo-oligosaccharides (FOS) et les xylo-oligosaccharides (XOS) ont été utilisés comme témoins.

Fractions issues de grains de blé : WC-1 et WC-2 : fractions pilotes blé contrôle insolubles en éthanol 0-65 % et 65-80 %, respectivement ; WE-1 et WE-2 : fractions pilotes blé enzymé insolubles en éthanol 0-65 % et 65-80 %, respectivement ; MEP-treated et MEP-untreated : fractions obtenues avec et sans enzyme à l'échelle laboratoire ; HMW: masse moléculaire élevée ; LMw: faible poids moléculaire.

	Lactate	Acetate	Propionate	Butyrate	AGCC
NC	$0,55^{g}$	$5,48^{g}$	$0,12^{d}$	$0,87^{f}$	$7,01^{f}$
FOS	$2,94^{de}$	$24,67^{ab}$	$2,63^{ab}$	$7,58^{b}$	$37,82^{b}$
XOS	$3,27^{cd}$	$23,69^{b}$	$2,30^{b}$	$7,28^{b}$	$36,54^{b}$
WC-1	$2,81^{de}$	$18,96^{ef}$	$1,32^{c}$	$4,71^{d}$	$27,80^{d}$
WC-2	$3,93^{b}$	$20,08^{de}$	$1,34^{c}$	$3,53^{e}$	$28,88^{d}$
WE-1	$4,93^{a}$	$25,54^{a}$	$2,83^{a}$	$8,41^{a}$	$41,71^{a}$
WE-2	$3,59^{bc}$	$21,99^{c}$	$2,49^{ab}$	$4,40^{d}$	$32,47^{c}$
MEP-untreated HMw	$1,99^{f}$	$18,03^{f}$	$1,25^{c}$	$3,48^{d}$	$24,74^{e}$
MEP-untreated LMw	$2,13^{f}$	$19,35^{ef}$	$1,37^{c}$	$4,30^{e}$	$27,15^{e}$
MEP-treated HMw	$2,47^{ef}$	$20,79^{cd}$	$1,50^{c}$	$6,45^{c}$	$31,20^{c}$
MEP-treated LMw	$3,18^{cd}$	$23,82^{b}$	$2,23^{b}$	$7,22^{b}$	$36,45^{b}$
ETM*	0,565	0,394	0,116	0,196	0,154
P-value	0	0	0	0	0

$^{a-g}$ Les valeurs de la même colonne avec des exposants différents sont significativement ($P < 0,05$) différentes.

*Erreur type de la moyenne

3- Les arabinoxylanes à chaîne courte produits par traitement enzymatique du grain de blé exercent un effet prébiotique pendant la période de démarrage des poulets.

L'objectif de **la troisième partie** de ce travail était d'étudier les effets des AX préparés à l'échelle pilote (WC-1 et WE-1) sur les performances des animaux et leur santé intestinale. Les fractions ont été incorporées (0,1 % p/p d'aliment) dans un régime à base de

blé pour nourrir des poulets de chair (Ross PM3; 12 poulets x 8 enclos x 3 groupes) pendant 2 semaines. Les groupes supplémentés ont été comparés à un groupe témoin non supplémenté (negative control, NC). Le gain de poids (GP) et l'indice de conversion (IC) ont été calculés. A la fin de l'essai (j 14), des échantillons des contenus digestifs et de tissus iléaux et caecaux ont été prélevés sur 24 animaux par groupe. Les tissus iléaux et caecaux ont été utilisés pour mesurer le taux d'infiltration des lymphocytes T. Les tissus iléaux ont été utilisés pour mesurer la densité des cellules L au niveau des cryptes et des villosités ainsi que la longueur des villosités. La concentration en AGCC a été mesurée directement sur les digestats. La fraction WE-1 augmente significativement ($P \leq 0.05$) le GP de 14,7 % après la première semaine après éclosion, et de 5,4 % sur une période de deux semaines, par rapport au NC. Elle augmente aussi la quantité d'aliment ingérée mais ne modifie pas l'IC. L'ajout de WE-1 au régime de base diminue significativement l'infiltration des lymphocytes T dans l'iléon (8,14 % *vs.* 15,86 %) et dans le caecum (6,96 % *vs.* 15,68 %) par rapport à WC-1. Les deux fractions de blé sans et avec traitement enzymatique n'ont pas d'effet sur la longueur des villosités. Les microbiotes caecal et iléal ont été analysés par séquençage 16S en utilisant la région hypervariable V3-V4. Le pyroséquençage montre que, dans le caecum, la fraction WE-1 a augmenté l'abondance de bactéries des familles *Lachnospiraceae* et *Ruminococcaceae*, auxquelles appartiennent des bactéries productrices de butyrate. WE-1 augmente également les espèces *Enterococcus durans* et *Candidatus arthromitus* dans l'iléon. En présence de WE-1, la concentration en AGCC augmente dans le caecum (principalement le butyrate et l'acétate). Cette fraction diminue significativement l'infiltration des lymphocytes-T au niveau des muqueuses caecale et iléale et augmente la densité des cellules-L dans l'épithélium iléal.

Ces résultats montrent que les AX à chaînes courtes présents dans la fraction WE-1 ont modifié la composition du microbiote caecal en faveur des bactéries productrices de butyrate. Le butyrate est connu pour avoir des effets anti-inflammatoires et pour stimuler les cellules L à produire des hormones telles que le glucagon-like peptide-2. Ce dernier est impliqué dans le contrôle de l'intégrité, de la motilité et de la sécrétion de l'épithélium gastro-intestinal, du flux sanguin local, et de l'absorption des nutriments.

4- Conclusion et perspectives

Cette thèse nous a permis dans un premier temps, de caractériser les produits de dégradation du grain de blé par la préparation enzymatique Rovabio®, et ensuite d'étudier

leurs effets sur les performances des poulets pendant la période de démarrage. Les résultats obtenus ont permis d'établir que la préparation enzymatique augmente la solubilizationdes AX, et réduit leur masse moléculaire et leur degré de polymérisation, sans toutefois produire d'oligosaccharides. L'incorporation de la fraction d'AX produite par traitement enzymatique à l'alimentation de poussins, améliore le gain de poids et modifie également le microbiote intestinal pour conduire à une amélioration de la santé digestive.

Une caractérisation poussée des fractions actives nous a permis de déterminer que l'effet positif de la préparation enzymatique sur les performances des poussins est lié à la formation de fractions AX à chaines courtes (SC-AX) et ne nécessite pas la production d'oligosaccharides. Utiliser du blé prétraité en ration de base pour les poussins en phase de démarrage pourrait être une démarche prometteuse. Le fait de prétraiter le blé permettrait d'assurer un taux optimal d'AX à chaînes courtes dans l'aliment. Ces derniers en modifiant le microbiote et en favorisant le développement des bactéries bénéfiques (surtout les productrices de butyrate) favoriseront la croissance des poussins. Cependant, il sera intéressant de savoir comment maintenir cette amélioration de performance tout au long du cycle de production.

Pour mieux comprendre les mécanismes d'action de ces préparations enzymatiques, il pourrait être également intéressant de vérifier l'hypothèse formulée par plusieurs auteurs sur « l'effet cage » exercé par les parois cellulaires. Cette hypothèse suggère que les préparations enzymatiques en dégradant les parois cellulaires, permettent de libérer le contenu cellulaire et augmentent ainsi l'accessibilité des enzymes de l'animal à leurs substrats et par conséquent la disponibilité des nutriments. Néanmoins, cet effet dépend étroitement de l'organisation des tissus dans les matières premières et de la capacité de la préparation enzymatique à dégrader efficacement les structures pariétales des différents tissus des différentes matières premières utilisées dans le régime de base. Dans le cas du blé, la capacité des enzymes à libérer des nutriments de la couche à aleurone reste à étudier.

Afin d'améliorer l'efficacité des enzymes, de nouvelles préparations plus ciblées avec plus d'activités synergiques efficaces sur un spectre plus large de matières premières, céréales (maïs, orge, seigle, blé,…) ou autres pourront être développées grâce au séquençage du génome *Talaromyces versatilis* (www.ncbi.nlm.nih.gov). Ces nouvelles préparations en combinaison avec de nouvelles variétés de céréales (à viscosité faible et énergie métabolisable élevée) pourront améliorer les performances des animaux et réduire les coûts de production.

Titel:

Water-oplosbare kortketenige arabinoxylanen afkomstig van de enzymatische behandeling van tarwe bevorderen de groei en de darmgezondheid bij vleeskuikens.

Samenvatting

Multi-enzym preparaten (MEP) die koolhydraten afbreken, worden vaak gebruikt om de prestaties van vleeskuikens te verbeteren. Het werkingsmechanisme van deze producten is ingewikkeld en nog niet volledig opgehelderd. In het eerste deel van dit doctoraat werd het effect bestudeerd van MEP behandeling op de wateroplosbare bestanddelen die kunnen geëxtraheerd worden uit tarwe. De wateroplosbare fracties (met en zonder MEP voorbehandeling) werden aangemaakt op laboratorium schaal en op semi-industriële (piloot) schaal. Deze fracties werden vervolgens toegevoegd aan een vleeskuiken voeder (op basis van een tarwe – soja formule) aan een concentratie van 0,1 % (in gewicht). Deze rantsoenen werden gevoederd aan vleeskuikens in de eerste veertien dagen na uitkippen. Het effect van deze rantsoenen op de vorming van korte keten vetzuren en op de microbiota samenstelling in de caeca werd onderzocht.

Uit de resultaten van deze onderzoeken bleek dat MEP voorbehandeling van tarwe resulteerde in een toename van de hoeveelheid en in een verlaging van het gemiddeld moleculair gewicht van de wateroplosbare arabinoxylanen (AX) die konden geëxtraheerd worden uit de tarwe. Deze wateroplosbare arabinoxylanen waren echter volgens de definitie nog steeds (weliswaar kortketenige) polymeren (SC-AX / polymerisatiegraad > 10) en geen oligosacchariden. Wanneer deze SC-AX toegevooegd werden aan het voeder van vleeskuikens in de eerste veertien dagen na uitkippen, dan kon vastgesteld worden dat deze kuikens snelle toenamen in gewicht. De microbiota samenstelling in hun caeca was gekenmerkt door een toename in abundantie van de *Lachnospiraceae* en *Ruminococcaceae* families. Deze beide families zijn allebei boterzuurproduceerders. Tegelijk was er ook een toename van de vluchtige vetzuren in de caeca. Er was meer in het bijzonder vooral een toename van acetaat en butyraat. Bovendien was er in de mucosa van de caeca en van het ileum een significante vermindering van het aantal T-lymfocyten. In het epitheel van het ileum was de densiteit van de L-cellen ook significant toegenomen.

Uit deze resultaten kon besloten worden dat de SC-AX die ontstaan bij MEP behandeling van tarwe, wanneer ze toegevoegd worden aan 0,1 % in het voeder van vleeskuikens, de vermeerdering van boterzuur producerende bacteriën in de caeca bevorderen en de inflammatie in de darm verminderen. Dit gunstig effect kan verklaard worden doordat

boterzuur een stimulerend effect heeft op de entero-endocriene L-cellen en een anti-inflammatoire werking heeft. L-cellen produceren hormonen, waaronder het glucagon-like peptide-2, dat de werking van de dunne darm bevordert, wat op zijn beurt kan leiden tot betere absorptie van nutriënten en daardoor betere groei.

Abstract

Carbohydrate-degrading enzyme preparations (MEP) are used to improve broilers performance. Their mechanisms of action are complex and not fully understood. In this PhD work, we first studied the effects of MEP on wheat grain by characterizing the different water-soluble fractions produced with and without enzymatic treatment. These fractions were incorporated in a wheat-based diet (0.1 % w/w) to feed broilers during the first 2 weeks post-hatch to investigate the production of short-chain fatty acids (SCFA), the diversity of intestinal microbiota, and finally the animal performance and intestinal health.

Enzymatic treatment increased the amount of water-soluble arabinoxylans (AX) and reduced their molecular weight. These short chain AX polymers (SC-AX) significantly increased body weight gain and the abundance of bacteria within the *Lachnospiraceae* and *Ruminococcaceae* families in the ceca. Accordingly, the concentration of SCFA, mainly butyrate and acetate, increased in the ceca. In addition, the T-lymphocyte infiltration decreased in the cecal and ileal mucosa while the L-cell density increased in the ileal epithelium.

These results indicate that SC-AX favored the growth of butyrate producing bacteria and decreased inflammation in the intestinal tract. This effect was likely due to butyrate that is known to have anti-inflammatory effects and to stimulate enteroendocrine L-cells. These cells produce hormones with beneficial effect on small intestine function, hereby improving animal performance.

Résumé

Les préparations enzymatiques (MEP) qui dégradent les polysaccharides pariétaux sont utilisées pour améliorer les performances des poulets de chair. Leurs mécanismes d'action sont complexes et encore peu expliqués. Dans ce travail de thèse, nous avons d'abord étudié l'effet de MEP sur le grain de blé. Différentes fractions hydrosolubles produites avec ou sans traitement enzymatique ont été isolées puis caractérisées. Ensuite elles ont été incorporées dans un régime à base de blé pour alimenter des poulets pendant 2 semaines. Leurs effets sur les performances animales et la santé intestinale, ainsi que sur la production des acides gras à chaîne courte (AGCC) et la diversité du microbiote intestinal ont été étudiés.

L'utilisation de MEP augmente la quantité d'arabinoxylanes hydrosolubles en réduisant leur poids moléculaire. Ces arabinoxylanes à chaînes courtes (SC-AX) augmentent significativement le gain de poids des poussins et l'abondance de bactéries des familles *Lachnospiraceae* et *Ruminococcaceae* dans le caecum. En conséquence, la concentration en AGCC, notamment butyrate et acétate, augmente. En outre, l'infiltration des lymphocytes T a diminué dans les muqueuses caecales et iléales tandis que la densité de cellules L a augmenté dans l'épithélium de l'iléon.

Ces résultats indiquent que les SC-AX favorisent la croissance des bactéries butyrogènes et diminuent l'inflammation de l'intestin. Cet effet est probablement dû au butyrate connu pour avoir des effets anti-inflammatoires et pour stimuler les entérocellules-L. Ces dernières produisent des hormones bénéfiques sur la fonction de l'intestin et sur la performance de croissance du poulet en phase de démarrage.

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Thèse de Doctorat

Nadia YACOUBI

Water-soluble short-chain arabinoxylans produced by enzymatic treatment of wheat grain improve growth performance and intestinal health of broilers

Résumé

Les préparations enzymatiques (MEP) qui dégradent les polysaccharides pariétaux sont utilisées pour améliorer les performances des poulets de chair. Leur mode d'action est complexe et encore peu expliqué. Dans ce travail de thèse, nous avons d'abord étudié l'effet de la MEP sur le grain de blé. Différentes fractions hydrosolubles produites avec ou sans MEP ont été isolées puis caractérisées. Ensuite elles ont été incorporées dans un régime à base de blé pour alimenter des poulets pendant 2 semaines. Leurs effets sur les performances animales et la santé intestinale, ainsi que sur la production des acides gras à chaîne courte (AGCC) et la diversité du microbiote intestinal ont été étudiés. La MEP augmente la quantité d'arabinoxylanes à chaine courte (SC-AX) hydrosolubles en réduisant leur poids moléculaire. Les SC-AX produits par traitement enzymatique augmentent significativement le gain de poids des poussins et l'abondance de bactéries des familles Lachnospiraceae et Ruminococcaceae dans le caecum. En conséquence, la concentration en AGCC, notamment butyrate et acétate, augmente. En outre, l'infiltration des lymphocytes T a diminué dans les muqueuses caecales et iléales tandis que la densité de cellules L a augmenté dans l'épithélium de l'iléon. Ces résultats indiquent que les SC-AX favorisent la croissance des bactéries butyrogènes et diminuent l'inflammation de l'intestin. Cet effet est probablement dû au butyrate connu pour avoir des effets anti-inflammatoires et pour stimuler les entérocellules-L. Ces dernières produisent des hormones bénéfiques sur la fonction de l'intestin et l'amélioration des performances du poulet en phase de démarrage.

Mots clés
Xylanase**,** butyrate, Lymphocyte-T, Cellule-L, microbiote

Abstract

Carbohydrate-degrading enzyme preparations (MEP) are used to improve broilers performance. Their mode of action is complex and not fully understood. In this PhD work, we first studied the effects of MEP on wheat grain by characterizing the different water-soluble fractions produced with and without MEP. These fractions were incorporated in a wheat-based diet (0.1% w/w) to feed broilers during the first 2 weeks post-hatch to investigate the production of short-chain fatty acids (SCFA) and the diversity of intestinal microbiota, and finally the animal performance and intestinal health. Enzymatic treatment increased the amount of water-soluble arabinoxylans (AX) and reduced their molecular weight. Nevertheless, degradation products were short chain AX polymers (SC-AX) and not oligosaccharides. SC-AX produced by enzymatic treatment significantly increased body weight gain and the abundance of bacteria within the Lachnospiraceae and Ruminococcaceae families in the ceca. Accordingly, the concentration of SCFA, mainly butyrate and acetate, increased in the ceca. In addition, the T-lymphocyte infiltration decreased in the cecal and ileal mucosa while the L-cell density increased in the ileal epithelium. These results indicate that SC-AX favored the growth of butyrate producers and decreased inflammation in the intestinal tract. This effect was likely due to butyrate known to have anti-inflammatory effects and to stimulate enteroendocrine L-cells that produce hormones with beneficial effect on small intestine function, hereby improving animal performance.

Key Words
Xylanase, butyrate, T-lymphocyte, L-cell, microbiota