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The use of exogenous xylanase and dietary fibre for modern broiler chicken production

By

Katya Petkova Dimitrova

Department of Animal Production, Welfare and Veterinary Sciences

Director of Studies: Professor Vasil Pirgozliev
Supervisors: Dr Paul Rose and Dr Stephen Mansbridge

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Abstract

Xylanase is extensively used as an exogenous enzyme in poultry diets due to its ability to reduce digesta viscosity, increase energy and nutrient availability and promote beneficial caecal fermentation through the generation of prebiotic xylooligosaccharides (XOS). Based on the premise that released XOS from feed ingredients may be more important for gut health and bird performance than the removal of antinutritional factors alone by xylanase, the objective of this thesis was to evaluate the best strategy for using xylanase for broiler chicken production. Parameters assessed included: growth performance, nutrient availability, metabolisable energy, gastrointestinal tract development and caecal short-chain fatty acids (SCFAs) concentration in Ross 308 male broiler chickens. In low fibre diets (wheat-maze based), both xylanase and SIGNIS® fed at commercially recommended levels provide beneficial effects through better utilisation of nutrients and increased metabolisable energy. However, this may not directly translate to improved bird growth performance. Changes in caecal SCFAs concentration may be indicative of the prebiotic effect on the microflora associated with XOS generated through xylanase supplementation or as supplied in SIGNIS®. Further research on the impact of dietary fibre on digesta transit time, endogenous enzyme activity and nutrient utilisation might better elucidate the mode of action of dietary fibres for improving gut health in poultry.

Declaration and Acknowledgements

I declare that the work presented was carried out by the author alone. It has not previously been submitted before to qualify for any other academic degree. The thesis contains the author's interpretations and suggestions about the subject. The references and any other used materials were cited according to the recommendation of HAU Guide to Referencing - 2018/2019. Some of the results from the study were presented at the WPSA (UK Branch) Annual Meeting (held in Edinburgh, Scotland, 10th/11th April 2019):

K. Dimitrova, S.P. Rose, S.C. Mansbridge, G. Gonzalez, M.R. Bedford, V. Pirgozliev, Feeding exogenous xylanase to young broiler chickens. Proceeds to the World`s Poultry Science Association (UK branch). *British Poultry Abstracts*, 2019, 15(1), p.39.

One abstract was accepted for poster presentation for the WPSA (UK Branch) Annual Meeting (Nottingham, UK which was planned on 30thMarch/1stApril but due to COVID-19 pandemic was cancelled):

Dimitrova, K., Gonzales-Ortiz, G., S.P., Mansbridge, Rose, S.C., Bedford, M.R., Pirgozliev, V. The impact of dietary xylanase on energy availability and caecal fermentation when fed to broilers

The following abstracts were accepted by The Scientific Committee for poster presentation at World Poultry Congress, Paris which due to COVID-19 pandemic was postponed until August 2021:

Dimitrova, K., Gonzales-Ortiz, G., S.P., Mansbridge, Rose, S.C., Bedford, M.R., Pirgozliev, V. Xylanase can improve metabolisable energy of the wheat-maze diet and increase caecal contents of short-chain fatty acids when fed to broiler chicken (reference number n 261)

Dimitrova, K., Gonzales-Ortiz, G., S.P., Mansbridge, Rose, S.C., Bedford, M.R., Pirgozliev, V. Combination of xylanase and xylooligosaccharides improves feed efficiency in broilers" (reference number n 262)

List of common abbreviation

AA	Acetic acid
AGPs	Antibiotic growth promotors
AME	Apparent metabolisable energy
AMEn	Nitrogen corrected apparent metabolisable energy
ANF	Anti-nutritional factors
ANOVA	Analysis of variance
BA	Butiric acid
BW	Body weight
CD	Crypth depth
CF	Crude fibre
CW	Crypth width
DF	Dietary fibre
DM	Dry matter
DMR	Coefficient of dry matter retention
EFSA	European Food Safety Authority
FI	Feed intake
FR	Fat retention
GIT	Gastrointestinal tract
LA	Lactic acid
NR	Nitrogen retention
NSP	Non-starch polysaccharides
P	Phosphorus
PA	Propionic acid
SCFAs	Short-chain fatty acids
VA	Valeric acid
VFAs	Volatile fatty acids
VH	Villus hight
VH:CD	Villus hight to crypth depth ratio
VW	Villus width
XOS	Xylooligosaccharides

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Chapter 1. General introduction

Feed represents approximately 70 % of the total cost of broiler chicken production (Acamovic, 2001). Increasing prices of conventional feedstuffs, e.g. soya beans, wheat and maize, makes alternative crops and by-products attractive options (Alagawany *et al.*, 2018). Nutrient availability in raw feedstuffs can be reduced due to complex cell wall structure and anti-nutritional factors (ANF) such as phytate, protease inhibitors and non-starch polysaccharides (NSP). The main impediment for complete digestion of these ingredients is the insufficient quantity or the absence of specific endogenous enzymes in the chicken gastrointestinal tract (GIT) (Acamovic, 2001). The use of exogenous enzymes is efficient at alleviating the negative effects of ANF in the feed (Aftab and Bedford, 2018).

Pioneering research on dietary supplemented exogenous enzymes started in the 1950s and are among the most widely studied and reported subjects in animal science. Exogenous feed enzymes affect energy, nitrogen, amino acid and mineral metabolism (Adeola and Cowieson, 2011), thus are expected to improve animal growth performance (the most important for the producer), nutrient utilisation and reduce nutrient excretion (Bedford, 1995). The most important effects of exogenous enzymes are: 1) hydrolysis of specific chemical bonds in feedstuffs; 2) alleviation of nutrient-encapsulating effect (cage effect); 3) breakdown of anti-nutritional factors; 4) solubilisation of insoluble NSP.

Global enzyme demand was forecast to increase from $5.8 billion to $11.3 billion, from 2010 to 2020 as the demand for industrial enzymes increases due to the animal feed, food and biofuel market segments (Kumar *et al.*, 2014).

According to Costa *et al.* (2008) there are at least two different practical approaches to enzyme incorporation into the feed. In the simpler approach, enzyme is provided in addition to the complete formulated feed (on the top) without any changes to the nutrient matrix. The second approach involves substitution of nutrients with the enzyme to obtain the same animal performance expected from the completely formulated diet. Zou *et al.* (2013) assessed the benefit (in %) of two basal diets fed to broiler chickens – one deficient in metabolisable energy (NC) and the other energy sufficient (PC). Both diets were designed with or without enzymes such as

β-mannanase (BM), α-galactosidase (AG) and a combination of xylanase and β-glucanase (XG). The effect of enzymes was pronounced in diets deficient in metabolisable energy, with the greatest profit obtained with xylanase and β-glucanase (XG) (Figure 1.1).

Deficient diet (NC)   vs.   Sufficient diet (PC)
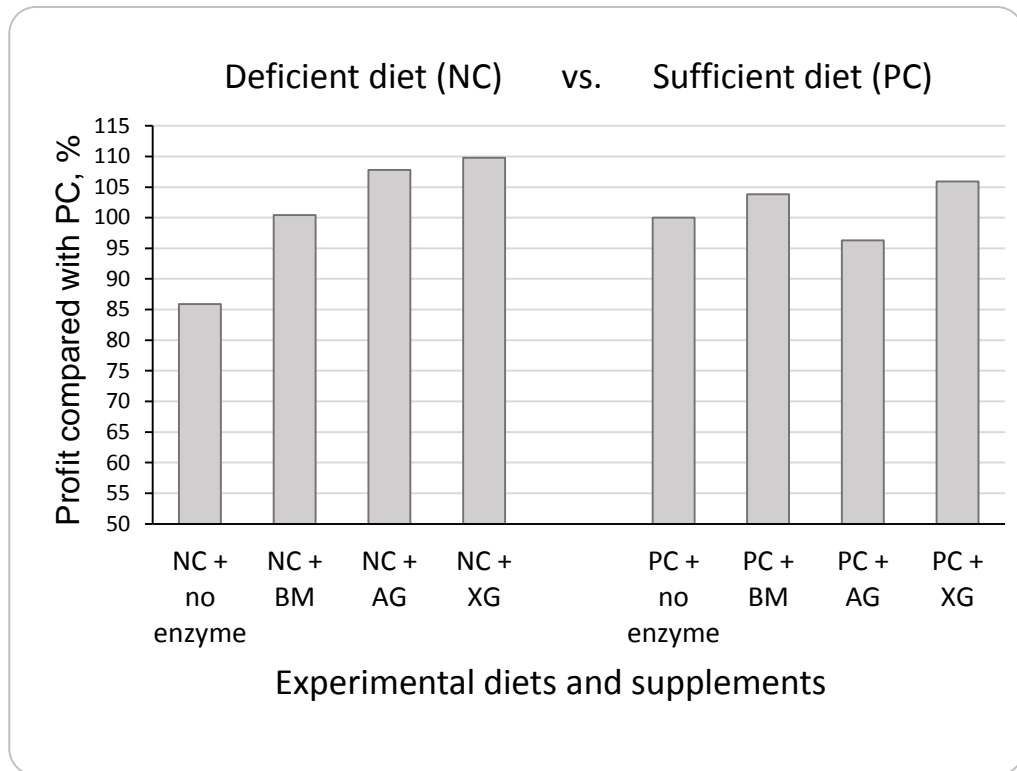
(Source: adapted from Zou *et al.,* 2013)

Figure 1.1: Comparison of economic profit of diets supplemented with β-mannanase (BM), α-galactosidase (AG) or combination of xylanase and β-glucanase (XG) when fed to broiler chickens.

A simple search of the European patent office using the key word "xylanase" provided 19372 results (Espacenet, not dated). The number of patents indicates that there is interest in xylanase and its applications. Xylanase as a feed additive is beneficially associated with increased nutrient and energy utilisation, reduction of gut viscosity, improving dietary nutrient digestibility, modulating the gut microbial population and increased caecal fermentation (Bedford, 1997; Choct *et al.*, 1999; Khadem, 2016a). Nian *et al.* (2011) found that xylanase increases the metabolisable energy of feed likely due to increased hemicellulose digestibility. Exogenous xylanase can reduce gut viscosity through its activity on β-glucans and arabinoxylans. In addition to the complete hydrolysis of fibres, there is a release of

xylooligosaccharides (XOS). These oligosaccharides can be suitable substrates for resident gut microflora, supporting their metabolism (Bedford, 1995). Other factors impacting enzyme activity are feed particle size and the addition of different dietary fibre substrates (Amerah *et al.*, 2007). Kheravii *et al.* (2017) found an improvement in feed conversion ratio, nutrient digestibility and litter quality when birds were fed pelleted diets containing coarsely ground corn and lignocellulose. The interaction between xylanase and other exogenous enzymes is possible and may be synergistic with endogenous enzymes (amylase, protease, lipase etc.) (Simon, 1998; Kiarie *et al.*, 2013). Further research will increase the understanding of exogenous feed enzymes on animal health, nutrition and performance (Adeola and Cowieson, 2011; Sarrouh *et al.*, 2012).

Proliferation of a beneficial microbial population in the chicken digestive tract is considered to be an essential mechanism which does not simply affect efficient nutrient utilisation but also affects bird health (Stanley *et al.*, 2014). Currently, along with exogenous enzymes, the importance of prebiotics (dietary fibre that selectively stimulate beneficial gut bacteria), probiotics (viable beneficial microorganisms), nutraceuticals (compounds that have beneficial effects) and dietary fibre (DF), have increased immensely. As feed additives, they could be promising alternatives to antibiotic growth promoters (AGPs) (Huyghebaert *et al.*, 2011). However, the mode of action of both feed additives and AGPs are not fully understood (Bedford, 2000b). Despite the well documented efficacy of xylanase there are unanswered questions about the dose dependant effects and enzyme mode of action (Kiarie *et al.*, 2013). In addition, other difficulties arise because xylanase, similar to other feed additives, acts in the gastrointestinal ecosystem which is a highly complex and intricate environment comprising of epithelial cells (the mucosal barrier), mucosal immune system, microbiota and its products (Allen *et al.*, 2013; Ari *et al.*, 2016).

Xylanase and other exogenous enzymes begin to exert their activity on substrates contained in the feed in the crop. However, their interactions with dietary ingredients and especially with the fibrous materials continue through the intestines and as a result could increase fermentation in the caeca. The appropriate blend of exogenous enzymes and feed materials can result in a significant increase in total volatile and short-chain fatty acids (Choct *et al.*, 1999). Caecal fermentation may not be important during the first weeks of life of the broiler chicken (Svihus *et al.*, 2013).

However, there is data to suggest that early supplementation of xylanase may elicit a greater response during the first four weeks of age because the gut of the younger chickens is underdeveloped and struggles with the utilisation of diets with high cereal content (Chiang *et al.*, 2005). Research has been done to test the effects of the direct addition of exogenous enzymes to animal diets, but *in vivo* results may not be parallel to all of the *in vitro* findings (Graminha *et al.*, 2008). Considering limitations and constraints of gut microbiota analysis, the caeca fermentation products such as short-chain fatty acids (SCFAs) can be successfully used for assessment of processes in the gut (Rychlik, 2020). Aftab and Bedford (2018) also considered that analysis based on alternative responses e.g. gut morphology, nutrient or energy digestibility, gut-flora and its metabolites or fermentation profiles could be very useful to develop a wider understanding of usefulness of a particular enzyme. However, the interpretation of data requires clear and systematic criteria.

1.1.   Objective of the study

Based on the premise that released XOS from feed ingredients may be more important for gut health and bird performance than the removal of ANF by xylanase enzymes alone, the objective of this thesis is to evaluate the best strategy for using xylanase for broiler chicken production. Specifically, the hypotheses being tested in this thesis are:

1. Supplementing a combination of xylanase and XOS in wheat-maze based diets could have a more pronounce effect than xylanase alone, on broiler production performance, gut health and dietary nutritional value.

2. Increasing dietary NSP content as a substrate for the commensal gut microflora improves gut health and bird performance, also enhancing the efficacy of supplementary xylanase and XOS.

Chapter 2. Lierature review

2.1.   Introduction

The poultry sector is considered the fastest growing and the most adaptive of all livestock sectors (McLeod *et al.*, 2009). This trend is driven by the global demand for meat particularly due to the consumer perception that poultry meat is a good source of protein with low-fat content and is an attractive choice in a healthy diet (Walley *et al.*, 2014). The steady increase of poultry meat consumption (Figure 2.1) shows that until 2030, in comparison to 1964/66, consumption in developing countries is expected to increase from 1.2 to 14 kg per capita.
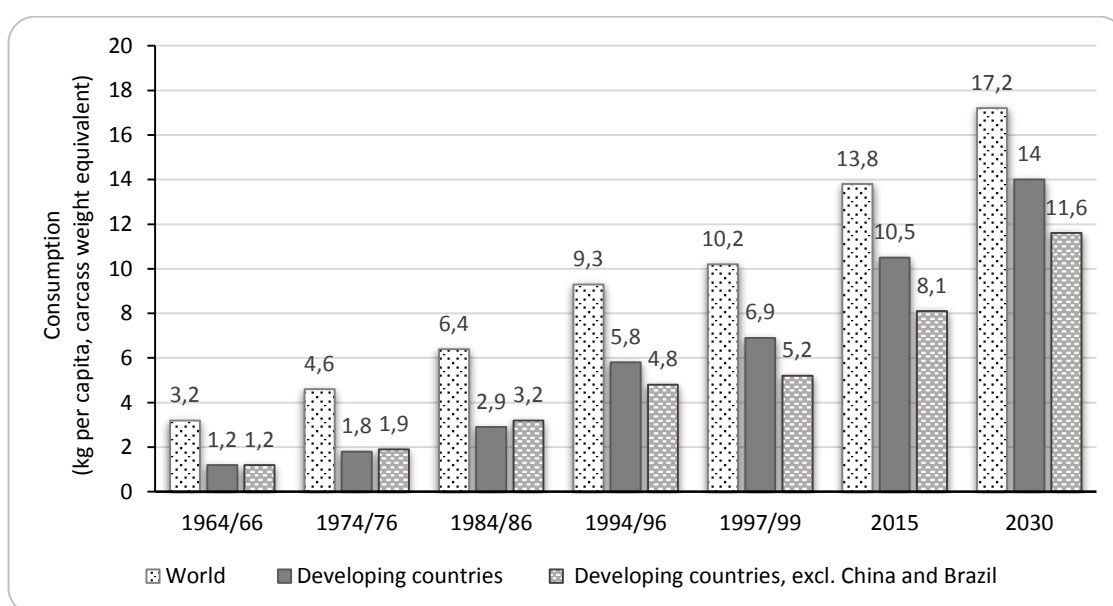
(Source: adapted from Bruinsma, 2003)

Figure 2.1: Global trend in poultry meat consumption (per capita) till 2030.

Annual poultry meat consumption per capita in 2018 in the UK exceeded pork and beef/ veal consumption by 10.8 kg and 18.3 kg, respectively (Figure 2.2).
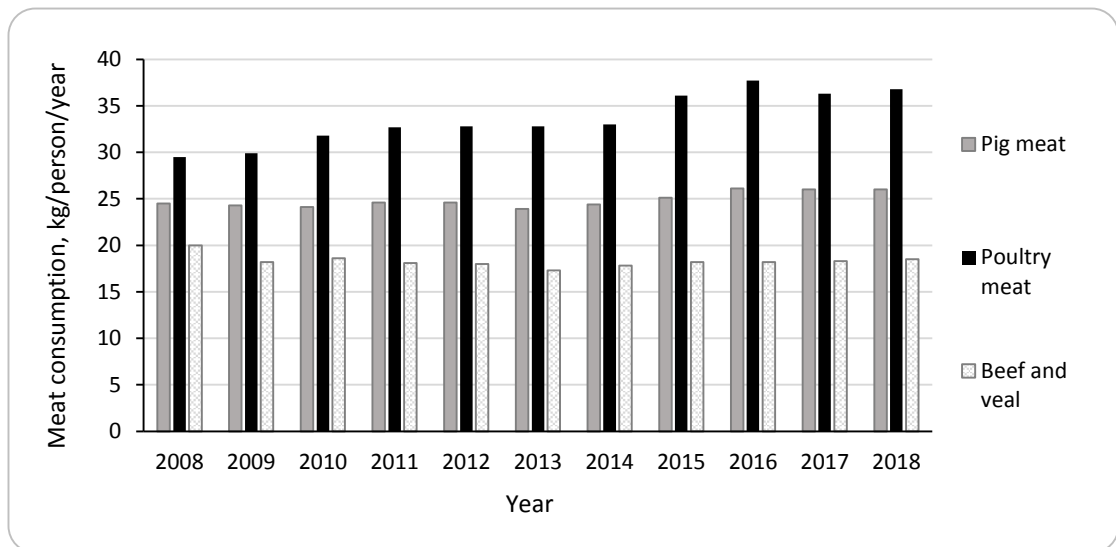
Figure 2.2: Annual consumption of major meats in UK (kg/person).

Table chickens (broilers) comprise 65 % of the total UK poultry population (Figure 2.3).
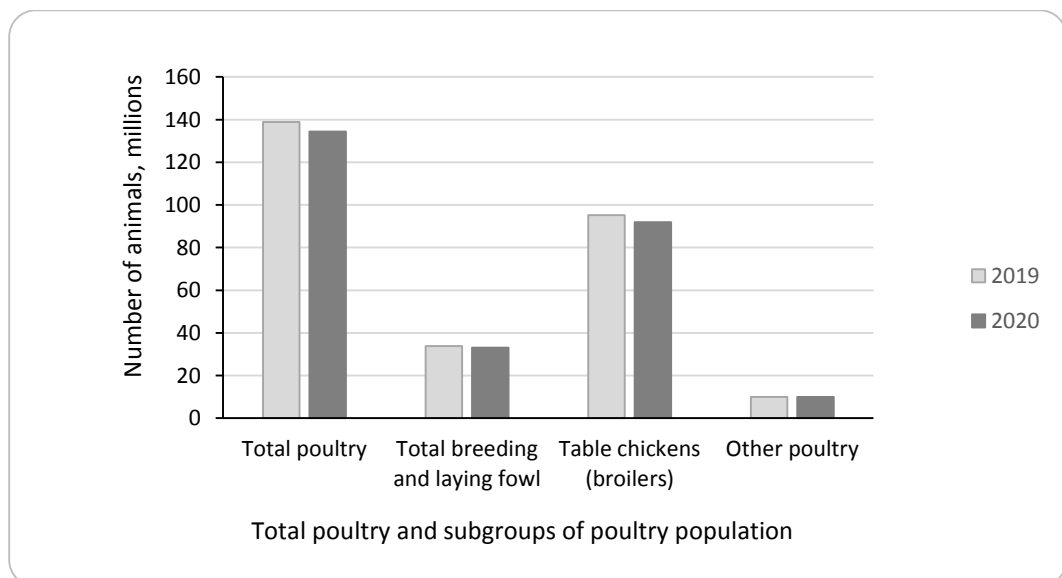
(Source: DEFRA - farming statistics, 2020)

Figure 2.3: Total poultry and subgroups of poultry population at June 2019 in UK commercial agricultural holdings.

According to Godley and Williams (2007) the poultry industry has been transformed due to series of critical innovations: in poultry breed selection, in nutrition, in accommodation of larger flocks, in slaughtering and processing, in retailing. The genetics of commercial broilers have been improved by implementation of successful breeding programmes. The precision of feed formulation has been increased through better knowledge of the physiological role and appropriateness of different ingredients, along with the choice of vitamins and mineral supplements. Vaarst *et al.* (2015) discussed the importance and sustainability of poultry production through environmental, social, institutional and economic aspects.

Havenstein *et al.* (2003) illustrates the margin of improvement in broiler performance parameters accomplished over a 40-year period between 1957 and 2000 – increased body weight (0.814 vs. 3.538 kg) and better feed conversion ratio (2.78 vs. 1.98). Havenstein *et al.* (2003) reported not only significant improvements in performance parameters but also an increase in mortality by approximately 4 % in 2001 compared to 1957. The higher mortality of strain representing 2001 were associated primarily with severe leg problems. In general, such problems are very common for contemporary fast-growing strains widely used in the modern poultry farms. In accordance with these findings, Tallentire *et al.* (2018) suggested that further improvement in body weight of broilers is not possible because of natural biological limits (e.g. genetic potential). The increasing number of health issues such as sudden death syndrome, ascites, lameness and contact dermatitis directly detracts from optimal broiler performance (Knowles *et al.,* 2008; Bessei, 2006). These welfare problems challenge the broilers' ability to withstand any additional internal and external stress factors resulting in new concept such as maintaining gut health (Kogut and Arsenault, 2016) and environmental enrichment (Riber *et al.,* 2018).

In poultry husbandry, expenditure on feed is usually estimated at about 70-75 % of the total production cost (Ravindran, 2013) but in some cases it can reach 95 % (Hussein *et al.,* 2014). These data clearly indicate the necessity to find, evaluate, and use alternative and cheaper feed ingredients in poultry feed or to increase the nutritional value of the available ones (Sheldon, 2000; Walters *et al.,* 2018). The proper and appropriate choice of available feedstuffs and feed additives can result in the production of new and improved feed formulations.

Laboratory analysis reveals that there are considerable variations of the feed constituents even when conventional ingredients are used (Mateos *et al.*, 2019). This variability is due to factors such as plant cultivar, growing conditions, harvesting year and processing (Aftab, 2012; Azhar, 2019). To deal with the variability in quality of feed ingredients, the use of exogenous enzymes is one of the best options. Exogenous enzymes, in particular carbohydrases, can increase the availability of energy and nutrients and thus increase chicken performance (Aftab, 2012). During the last decade phytase and xylanase became important feed supplements for non-ruminant animals (Mullaney *et al.*, 2000; Dersjant-Li *et al.*, 2015). The use of exogenous xylanase is associated with three main outcomes: reduced viscosity, disruption of the plant cell wall and the generation of prebiotics (Bedford, 1995; Acamovic, 2001; Aftab, 2012). However, some of the effects of exogenous enzymes have been explained solely on the basis of empirical data obtained from performance objectives experiments. In order to reveal underlying mechanisms further research will need to deal with biochemistry and genetics aspects.

2.2.   Feed materials – carbohydrates and fibres

Main materials used in the manufacture of poultry feed can be summarised into several categories (Figure 2.6). Feed materials supply energy, protein, fat and carbohydrates which are needed either for the metabolism and maintaining the physiological functions of the body or to serve as building blocks for bones and muscles. Traditionally, the most used energy and protein sources are maize and soybean meal. Cereals, like wheat and sorghum, and some plant protein meals are also used globally. Cereal grains, with their high content of carbohydrates, are the main source of energy in the feed (Pirgozliev *et al.*, 2010). Soybean meal (SBM), typically with a crude protein content of 40–48 % (depending on the quantity of hulls removed and the oil extraction process) is also a preferred protein source used in poultry feed manufacturing (Beski *et al.*, 2015). Soybean protein in comparison to the protein meal of other oilseed grains is used due to its balanced amino acid profile (Ravindran, 2013). A variety of feed additives, vitamin and mineral premixes and source of dietary fibre are an important part of modern poultry feed formulations (Pirgozliev *et al.,* 2019; Aviagen, 2019).
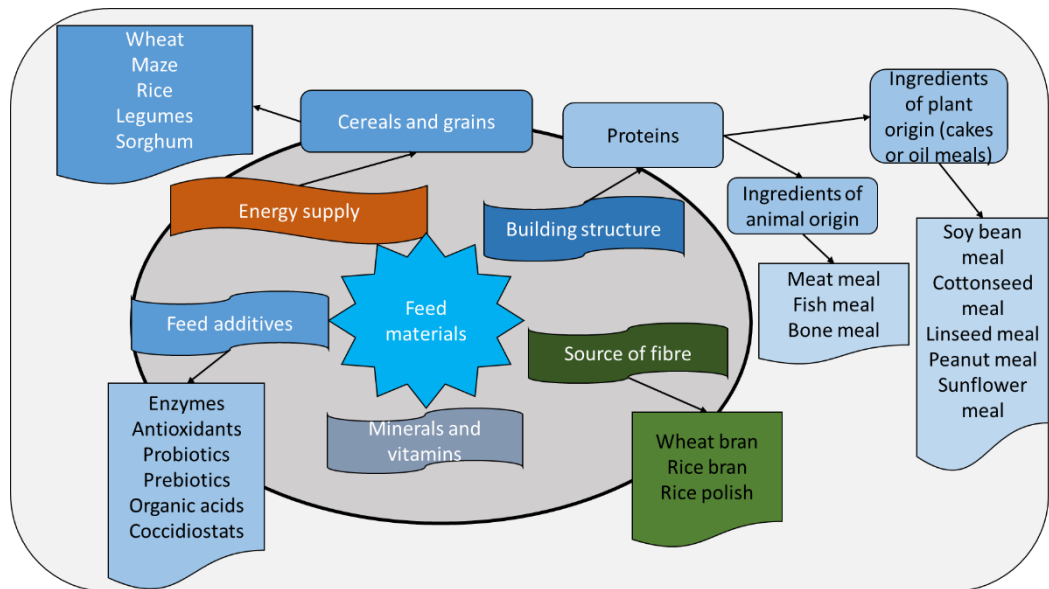
Figure 2.4: Main materials in poultry feed.

Grains are an indispensable part of the poultry feed formulation thus the structure and chemical composition of the grain seed attracts lot of attention (Figure 2.5). The seed is divided into embryo, aleurone or bran, endosperm and seed coat/hull and each seed layer contain different proportions of starch granules and fibres (Raza *et al.,* 2019). The use of new alternative ingredients in poultry feed depends on the contents of ANF and/or fibrous materials in them, biological benefits, market costs and availability (Ravindran, 2013; Abdollahi *et al.*, 2016).
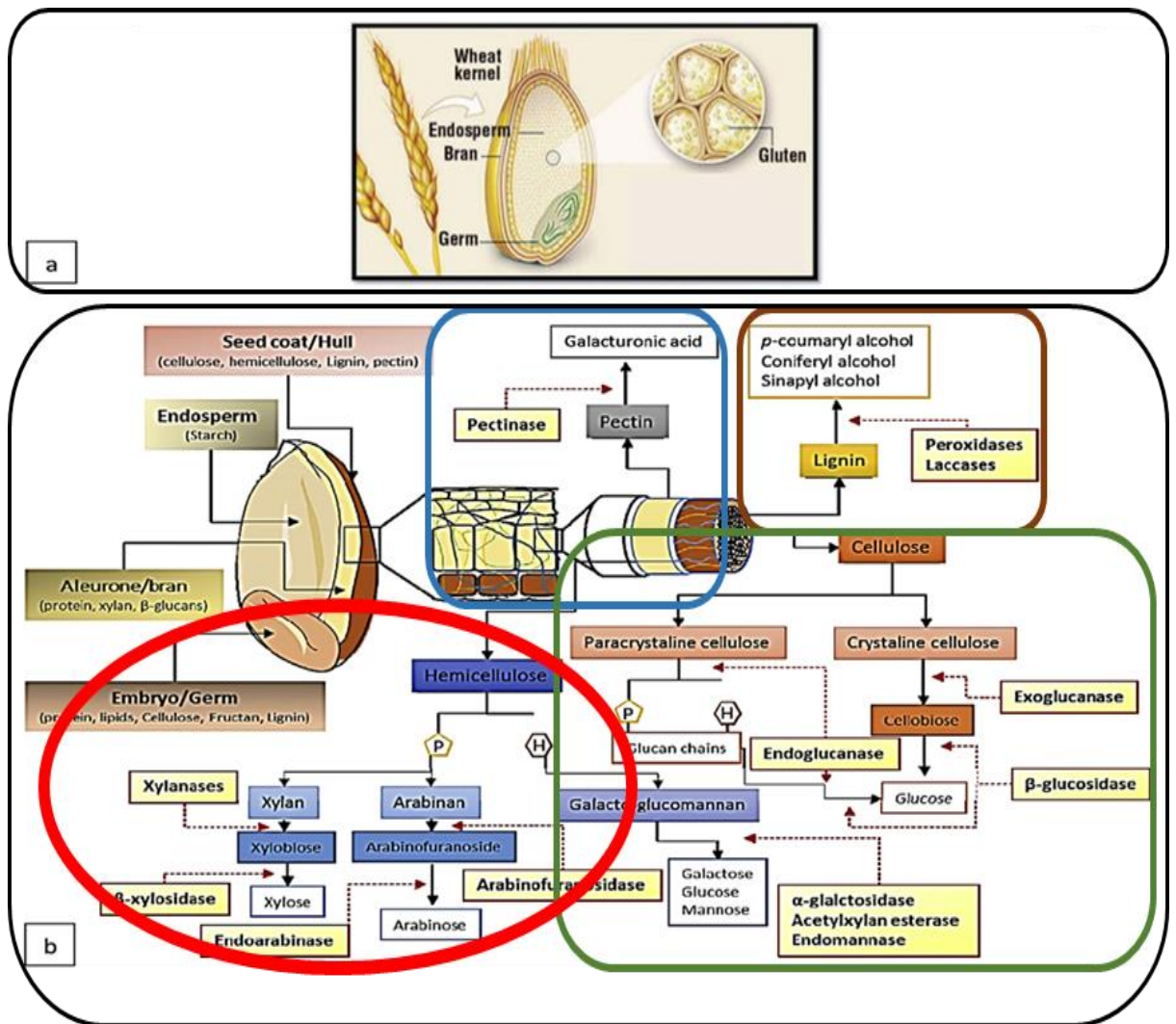
Figure 2.5: Wheat grain general view (a) and the main hydrolytic enzymes which decompose wheat grain materials (b).

Bach Knudsen (1997) analysed 115 samples of 38 different feed materials from the Danish and European feedstuff market for dietary fibre (DF), Klason lignin and total NSP content by applying different methods (Figure 2.6). The soluble and insoluble non-cellulosic polysaccharides in NSP content was estimated in free starch extract and after removal of cellulose.

Starch, lignin and dietary fibre content in whole grain cereals
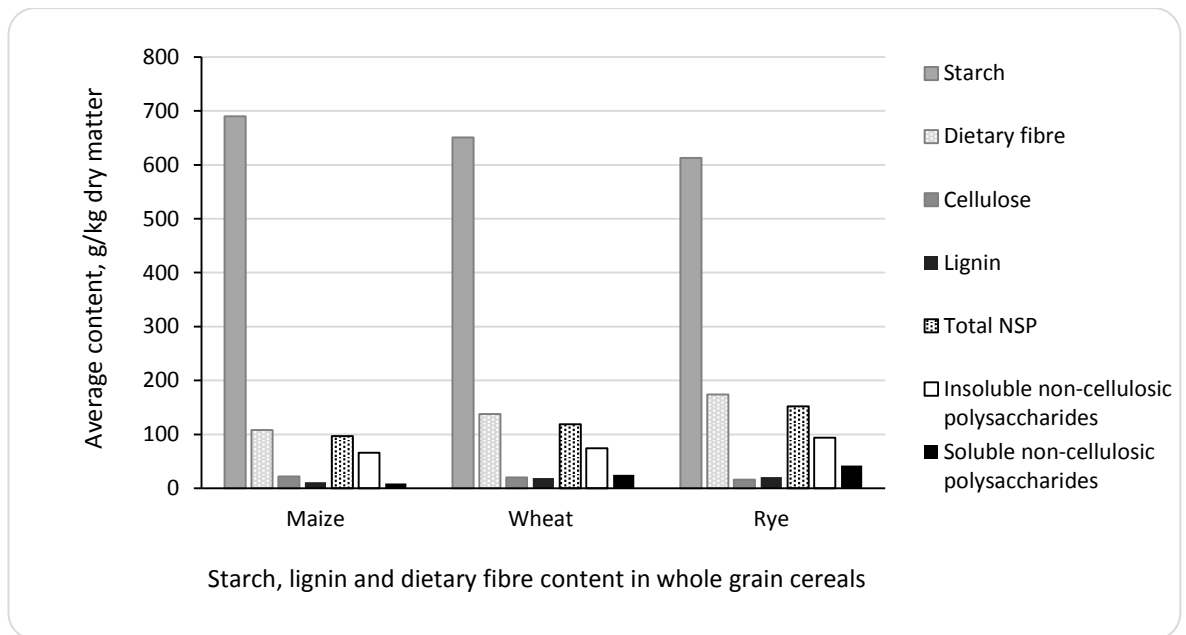
Figure 2.6: Content (g/kg dry matter) of starch, lignin and dietary fibre (DF) in selected whole grain cereals.

Choct (2015a) reviewed the current progress in classification, methods of analysis and function of dietary fibre. He defines DF as the sum of NSP and lignin, and the entity of NSP is comprised of cellulose, non-cellulosic polysaccharides and pectic polymers. Furthermore, the author stressed that non-cellulosic polysaccharides include, but are not limited to arabinoxylans (pentosans), mixed-linked b-glucans, mannans, galactans, xyloglucans and fructants.

The primary choice for inclusion of feed materials in poultry diets is based on their importance as a source of energy and proteins. However, the specific content of fibre and/or some other components should also be considered. Based on the predominant type of extractable fibre, cereal grains have been classified into – viscous and non-viscous. Wheat, rye, barley, triticale and oats are considered viscous cereals, whereas maize, sorghum, rice and millet are considered non-viscous (Choct, 2015a). However, the digestibility of NSP does not depend solely on viscosity but also on other factors such as processing technology, particle size, solubility and hydration properties (De Vries *et al.*, 2012). Dietary NSP contain ANF properties, which can cause adverse effects such as reduced nutrient and energy availability. Table 2.1 presents the most important ANF found in plant materials which are used in poultry feed.

Table 2.1: Anti-nutritional factors in poultry feed materials

Feed material	Anti-nutritional factors
Maize	lectins, phytate, resistant starch, trypsin/a-amylase inhibitors
Wheat	arabinoxylans, wheat germ agglutinin, phytate, resistant starch, trypsin/a-amylase inhibitors, tannins
Barley	β-glucans, resistant starch, trypsin/a-amylase inhibitors, tannins
Rice	phytate, arabinoxylans, trypsin/a-amylase inhibitors
Sorghum	tannins, resistant starch
Rye	arabinoxylans, polyphenols
Triticale	phytate, NSPs
Soybean meal	oligosaccharides and NSPs, trypsin and chymotrypsin inhibitors, lectins
Peas	resistant starch, proteins, saponins, , trypsin inhibitors
Oat	phytate, β-glucans, resistant starch
Beans	tannins, trypsin inhibitors, lectins, oligosaccharides, NSPs, α-amylase inhibitors
Lupins	oligosaccharides, NSPs, phytate, phenolic compounds, some proteins
Rapeseed meal	oigosaccharides, NSPs, tannins, glucosinolates
Sunflower meal	oligosaccharides, NSP

(Source: Acamovic, 2001; Madsen and Brinch-Pedersen, 2016)

2.3. Cell wall components as a major source of fibre

The plant cell wall is arranged in layers and contains NSP (up to 90 %) and lignin. Very often cellulose, hemicellulose and lignin as cell wall building polymers are defined with the term lignocellulose (Houfani *et al.*, 2020). The term hemicellulose was originally proposed in 1891 by Schulze to designate extractable by aqueous alkaline solutions polysaccharides from higher plants through a method which was not applicable for the extraction of cellulose. These extractable polysaccharides were mistakenly regarded as the precursors of cellulose, but the term hemicellulose is still commonly used. Nowadays the term does not include pectic polysaccharides which can be extracted from plant materials by hot water, weak acids or chelating agents (Ebringerova *et al.*, 2005). According to Caprita *et al.* (2010) the types of plant material that are included within the definitions of DF may be divided into two

groups based on their water solubility. The first group - insoluble dietary fibre (IDF) - includes celluloses, some hemicelluloses and lignin and the second group - soluble dietary fibre (SDF) - includes *β*-glucans, pectins, gums, mucilages and some hemicelluloses. The IDF and SDF compounds, apart from lignin, are known collectively as non-starch polysaccharides (NSP). Englyst *et al.* (2007) referred to NSP, resistant short-chain carbohydrates and sugar alcohols as resistant carbohydrates. Main characteristics of resistant carbohydrates includes resistance to digestion in the small intestine, slow metabolism and/or poor absorption, specific functional properties. Figure 2.7 illustrates the main constituents of the NSP entity based on their water solubility.
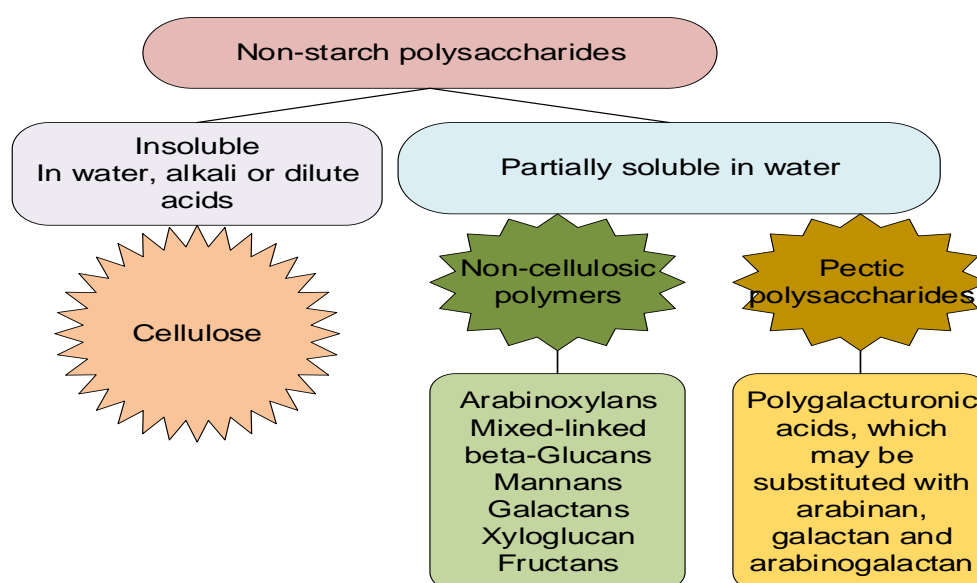
(Source: adapted from Choct, 2002)

Figure 2.7: Non-starch polysaccharides (NSP) classification based on their water solubility.

The study of Austin *et al.* (1999) analysed 12 grain samples and estimated the quantity of total NSPs within the range 87.6–129.2 g/kg, with a mean value of 104.3 g/kg/dry matter. The NSP content varies not only between different feed ingredients but also within the same ingredient due to geographical location where the plant (cereal grains, legumes or oil seed) is grown (Choct, 1997). Cell wall polysaccharides are built from a limited number (around 10) of common monosaccharides but a huge variety in their chemical composition is possible. Firstly, each of the monosaccharide can exist in 2-ring isomer form (pyranose and furanose); secondly, these residues can be linked through glycosidic bonds at any

one of their 3, 4, or 5 available hydroxyl groups; and thirdly, the residues attached to the molecule bone can be placed either in 2 orientations (*α* or *β*). As a result, polysaccharides form different stereoisomers or 3-dimensional shapes and therefore a lot of functional surfaces became available for further interactions. Hydrophobic surfaces, as an example, are shaped when NSP are linked to lignin and suberin. In addition, the charged groups on polysaccharides (i.e., the acid group on uronic acids) and their degree of the esterification affect the ionic properties of the polysaccharide chain (Bach Knudsen, 2014). Some researchers added lignin to the water-insoluble fraction along with galactomannans, xylans, xyloglucans despite the fact that lignin is not a carbohydrate (Caprita *et al.,* 2010).

Hemicelluloses are branched polymers of low molecular weight with a degree of polymerization of 80-200 and are comprised of various different sugar units, arranged in different proportion and with different substituents. The principle sugars are pentoses (D-xylose and D-arabinose) and/or hexoses (D-mannose, D-glucose, and D-galactose) with xylose as the most abundant sugar (Houfani *et al.*, 2020). In addition, uronic acids (D-glucuronic, D-galacturonic, and methylgalacturonic acids) and to a lesser extent, L-rhamnose, L-fucose, and various O-methylated neutral sugars may also be present in branched chains (Sun *et al.*, 2003). When the main backbone is comprised of *β*-1,4-linked D-xylopyranosyl residues the most common substituents which can be attached are acetyl, arabinosyl, and glucuronosyl residues (Wong *et al.*, 1988).

On the basis of their chemical and structural characteristics hemicelluloses are divided into four general classes: (a) xylans, (b) mannans, (c) xyloglucans and (d) *β*-glucans with mixed linkages (Ebringerova *et al.*, 2005). Considering the lack of scientific agreement in terminology and clear link between the term NSP and hemicellulose the main classes of hemicellulose seem to cover primarily non-cellulosic polymers of NSP. Table 2.2 presents, in brief, the four general classes of hemicelluloses with their main characteristics such as the residues which build the main backbone; the carbohydrate residues which participate in the side chains; the type of chemical linkage between residues; plants or plant materials as a source of polysaccharides of a particular type; and their function in the cell wall.

Table 2.2: Some important characteristics of the main groups of hemicellulose

Order	Name	Main backbone	Side chain residues	Types	Source	Role
1.	D-xyloglycans (xylan)	β- (1 → 4) D-xylan	D-glucoronic acid (and its 4-O-methylether) L-arabinose D- and L -galactose D-glucose	1.1.Homoxylans; 1.2.Heteroxylans	about 20–30% of the biomass of dicotyl plants (hardwoods and herbaceous plants); up to 50% in some tissues of monocotyl plants (grasses and cereals)	
1.1.	Homoxylan	β-(1→3) or mixed β-(1→ 3, 1 → 4) D-xylan	No side chain	X$_3$ (β- (1→ 3) linkage) Xm (mixed 1 → 3, 1 → 4)	seaweeds of the *Palmariales* and *Nemaliales*	structural function in the cell-wall architecture
1.2.	Heteroxylans	β- (1 → 4) D-xylan		1.2.1. Glucoronoxylans; 1.2.2.(Arabino) glucoronoxylans; 1.2.3.(Glucorono) arabinoxylans; 1.2.4. Arabinoxylans; 1.2.5.Complex heteroxylans		
1.2.1.	Glucoronoxylans	β- (1 → 4) D-xylan	Position C2 single 4-O-methyl-α-D-glucoronic acid residues (both 4-O-methylated and non-methylated forms are possible)		the main hemicellulose component of hardwoods; fruits and storage tissues	

Order	Name	Main backbone	Side chain residues	Types	Source	Role
1.2.2.	(Arabino) glucoronoxylans	β-(1 → 4) D-xylan	Position C2 single 4-O-methylglucoronic acid residues		coniferous species; lignified supporting tissues of grasses and cereals	
1.2.3.	(Glucorono) arabinoxylans	β- (1 → 4) D-xylan	Position C3 single α-L-arabinofuranose residues		non-endospermic tissues of cereal grains such as in wheat, corn, and rice bran	
1.2.4.	Arabinoxylans	β- (1→ 4) D-xylan	α-L-arabinose; Monosubstituted position O-2 or O-3 Disubstituted both O-2 and O-3 and α-L-arabinose esterified with ferulic and cumaric acid (position O-5)	(a) water-insoluble monosubstituted (Ara : Xyl ~ 0.2–0.3); (b_1) water-soluble (Ara:Xyl - 0.3 and 1.2; (b_2) water-soluble (Ara: Xyl - 0.5 – 0.9)	cereals: wheat, rye, barley, oat, rice, corn, sorghum; rye grass, bamboo shoots, pangola grass	bread-improving properties
1.2.5.	Complex heteroxylans	β-(1 → 4) D-xylan	Single uronic acid Arabinose residues Mono- and oligoglycosyl side chain		cereals, seeds, gum exudates, mucilages; leaves and barks of tropical dicots;	
2.	D-mannoglycans (mannans)	β- (1→ 4) D-mannose (1 → 4)- β- D-glucose	Single D-galactose residues	2.1. Galactomannans 2.2. Glucomannans		
2.1	Galactomammans	β- (1 → 4) D-mannose	Single D-galactose residues	Water insoluble (~4% galactose) Water soluble (30-96% galactose residues)	seed endosperm of vegetable ivory nut; date; green arabica coffee beans; storage tissues (endosperm, cotyledons, perisperm) of seeds	from the seeds of various plants used as traditional food or medicines

Order	Name	Main backbone	Side chain residues	Types	Source	Role
2.2.	Glucomannas (galactoglucomannas)	β- (1 → 4) D-mannose and β- (1 → 4) D-glucose	D-galactose		secondary cell walls of softwoods; bulbs, tubers, seeds, roots, and leaves of some non-gramineous monocotyl plants	immunostimulatory activity (the filet and the skin of aloe vera leaves); Japanese food applications
3.	D-xylo-D-glucans (xyloglucans)	(1 → 4)- β- D-glucose	Position C6 α-D-xylose and further (in position 2) attached β- D-galactose, gucose and arabinose are also possible	XXXG XXGG	20–25% of the primary cell wall in dicotyledonous angiosperms such as Sycamore or Arabidopsis thaliana; 2–5% in grasses (monocotyledonous angiosperms); 10% in the bulb cell walls of onion (a monocotyledonous angiosperm); about 10% in the primary cell walls of fir trees (gymnosperms)	building material of the primary cell wall; storage xyloglucan–cellulose interactions; thickening, stabilizing and gelling agent in food, in textile sizing and weaving, and as an adhesive or binding agent in industry
4.	β –D –glucans (β-glucans)	(1 → 3, 1 → 4) β- D-glucose	No side chain	Cellotriosyl and cellotetraosyl cellulose like segments	subaleurone and endospermic cell walls of cereal grains	regulation of postgrandial serum glucose levels in humans and animals; immunostimulatory activity

(Source: Ebringerova *et al.*, 2005)

The chemical and structural variety among hemicelluloses is vast and it is beyond the scope of this review to present in detail all existing forms and structures which have been extracted and studied so far. Considering all the limitations of the schematic view, Figure 2.10 presents only the basic features of the main four groups of hemicellulose.
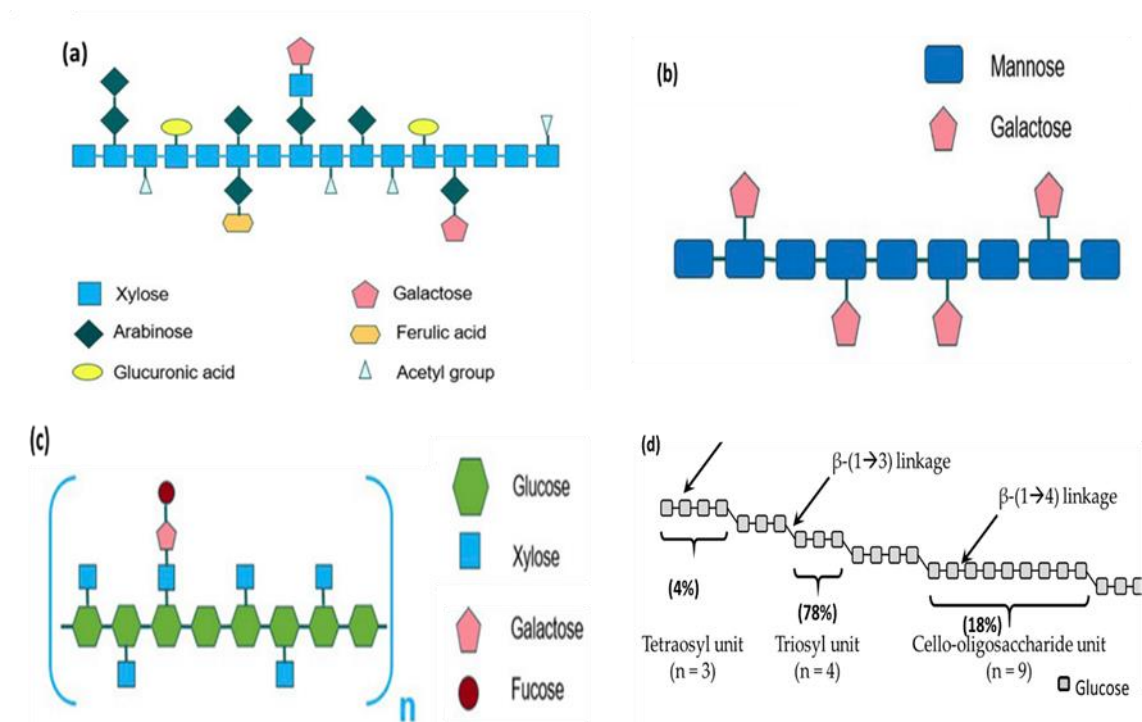
(Source: Ebringerova *et al.*, 2005; Henrion *et al.*, 2019; Houfani *et al.*, 2020)

Figure 2.8: Schematic view of the main four hemicellulose groups: (a) D-xyloglycans (xylan); (b) D-mannoglucans (mannan); (c) D-xylo-D-glucans (xyloglucan); (d) *β*-D-glucans.

2.4.   Approaches towards characterisation of dietary fibre

Dietary fibre is the main non-digestible component in the diet of monogastric animals (Williams *et al.*, 2019). Initially, the interest in DF came as a result of indications that they exert some beneficial effects in human nutrition and digestion (Van Soest *et al.,* 1991). The subject of DF, including the evolution of its definition and important methods of analysis are still a matter of debate and are well discussed in review articles (De Vries *et al.,* 1999; Tungland and Meyer, 2002; Caprita, A. *et al.,* 2010; Choct, 2015a). As a part of poultry feeds, NSP are analysed either as a single

ingredient (Annison and Choct, 1991) or as a combination of molecules from more complex feed matrixes, which fall in two categories of raw materials - "conventional" or "non-conventional" (Bach Knudsen, 2001; Choct, 2015a).

Analysis of the contents of total DF in human foods, according to Mertens (2003), included two types of methods: 1) enzymatic gravimetric and 2) enzymatic chemical. Classification by other authors included three groups of analytical methods: chemical-gravimetric, enzymatic-gravimetric and enzymatic-chemical methods (Elleuch *et al.,* 2011; Agyekum and Nyachoti, 2017). Most of the methods of analysis have reproduced the processes and conditions which exist in the gastrointestinal tract during digestion (Malathi and Devegowda, 2001). However, due to complexity of digestion, it is difficult to reproduce fully the process in the laboratory *in vitro* conditions (Mertens, 2003). For that reason, the choice of specific method for fibre analysis is usually dependent on the nature of source material, expected quantity and type of fibre in the material and their chemical structure. Some possible interactions with other components in the source material are also considered, along with other specific requirements or limitations of the analysis.

Englyst *et al.* (2007) explained that for the majority of products, analysis of the total NSP provides a close measurement of dietary fibre which also conform to the concepts of the general definition of DF – their digestion (physiological fate) in the gut and their chemical structure. The authors considered the site of the fibre digestion in particular compartments of the gut as the main feature for their nutritional classification. According to the physiological aspect, the definition of DF is dietary ingredients which are resistant to degradation by digestive enzymes produced by vertebrate animals and the relevant chemical entity of DF is regarded as the sum of NSP and lignin (Angel and Sorbara, 2014). Choct (2015a) suggested that, in comparison to other unnecessary complicated ones, this simple way of explaining DF provided a better practical definition for poultry nutritionists. Currently, fibre analysis is based on sample treatments which aim to obtain different fractions of dietary fibre, namely crude fibre (CF), acid detergent fibre (ADF), neutral detergent fibre (NDF) and acid detergent lignin (ADL). These fractions are not separate or completely independent because they overlap on some NSP, e.g. cellulose, lignin and polyphenolic compounds (Choct, 2015a). Furthermore, the term CF is not accurate because it refers to the remnants of plant material after extraction

with acid and alkali and includes variable portions of the insoluble NSP (Choct, 1997). For the purpose of simplification, in Figure 2.10 CF analysis is not included because it has been replaced by NDF, ADF and ADL analysis, which can be performed either consecutively or separately. The choice between these methods depends on the sample material and purposes of the analysis. Digestion with neutral detergent solution leaves in the filtrate (mainly starch), but also sugars, organic acids, proteins, and pectin. From the physiological point of view, starch is easily digestible and is the main source of energy in feed and food. The remaining pellet is comprised of hemicellulose, cellulose and lignin. Hemicellulose and cellulose are less digestible fibres and along with lignin are considered to provide bulk in the feed. Further digestion with acid detergent solution dissolves hemicellulose, which are expelled in the liquid phase. Determination of ADF, which has been adopted for animal feeds, utilises strong acid to hydrolyse all polysaccharides, except cellulose and lignin, which are therefore the only components in ADF (Caprita and Caprita, 2011). In order to obtain only lignin, the pellet is digested with sulphuric acid. Lignin also can be analysed by two methods – Klason lignin (KL) and ADL method. Experimental data with different grass showed that even for lignin it is possible to obtain varying content. Klason lignin typically shows higher content which is explained likely by the greater solubilisation of lignin components by the ADL treatment (Hatfield *et al.,* 1994). Similarly, other experiments reported no correlation between CF and the other methods of fibre analyses, which suggests that CF comprises a variable part of the true fibre content (de-Oliveira *et al.*, 2012).
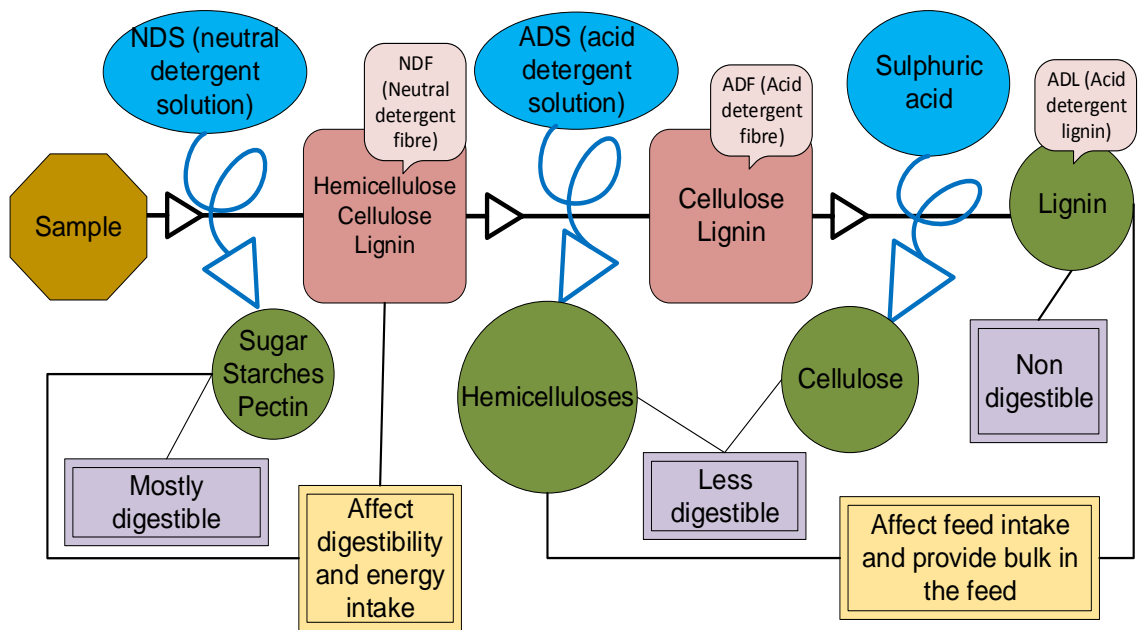
(Source: Choct, 2015a)

Figure 2.9: **Simplified scheme about the link between digestibility, physiological function and detergent method analysis for dietary fibre**.

Irrelevant to the applied method of analysis, the total sum of fibres extracted from the final feed mixture never reaches 100 % accuracy because calculation errors can reach approximately 10 %, due to incomplete and/or incorrect analysis of CF content (Choct, 2015b). Mertens (2003) also considered the use of the term CF incorrect and improper. However, neither NDF nor ADF values cover a large proportion of soluble fibre, for example, in leguminous crops that contain a high level of pectic polysaccharides (Choct, 2015a). Englyst *et al.* (2007) also agreed that the primary definitions which dealt mostly with the physiological effect of the fibre did not provide enough information about their chemical structure which made their proper characterisation difficult and inconsistent.

2.5. Soluble and insoluble NSP

Solubility of the fibre defines their physico-chemical characteristics and nutritional properties (Choct, 2015b). The early methods for the extraction of fibres (soluble and insoluble) applied different chemical solutions with a specific pH value (Gray, 2006). Soluble and insoluble NSP exert different effects on nutrient digestion, intestinal function, gizzard development, digesta transit time, cannibalism,

behaviour and welfare of birds (Hetland *et al.*, 2004; Van Krimpen *et al.*, 2009). Fibres with a viscous nature and ability to form gels, which also can affect glucose and fat absorption, are considered as soluble. The ANF effects of soluble NSP have been associated with increased viscosity, undesirable changes to digestive and absorptive dynamics of the poultry gut and a shift in the gut microflora. The overall result is poor efficiency in nutrient assimilation and impaired growth performance of the animal (Choct, 1997). The insoluble NSP have mainly been regarded as a nutrient diluent in the diet. They can affect bowel function through digesta transit time and gut motility (Choct, 2006).Table 2.3 presents total, soluble, and insoluble non-starch polysaccharides (NSP) in selected poultry feed ingredients.

Table 2.3: Total, soluble, and insoluble non-starch polysaccharides (NSP) in selected poultry feed ingredients

Ingredients	NSPs (g/kg as fed)		
	Total	Soluble	Insoluble
Barley	159.0	47.0	112.0
Soybean	152.5	36.5	20.0
Rye	128.0	49.0	80.0
Wheat	99.0	26.0	74.0
Maize	64.0	9.0	55.0

(Source: adapted from Choct *et al.*, 2010; Amerah, 2013)

Dhingra *et al.* (2011) confronted the general assumption that the digestion of soluble substances is easier and more rapid than insoluble. Williams *et al.* (2017) also emphasised that physiological distinction between soluble and insoluble fibre can change through acquisition of new data which implies that some insoluble fibre can also be a subject of fermentation. As an example, Dhingra *et al.* (2011) pointed out that some un-lignified amorphous cellulose in vegetable wastes (insoluble carbohydrates) can be more rapidly fermented than some modified starches and hemicelluloses (soluble carbohydrates). Being a very broad parameter, solubility does not provide sufficient information about the digestibility of the specific fibre. Gidley and Yakubov (2019) stressed that there is a whole continuum from highly soluble to completely insoluble fibre and a lot of other structures in-between these two boundaries. They also suggest that in order to predict the possible effects of the

fibres, more than a single approach has to be used. The complex nature of the fibre probably requires a combination of methods and approaches which would consider the fibre chemical structure, nutritional functionality and potential health outcomes and treat all these methods and approaches as inseparable and interrelated.

2.6. Fermentable and non-fermentable carbs/fibres

Dietary fibres, as a rule, are not hydrolysed in the stomach or in the small intestines of mammals (Jones, 2014), so they reach the lower part (caeca and colon) of the gastrointestinal tract (GIT). In poultry, due to the differences in the anatomy of the foregut and hindgut, fibres could affect the length and weight of the caeca (Jorgensen *et al.*, 1996). Mtei *et al.* (2019) showed that the utilisation of fibre could be different in the gut of pullets, hens and broilers. Some researchers (Englyst and Englyst, 2005) claimed that the introduction of the term "unavailable carbohydrates", instead of "insoluble carbohydrates'', seems more convenient and a practical alternative. The distinction between unavailable and available carbohydrates (or glycaemic carbohydrates) is based on their effect on blood glucose level. Additionally, available carbohydrates are digested and absorbed more easily (Gray, 2006). However, Comino *et al.* (2018) found that there was no significant difference in the fermentation kinetic of soluble and insoluble cereal dietary fibre. The authors stressed that the most important for the kinetics were the source and specific cell wall structure of the cereal grains. All these arguments indicate that the use of a variety of terms and approaches to explain and define fibre are in fact mutually related and instead of regarding them as contradictory, the terms and approaches should be considered different aspects which make defining the fibre more complete.

2.7. Enzymes as a part of nutrition-based health approach

According to Regulation (EC) No 1831/2003 of the European Parliament, feed additives are *"substances, micro-organisms or preparations, other than feed material and premixtures, which are intentionally added to feed or water in order to perform, in particular, one or more of the functions"*. The specific functions of the feed additives are presented in Figure 2.10.
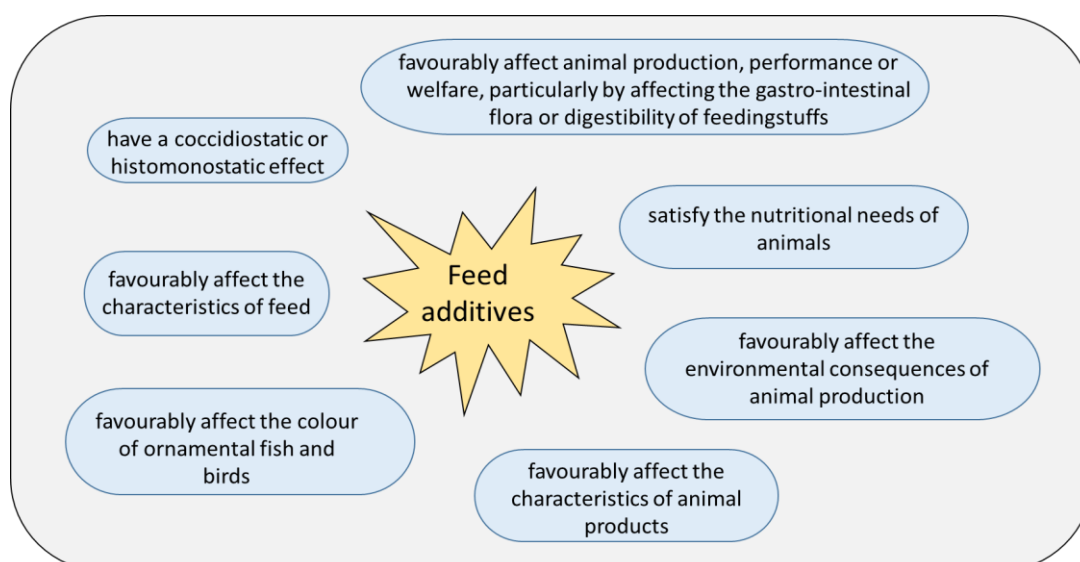
Figure 2.10: Functions of additives in animal feed.

Currently, the approved feed additives in the EU register are placed in more than ten different categories including: (1) substances that add or restore colour in feeding stuffs; (2) amino acids, their salts and analogues; (3) digestibility enhancers; (4) coccidiostats and histomonostats; (5) hygiene condition enhancers; (6) gut flora stabilisers; (7) zootechnical additives (improvement of zootechnical performance); (8) preparation of muramidase (EC 3.2.1.17) (lysozyme); (9) natural products – botanically defined; (10) gelling agents; (11) flavouring compounds; (12) trace elements; (13) vitamins, provitamins and chemically well-defined substances having a similar effect; (14) compounds of trace elements. Currently, the exogenous enzymes, e.g. xylanase, phytase etc., are placed in the category *digestibility enhancers*.

In contemporary scientific literature, some authors referred to supplementary enzymes as nutraceuticals (Sugiharto, 2016). According to Bender`s dictionary nutraceuticals are "*compounds in foods that are not nutrients but have (potential) beneficial effects*" (Bender, 2006). The same dictionary lists another term nutricines – "*biologically active ingredients in animal feedstuff used to promote nutrition-based health*". The research on health promoting substances in food triggered the development of the functional food concept. However, relating to animal feed, the terms "*a nutrition-based health (NbH) approach*" (Adams, 2006) or "*immuno-nutrition*" have been used (Beski *et al.,* 2015). Currently, the term "nutricines" is

neither very popular nor often used in comparison to "nutraceuticals". However, the use of the term nutraceuticals is broad and non-specific allowing a variety of substances to be covered. These substances could be organic acids (Khan and Iqbal, 2016), enzymes (Bedford, 2000a), probiotics (Jadhav *et al.,* 2015), prebiotics (Teng and Kim, 2018), synbiotics (Maiorano and Bednarczyk, 2016), phytogenics (Yitbarec, 2015), antimicrobial peptides and bacteriophages (Gadde *et al.,* 2017). Such substances are under considerable scientific research because they are associated with potential benefits for poultry performance and health. It has been theorised, that they do not possess side and/or residual effect and thus are generally characterised as non-hazardous and ecologically friendly (Yitbarec, 2015).

Elwinger *et al.* (2016) indicated that Clickner and Follwell (1925) first reported the use of a mixture of enzymes from *Aspergillus orizae* as an exogenous supplement in poultry feed. Since the decision of the European Union in (Regulation (EC) No 1831/2003) to ban the use of antibiotics as growth promoters (on January 1st, 2006), the search for successful alternative ingredients has increased (Suresh *et al.,* 2018). Supplementing enzymes in animal feed is beneficial not only for animal performance but also in dealing with some important environmental issues and has become a well-accepted practice for the last twenty years (Mullaney *et al.*, 2000; Bedford, 2018). Expected benefits of exogenous enzymes in feed are presented in Figure 2.11.
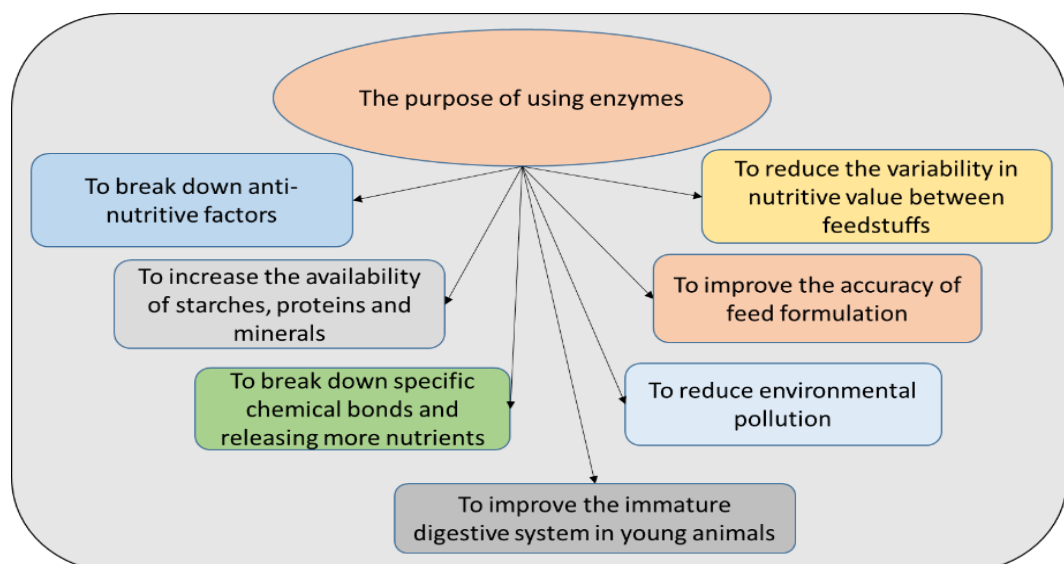
(Source: Sheppy, 2001)

Figure 2.11: The benefits of enzymes in animal feed

Before or after particular technological steps, enzymes can be supplemented to the feed in several forms such as: (a) powder (before mixing and pelleting), (b) granules (before mixing and pelleting) or (c) liquids (after pelleting) (Acamovic, 2001). Recently, research has not only be limited to enzymes in poultry and pig feed but has expanded further towards aquaculture (Zheng *et al.*, 2020). A variety of enzymes, such as carbohydrase, protease and lipase, both alone or in different combinations are approved and used as feed additives in animal nutrition (Table 2.4).

Table 2.4: Feed enzymes and target feedstuffs

Enzyme	Target substrate	Target feedstuffs
Amylase	Starch	cereal grains, grains legumes
Lipases	Lipids	lipids in feed ingredients
Phytases	Phytic acid	all plant-derived ingredients
Protease	Proteins	all plant protein sources
α-Galactosidases	Oligosaccharides	soybean meal, grain legumes
β-Glucanases	*β*-glucan	barley, oats and rye
β- D-mannase	galactomannan-containing hemicelluloses	soya/maize-based feedstuffs

(Source: Ravindran and Son, 2011)

The use of exogenous enzymes turns out to be a successful strategy especially when added to wheat and barley or wheat and maize-based diet formulations (Bedford and Morgan, 1996; Bedford and Schulze, 1998). The arabinoxylans/xylans in grains are considered as ANFs and their main effect is related to the increase of gut viscosity, wet litter, impaired digestion and absorption of nutrients and reduced performance (Chotinsky, 2015). The benefit of using xylanases is immense because even partial hydrolysis of the polymer chain is very effective in elimination and/or diminishing the antinutritive effects (gel-forming ability, water absorption capacity, ability to immobilize nutrients) (Chesson, 1993).

The maize used in poultry feed formulation is a favourable choice because of its low content of ANFs and the use of exogenous enzymes may therefore seem unnecessary in maize based formulations. Research reveals that the use of exogenous enzyme (enzyme cocktail of xylanase, amylase and protease) in maize-based diets is also beneficial for animal performance since it improves weight gain and feed efficiency (Cowieson and Ravindran, 2008). Additionally, the work of Jasek *et al.* (2018) showed that the addition of carbohydrase enzymes only, such as a combination of *α*-galactosidase and xylanase, can improve nutrient and ileal amino acid digestibility in broilers. Further to that, Cowieson (2005) reported that xylanase alone can significantly improve FCR of broiler chickens fed maize-based diet. Masey O`Niell *et al.* (2014a) suggested that xylanase can act through different mechanisms - in the case of wheat, xylanase improves rates of digestion as a result of viscosity reduction, whereas in maize this may be due to the implementation of the ileal brake mechanism (the delay in gastric emptying). It is very likely that xylanase has an equal potential to improve production performance in broilers in either wheat- or maize-based diets.

2.8.   Enzymology of xylanase

2.8.1.   Xylanase producing organisms

Bacteria, algae, fungi, protozoa, gastropods, and arthropods have the ability to synthesise xylanases (Dekker and Richards, 1976). Microorganisms are the preferred choice for research and industrial applications because most of them excrete, in an abundant quantity, the xylanases into the medium and are convenient objects for cloning, sequencing, mutagenesis and manipulation (Kulkarni *et al.*, 1999). A review article by Sunna and Antranikian (1997) listed 60 xylanase producing species/strains belonging to 18 fungal and 9 bacterial genera. Haltrich *et al.* (1996) reviewed the most important conditions for solid and submerged fermentation processes in either small laboratory experiments or a large-scale fermentation for the production of xylanase. Recent xylanase producing microorganisms including fungi from the genera *Trichoderma*, *Aspergillus*, *Penicillium*, *Fusarium*, *Chaetomium*, *Humicola* and *Taloromyces,* and some bacterial species as *Bacillus subtilis* and *Acinetobacter* spp. (Sanchez, 2009; Pandey *et al.,* 2015) are used commercially. There are also some reports of the

importance of yeast as a valuable source of proteins, vitamins and xylanase for feed applications (Wang and Hong, 2018). In comparison to fungal, bacterial hemicellulases showed higher thermostability and thus have been extensively studied and isolated from thermophilic (*Geobacillus* sp.) (Bhalla *et al.*, 2015) and extremely thermophilic bacteria (*Caldicellulosiruptor owensensis*) (Peng *et al.,* 2015). There is a continuous process of isolating and characterising completely new and still unknown xylanase enzymes from different microorganisms (Wang *et al.*, 2019). These facts show that microorganisms are valuable, natural and indispensable sources of different hemicellulases. Due to their diversity and specificity the approach towards each enzyme should comprise careful research to obtain sufficient information required for their safe and profitable future applications.

2.8.2.   Classification of xylanases

Reilly (1981) suggested a classification of xylanases based on the type of the end products of hydrolysis. According to this approach three main groups shape the classification: *β*-xylosidases, exo-xylanases and endo-xylanases. The endo-xylanases group was further divided into four classes based on two main properties of enzymes: (a) an ability to cleave L-arabinose from xylan and (b) the length of the final fragments (xylose, xylobiose, xylotriose, oligosaccharides). This approach turned out to be very useful for screening among xylanase producing microorganism, which can be used commercially (Bastawde, 1992; Haltrich, 1996).

The review article by Wong *et al.* (1988) divided xylanases into two forms: (1) xylanases with low molecular weight (<30 kDa) and basic pH and (2) xylanases with high molecular weight (>30 kDa) and acidic pH. This classification of xylanase was based on enzyme multiplicity observed in genera *Bacillus*, *Clostridium*, *Streptomyces*, *Aspergillus* and *Trichoderma*. However, approximately 30 % of the currently identified xylanases, especially fungal xylanases, cannot be classified by this system (Collins *et al.*, 2005). Bhat and Hazlewood (2000) also distinguished two groups of xylanases but based on the type of chemical bond that they can hydrolyse: (1) specific and (2) non-specific. Specific endoxylanases were defined as those which were active on xylans with only *β*-1,4 linkages, whereas non-specific endoxylanases were able to hydrolyse *β*-1,3- and *β*-1,4- linkages of mixed xylans.

Xylanases exist in diverse forms displaying varying folds, mechanisms of action, substrate specificities, hydrolytic activities (yields, rates and products) and physicochemical characteristics and currently are classified in different families based on their amino acid sequence (Henrissat, 1991). Collins *et al.* (2005) estimated that research has mainly focused on only two of the xylanases from glycoside hydrolase families, namely families 10 and 11, but enzymes with xylanase activity also belong to families 5, 7, 8 and 43 and have been isolated and studied, but to a lesser extent. The current classification of carbohydrate-active enzymes collectively designated as (CAZymes) which comprise enzymes involved not only in breakdown (glycoside hydrolases, polysaccharide lyases, carbohydrate esterases) of oligo- and polysaccharides but also in their assembly (glycosyltransferases) can be found in the CAZy database (Lombard *et al.*, 2014). The CAZy database provides continuously updated online access to a sequence-based family classification (340 000 enzymes as of 2014) and its major contribution is the dissemination of stable nomenclature for these enzymes (Lombard *et al.*, 2014). Additionally, the important achievement of this classification scheme lays in its power to expand further and to incorporate into its hierarchal system new enzymes.

2.8.3.   Xylanolytic enzyme complex

The enzymes which are closely related to the hydrolysis of xylan comprises the so called xylanolytic enzyme complex (Figure 2.12). Researchers recognised two groups of enzymes in the complex – the main and accessory (branch point-degrading) enzymes (Moreira and Filho, 2016). Endo-1,4-β-xylanase (EC 3.1.2.8) and β-D-xylosidase (1,4-β-D-xylan xylohydrolase, EC 3.2.1.37) were designated as the main enzymes because their role for the degradation of the backbone chain of xylan (Thomas *et al.* 2013). According to Polizeli (2005) acetylxylan esterase (EC 3.1.1.6), exo-α-L-arabinofuranosidase (EC 3.2.1.55), endo-1,5-α-L-arabinase (EC 3.2.1.99) and α-glucuronidase (EC 3.2.1.-) are the accessory enzymes in the complex. The review article of Collins *et al.* (2005) listed the synonyms under which xylanase is denoted in the scientific literature: endoxylanase, endo-1,4-β-D-xylanase, β-1,4-xylanase, 1,4-β-D-xylan-xylanohydrolase or simply β-xylanase.
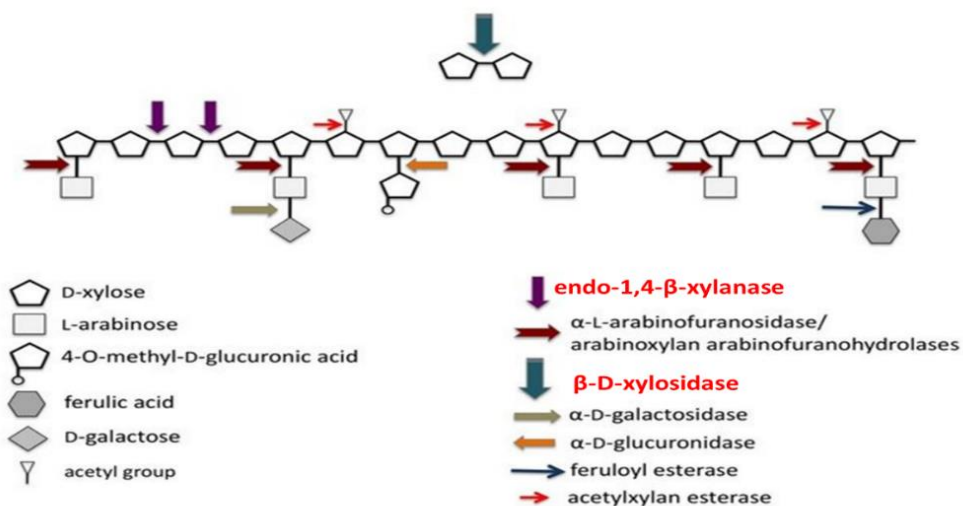
(Source: Beg *et al.,* 2001; De Souza *et al.*, 2013; Godoy *et al.*, 2018)

Figure 2.12: Xylanolytic enzyme complex and their possible site of hydrolytic activity on xylan main backbone and side chain moieties: schematic view of hydrolysis of xylan.

2.8.4. Substrate specificity and mode of action

The main and accessory enzymes of xylanolytic complex, the corresponding EC number - Enzyme Commission number for enzymes and a brief note about their specific site of hydrolytic activity and products are presented in Table 2.5.

According to their mode of action, hemicellulases can be two types: exo- and endo-enzyme. Exoenzyme degrades the polysaccharide by successive removal the terminal monomeric unit of the oligosaccharide chain and proceeds along the chain in a stepwise manner, usually from the non-reducing end. In contrast, endoenzyme hydrolyses polysaccharides at several inner sites of the main backbone and their activity leads to a significant decrease in the degree of polymerization (DP) of the polysaccharide. Endoenzyme activity usually ceases when shorter fragments or nondegradable products (usually mono- and disaccharides) are formed (Dekker, 1985).

Table 2.5: Xylanolytic enzymes complex and their hydrolytic activity

EC number	Enzyme	Enzyme hydrolytic activity and end products
EC 3.2.1.8	Endo-xylanase	hydrolyses mainly interior β-1, 4-xylose linkages of the xylan backbone
EC 3.2.1.72	Exo-xylanase	hydrolyses β-1, 4- xylose linkages releasing xylobiose; liberate xylose from reducing end of branched oligosaccharides
EC 3.2.1.37	β-Xylosidase	hydrolyses xylobiose and short xylooligosaccharides from the nonreducing end to xylose
EC 3.2.1.55	α-Arabinofuranosidase	cleaves arabinan at O-2 and O-3 positions on xylan back bone
EC 3.2.1.131	α-Glucuronidase	cleaves the α-1,2-glycosidic linkage between xylose and glucuronic acid or its 4-*O*-methyl ether
EC 3.1.1.6	Acetylxylan esterase	removes *O*-acetyl groups from the C-2 and C-3 positions of xylose residues in both xylan and xylooligosaccharides
EC 3.1.1.73	Ferulic acid esterase	cleaves ferulic acid side chain substitutions releasing ferulic acid
EC 3.1.1.x	*p*-Coumaric acid esterase	hydrolyses *p*-coumaryl ester bonds in xylans

(Source: Saha and Bothast, 1999; Juturu and Wu, 2013; Juturu and Wu, 2014)

The mode of action of endoxylanases has been extensively studied (Biely *et al.,* 1997). The simplest scheme presenting the breakdown of the substrate can be explained in two steps: the first step of hydrolysis ends with the availability of β-D-xylopyranosyl oligomers and after the second step of hydrolysis some small molecules such as mono-, di- and tri-saccharides are present (Polizeli *et al.*, 2005; Linares-Pasten *et al.*, 2018). Activity of the main enzymes in xylanolytic complex (i.e. xylanase and β-xylosidase) depends on the length of the XOS. Activity of

xylanase diminishes with decreasing length of XOS and xylanases are not active against oligosaccharides with DP 4 (X_4) or lower (Deshpande *et al.*, 1986; Garcia-Campayo *et al.,* 1993). However, Esteban *et al.* (1982) reported a bacterial xylanase, which actively yielded xylobiose from a fragment of four 1,4-linked-*β*-o-xylopyranoside units. On the contrary, *β*-xylosidase from *Trichoderma harzianum* strain was completely inactive towards longer oligosaccharides such as xylohexaose (X_6) or acetylated xylan (Silveira *et al.*, 1999). Some exceptions have also been reported - a *β*-xylosidase from *Trichoderma lignorum* was shown to be more active against longer oligosaccharides (X_6, X_7 and X_8) than shorter oligosaccharides and xylobiose (John and Schmidt, 1988). These data showed that there is a great diversity among the existing xylanases and despite their typical activity on the substrates, exceptions are possible.

On the other hand, the degree of substitution which influenced the xylan solubility can also impact hydrolysis (Wong, 1988; Tenkanen *et al.,* 1992; Silva *et al.*, 1999). When referring to the activity against the substituted backbone, endoxylanases can be: (1) non-debranching - which do not hydrolyse L-arabinose and (2) debranching - which hydrolyse arabinose or the side chains during the hydrolysis of arabinoxylans (Bastawde, 1992; Garcia-Campayo *et al.*, 1993). The GH10 family hydrolyse substituted heteroxylans because the structure of the active site can accommodate units from the side chain (Pell *et al.*, 2004; Pollet *et al.*, 2010). Reports on *β*-D-xylan xylohydrolysis concomitant with *α*-L -arabinofuranosidase activity was defined by some authors as a multifunctionality of xylanases (Herrmann *et al.*, 1997; Saha and Bothast, 1999). Additionally, xylanases showed specificity towards decorated substrates. Tenkannen *et al.* (1992) suggested that the preferred site of action of the enzymes was the xylan backbone near to the branch points. Pollet *et al.* (2010) explained that the GH10 xylanases hydrolyse the glycosidic linkage next to a single- or double-substituted xylose toward the non-reducing end and required two unsubstituted xylose residues between branched residues. Some other mechanisms were also possible as two other xylanases can cleave both substituents - at the reducing end and in the middle of the oligosaccharide chain (Dekker, 1985). However, Gallardo *et al.* (2003) studied xylanase which showed activity irrespective of the extent of substitution on birchwood, oat spelt and beechwood xylan, methylglucuronoxylan, rye or wheat arabinoxylan. The ability to

hydrolase not only the main backbone of the XOS but also to remove arabinose moieties is an important ability of xylanase which increases the structural diversity of the final products of hydrolysis and their metabolic fate.

Some authors suggested that all substituents should be removed from the backbone before the actual hydrolysis by endoxylanase could take place. However, they acknowledged that if such hydrolysis occurs, it would require a cooperative action of at least nine enzymes (Saka and Bae, 2016). The supposed mechanism seems very unlikely because some xylanases were able to hydrolyse a variety of substituted xylans with intact substituent groups (Tuohy *et al.*, 1994). Additionally, other data showed that a regularly distributed side chains serves as specific markers for the enzyme activity or even further - the enzyme active site has a specific fold that could accommodate the substituted residues and this interaction is a key determinant for the enzyme specificity (Nishitani and Nevins, 1991; Correia *et al.,* 2011). These experimental results suggest that enzyme activity and cooperation among enzymes in the complex environment when lots of different enzymes exist depend on the individual characteristics of enzymes, the substrate provided and probably some other physicochemical parameters.

Among the extensive data about xylanases it is worth mentioning that some endoxylanases can execute two differences in their natural enzymatic reactions i.e. hydrolysis and transferase (or transglycosylase) reactions (Christakopoulos *et al.,* 1996; Silva *et al.,* 1999). The essential difference between hydrolysis and transferase reactions is that, in the former, water acts as an acceptor of the glycosyl moiety, whereas in the latter, the acceptor is an alcohol or a sugar molecule (Bhat *et al.,* 2000). John and Schmidt (1988) presented a very detailed explanation of the mode of action of xylanase with high glycosyltransferase activity. The enzyme catalyses the transfer of glycosyl residues from a xylooligosaccharide donor to an acceptor molecule producing a series of homologous XOS. Xylopentaose (X_5) and xylodecaose (X_6) served as substrates for the reaction which can yield XOS with DP greater than 50. Authors also noticed that transglycosylation products of one xylanase could became a substrate for the hydrolysis of the second xylanase which was present in the reaction mixture and xylobiose and xylose were the final products of cooperative hydrolysis. The application for xylanase with high transferase activity in commercial enterprises could be dubious since it is going to change the substrate

and eventually the final products of hydrolysis. In this case, a careful selection of the enzymes would be advisory.

2.8.5.   Synergism with other enzymes

The interactions between enzymes in a xylanolytic complex are examples of synergy. According to Bhat *et al.* (2000) separation, identification and quantification of the products of hydrolysis should be used for assessing the type of the synergy. Three types of synergy have been suggested: (1) homeosynergy; (2) heterosynergy; and (3) antisynergy.

> ➢ Homeosynergy is defined as interaction between two or more different types of side-chain-cleaving enzymes or between two or more types of main-chain-cleaving enzymes. Example of homeosynergy can be found in the study of Despande *et al.* (1986). They showed that hydrolysis of xylan by fungal xylanase was estimated to be 18 % in 20 h but additional supplementation with two different *β*-xylosidases increased hydrolysis up to 48 % and 68 %.

> ➢ Heterosynergy is defined as synergistic interaction between side-chain- and main-chain-cleaving enzymes. Heterosynergy has been reported between ferulic acid esterases and endoxylanases (Faulds *et al.*, 2005; Wong *et al.*, 2013). Hashimoto and Nakata (2003) demonstrated that *α*-L-arabinofuranosidase was able to release twice the amount of arabinose moieties after fragmentation of the arabinoxylan backbone by xylanase and in turn the hydrolysis of the arabinoxylan backbone through xylanase accelerated by 1.5-fold when there was a pre-removal of the substituents.

> ➢ Antisynergy is defined as an interaction when an enzyme activity could prevent or hinder the activity of another enzyme (Bhat *et al.*, 2000). Although it is not certain what antisynergy would look like in vivo, it is observed during in vitro experiments where combinations of known quantity and types of enzymes are involved. This interaction could be extended also to some cases where the negative impact on enzymes is not so profound but there is a complete lack of synergy. As an example, Beaugrand *et al.* (2004) showed the lack of synergistic effect of xylanase from GH10 and GH11 on destarched wheat bran. Sørensen *et al.* (2003) observed no interaction and a weak

antagonistic effect of three enzyme preparations against arabinose-containing substrate.

This spectrum covering the whole scale from positive through neutral to negative interactions between enzymes of xylanolytic complex shows very complex and diverse interrelations between enzymes. The assessment of each of these interactions is going to be a multilevel process which probably is going to require an individual approach to each combination of interacting enzymes.

2.8.6.   Multiplicity of microbial xylanases

The hydrolysis of hemicellulose and xylan is a dynamic process and the accessibility of the chemical bonds depends on the consecutive changes of the substrate molecule. This explains the strategy of the microorganisms to synthesize a complete system of enzymes, all of which are related to the substrate to some extend but at the same time with a specific function to achieve complete hydrolysis (Wong, 1988). Wong *et al.* (1988) explained multiplicity of xylanase by the existence of major and minor forms of xylanases. The minor forms of xylanases were associated with hydrolysis which infrequently occurred in the substrate chemical bonds. The authors suggested that the small quantity of minor xylanases was the main impediment for their isolation and purification. Some other obstacles can also relate to: (1) the limitation of the purification procedures which favour the isolation of major xylanases; (2) insufficient quantities under the particular growth conditions; (3) loses from the culture filtrate due to degradation or adsorption onto insoluble growth substrates. In contrast, the major xylanases may be relatively overproduced under the growth conditions and as a result are easily isolated. The authors suggested some possible reasons for the existence of multiplicity such as: substrate cross-specificity; specific regulation of a particular gene; existence of several genes; existence of allozymes (products of different alleles of the same gene); post translational modifications of the enzymes. The authors mentioned, despite that they acknowledged it as an extreme option, that multiple xylanases could be artefacts due to some degradation in microbial culture filtrates. Bhat and Hazelwood (2000) explained difficulties of characterisation of multiplicity of xylanase with the lack a substrate specificity and/or lack of defined and structurally characterized substrate. Advances in the molecular techniques revealed that multiple xylanases were not just a simple result of expression of different genes but also underwent post-

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translational modifications (Liao *et al.,* 2015). The multiplicity of xylanase could be a result of an evolutionary mechanisms which microorganisms developed in order to deal with the structurally diverse and composite materials such as hemicellulose.

2.8.7. Feed supplemented xylanase - biological effects

Research into xylanase in animal feed experiments resulted in three main hypotheses which aimed to explain its biological effects: (1) reduction of gut viscosity, (2) release of encapsulated digestible components from the plant cell wall (diminishing of so-called cage effect), (3) generation of XOS with potential prebiotic effect (Masey O`Neill *et al.,* 2014b).

Bedford and Morgan (1996) reviewed the data about xylanase in wheat-based diets and addressed its beneficial effects in reducing gut viscosity and treating the problem of wet litter. The authors did not refer to the „cage effect", but mentioned cell wall perforation through the activity of xylanase and also possible changes in the gut microorganisms. Zyla *et al.* (1999) showed the relationship between reduced gut viscosity and improvement in weight gain of broilers. However, Amerah *et al.* (2008) did not find such relations but reported an improved AME after xylanase supplementation. The authors supposed that xylanase could release components from the vegetable materials that provide additional energy sources. Other researchers supposed that the complete physical disruption of cell walls through the activity of xylanase is very unlikely and any effects upon the cell wall matrix are partial and moderate (Bedford and Schulze, 1998). It is highly possible that under enzyme activity, some monosaccharides could be released. However, it is very unlikely that the quantity or the nature of these monosaccharides would be readily digestible substances and even more doubtful that they could become a substantial energy source for the host (Annison and Choct, 1991; Amerah *et al.,* 2008). It is more likely that these monosaccharides will serve as substrates for the gut microflora (Rowland *et al.,* 2018).

The effect of xylanase could be a combination of several mechanisms which work in a concomitant or consecutive manner and could depend on some other indirectly involved factors. Amerah *et al.* (2008) observed a decrease in the effectiveness of xylanase in a diet with medium size particles. The authors suggested that the accessible area on the particle surface increased and this facilitated gel formation,

thus the optimal activity of xylanase and other digestive enzymes was restrained. In maize-based diets, in comparison to wheat-based diets, the benefit of enzyme supplementation was not due to NSP hydrolysis (Cowieson and Ravindran, 2008). Authors attributed effects of the enzyme to the improvement in starch and resistant starch digestibility, changes to cell wall integrity, modification of the microbial community, improvement of solubility and digestibility of proteins and minimising the effect of ANFs.

The complexity of the effects of feed supplemented with xylanase involves the digestibility of proteins and fats. Zanella *et al.* (1999) found a beneficial effect through an increase (2.9 % absolute value and 3.6 % relative value) in overall crude ileal protein digestibility despite that the observed effect showed no consistency for all examined amino acids. Enzyme supplementation also improved body weight (BW) and feed conversion ratio (FCR). Additionally, Silva and Smithard (2002) found that the enzyme could retain up to 15 – 20 % of its activity in the chicken gut and suggested that xylanase may influence fat digestion and absorption, bile salts and protein metabolism. Amerah *et al.* (2008) supposed that xylanase was not directly involved in fat metabolism but may still improve nutrient digestibility. Engberg *et al.* (2004) found that xylanase addition increased pancreatic chymotrypsin and lipase activities.

Another important indirect mechanism of xylanase was related to the generation of XOS with potential prebiotic effect (Karlsson *et al.*, 2018). Zanella *et al.* (1999) and Kocher *et al.* (2002) suggested that improvement in dietary nutrient digestibility and better bird performance when fed maize-soy based diets was due to surmounting the *cage effect* or prebiotics. Waititu *et al.* (2018) reported the benefits of using xylanase along with other enzymes when soybean meal was replaced with low protein sunflower meal. The researchers did not find an effect on digestibility but explained a growth-promoting effect of enzymes through the release of prebiotics in the hindgut. However, Masey O'Neill *et al.* (2014b) suggested that feeding very young birds with high doses of xylanase did not have a positive effect on dietary nutrient digestibility. The lack of a positive effect was explained by an improper dose of the enzyme or unsuitable XOS. They also suggested that the beneficial effect of the supplemented prebiotics could be expected after stabilisation of gut microflora. These data clearly indicate that it is not always possible to demonstrate a direct

relationship between xylanase and digestibility. The potential positive effect of the prebiotics needs further research.

Some data suggested that xylanase can influence microbiota in the gut, gut hormones, GIT development and can have an antioxidant effect (Bao and Choct, 2010). It is also possible that xylanase can influence some gastrointestinal hormones like ghrelin, cholecystokinin (CCK), glucagon-like peptide 1 (GLP-1), and peptide tyrosine-tyrosine (PYY). Singh *et al.* (2012) found a release of peptide tyrosine-tyrosine in the response of xylanase supplementation. The main difficulties in this particular area of research emerge from the fact that the same hormones have different effects in chickens in comparison to mammals, which make the elucidation of their effects uncertain and prone to misinterpretation.

2.8.8.   Xylanase as subject of industrial production

Over 90 % of all broiler diets used in commercial production contains feed enzymes and up to 70 % of wheat and barley-based poultry feeds are supplemented with glycanases (xylanases and glucanases) globally (Ravindran and Son, 2011). Increased safety and advances in processing technologies are the main factors which have a significant impact on the market prospects for feed enzymes (Pariza and Cook, 2010; Torre and Kadowaki, 2017; Kumar *et al.,* 2018). Table 2.6 presents the main characteristics of some xylanase-based products which have been approved by the European Food Safety Authority (EFSA) and are currently available on the market.

Table 2.6: The main characteristics of some xylanase-based products approved by European Food Safety Authority (EFSA).

Xylanase-based product	Microorganism producer	Enzyme content	Application	EFSA Ref.
Endo-1,4-β-xylanase endo-1,3(4)-β-glucanase)	two strains of *Trichoderma reesei*	3000 XU 600 BGU/kg	chickens for fattening	2009a
Endo-1, 4- β-xylanase subtilisin α-amylase (amylase)	genetically modified strain of *Trichoderma reesei,* genetically modified strain of *Bacillus subtilis* genetically modified strain of *Bacillus amyloliquefaciens.*	intended dose of 300 xylanase, 4000 subtilisin and 400 amylase U/kg feed.	laying hens	2011
Endo-1,4- β-xylanase	*Bacillus subtilis*	10 IU per kg feed	chickens for fattening piglets pigs for fattening turkeys for fattening ducks	2006a
Endo -1, 4- β-xylanase endo – 1, 3 (4) – β - glucanase.	*Aspergillus oryzae* (DSM 10287) *Aspergillus aculeatus* (authorised as Energex)	6-18 FBG and 60-180 FXU	chickens for fattening piglets	2005a
Endo-1,4- β -xylanase	genetically modified *Trichoderma reesei*	1250-2500 U/kg	turkeys for fattening	2007b
Endo-1, 4- β-xylanase endo-1, 3(4)- β - glucanase	Two genetically modified strains of *Trichoderma reesei*	Endo-1,4- β -xylanase: 12200 U/g Endo-1,3(4)-β-glucanase: 1520 U/g	chickens and turkeys for fattening, laying hens,	2010

			piglets and pigs for fattening	
Endo-1,4-*β*-xylanase endo-1,3(4)-β-glucanase	*Trichoderma* reesei (CBS 529.94) *Trichoderma reesei* (CBS 526.94)	6 000 BXU - 1 500 BU 20000 BXU - 5000 BU	chickens for fattening turkeys for fattening	2005d
Endo-1,4-*β*-xylanase	genetically modified *Trichoderma reesei*	24000 BXU/kg	laying hens, minor poultry species (including ducks, geese, quails, pheasants and pigeons) and pigs for fattening	2008
Endo-1,3(4)-*β*-glucanase endo-1, 4-*β*-xylanase	*Aspergillus niger*	125 mg/kg supplies: endo-1,3(4)-*β*-glucanase EC 3.2.1.6: 138 U endo-1,4-*β*-xylanase EC 3.2.1.8: 200 U	chickens for fattening laying hens pigs for fattening minor poultry and porcine species	2004 2013 amdt 2017b
Endo-1,4-*β*-xylanase	non-genetically modified strain *Trichoderma citrinoviride*	1 050 EPU/kg 1 500 EPU/kg	turkeys for fattening chickens for fattening laying hens piglets (weaned) and pigs for fattening	2013b
Endo-1,3(4)-*β*-glucanase Endo-1,4-*β*-glucanase, *α*-amylase bacillolysin Endo-1,4-*β*-xylanase	*Aspergillus aculeatus Trichoderma longibrachiatum Bacillus amyloliquefaciens Bacillus amyloliquefaciens Trichoderma viride*	10 g/kg	laying hens turkeys for fattening chickens for fattening piglets	2005b

Endo-1, 4-*β*-xylanase endo-1, 4-*β*-glucanase	*Aspergillus niger*	280 TXU and 125 TGU per kg feed for fattening birds and ornamental birds and 560 TXU and 250 TGU per kg for laying birds	chickens reared for laying, turkeys for breeding purposes, turkeys reared for breeding, other minor avian species (other than ducks) and ornamental birds	2009b
Endo-1,4-*β*-xylanase	genetically modified *Aspergillus niger*	280 TXU/kg	chickens for fattening and ducks	2009b
Endo-1, 3(4)-*β*-glucanase endo-1, 4-*β*-xylanase	non-genetically modified strain of *Penicillium funiculosum (Talaromyces versatilis sp. nov.).*	1 900/1 100 U/kg	chickens and turkeys for fattening, laying hens, piglets (weaned) and pigs for fattening, ducks, guinea fowls, quails, geese, pheasants and pigeons	2013c
Endo-1,4-*β*-glucanase, endo-1,3(4)- *β*-glucanase endo-1,4-*β*-xylanase	*Trichoderma longibrachiatum* (ATCC 74 252)	400-1600U 900-3600U 1300-5200U	ducks	2005c
Endo-1,4-*β*-xylanase	*Trichoderma longibrachiatum* CL 847	1050 IFP kg- 1400 IFP kg- 700 IFP kg-1	chickens turkeys ducks	2007a
α-galactosidase, *α*-amylase, endo-1,3(4)-b-glucanase, mannan-endo-1,4-b-mannosidase, pectinase,	*Trichoderma citroviride Aspergillus niger Bacillus licheniformis Bacillus amyloliquefaciens*	50 mg/kg delivering: 4 GALU galactosidase 500 UA amylase 1 000 BU glucanase 50 UM mannanase 105 UP pectinase	chickens for fattening weaned piglets	2017a

protease, endo-1,4-β-xylanase		75 UPR protease, 160 UG cellulase, 8 000 BXU xylanase units		
Endo-1,4-β-xylanase endo-1,4-β-glucanase	*Aspergillus niger (CBS 600.94)*	endo-1,4-β-xylanase 4860-6000 FXU endo-1,4-β-glucanase 2025 -2500 BGU	chickens for fattening, turkeys for fattening and piglets ducks	2006b

2.9. Chicken gastrointestinal tract (GIT) anatomy and physiology

According to Svihus (2014), the benefit of dietary additives (e.g. enzyme and pre- or probiotics) is closely related to the optimal functionality of the digestive tract and therefore a suitable analysis of the morphology and physiology of the GIT should be part of each experimental design and interpretation of results.

Neves *et al.* (2014) described the digestive system of the chicken as simple, short, and extremely efficient. Choct *et al.* (2010) defined the digestion as disappearance of nutrients from the entire GIT as well as from specific parts of the tract, e.g. ileal digestibility. The process of digestion is achieved by different enzymes, some of which are secreted endogenously and others by the resident microflora (Bach Knudsen *et al.*, 2006). The enzymes brake down carbohydrates, proteins, and fats to monosaccharides, dipeptides and amino acids, free fatty acids, and monoglycerides that can be absorbed (Svihus, 2014). When the birds do not secreted enzymes for feed material digestion, which is the case for NSP and some oligosaccharides, the digestibility can be achieved partly by chemical degradation (acid in the proventriculus in chickens), or by microbial degradation (Choct *et al.,* 2010).

The intestinal tract of birds is shorter than that of mammals and consists of the following organs (Figure 2.13): oesophagus, crop, proventriculus, gizzard, small intestine (duodenum, jejunum, and ileum), caeca, colon, and cloaca (Pan and Yu, 2014). Anatomical structure and function of each organ provides a specific sequence of digestive events such as hydrolysing, acidifying, grinding, emulsifying etc. which are necessary for feed digestion and absorption (Klasing, 1999).
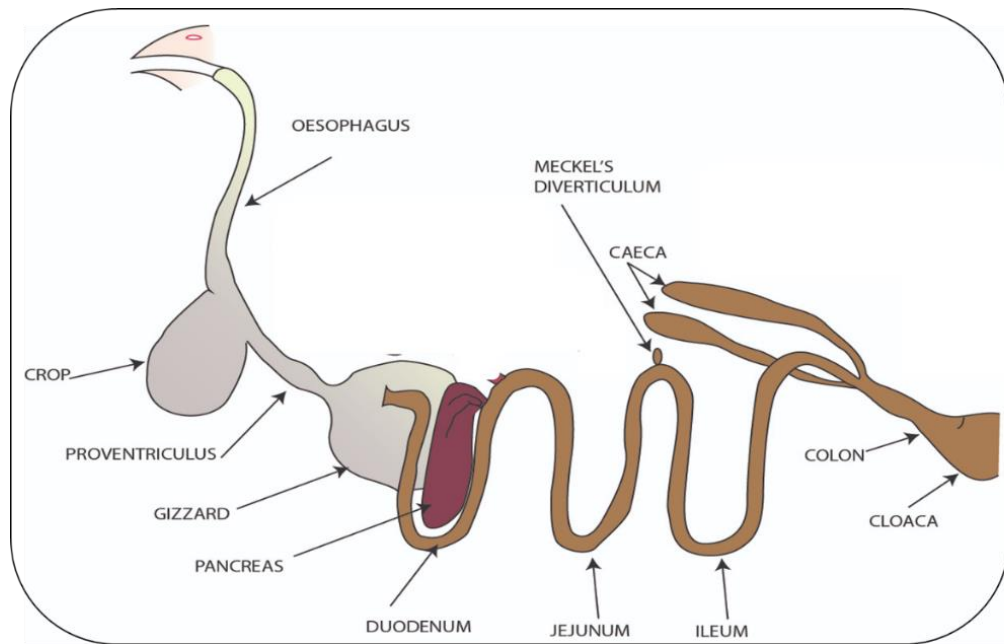
Figure 2.13: General scheme of the digestive system of a chicken.

Birds have no teeth and use the beak to collect the feed which is swallowed whole with a little saliva containing amylase. In adult birds, the salivary glands can be accompanied with some lymphoid tissue (Neves *et al.*, 2014). The oesophagus is a distensible tube which transports food from the pharynx to the crop. If the feed remains for some time in the crop it can be moistened. The oesophagus and crop are lined with incompletely keratinized stratified squamous epithelia into which numerous mucous glands open (Denbow, 2014). It is also possible that after swallowing the feed can pass directly to the proventriculus or gizzard when these sections of the digestive tract are empty (Svihus, 2014).

The stomach of the chicken is divided in two parts - the proventriculus (glandular stomach) and the gizzard (muscular stomach) (Klasing, 1999; Denbow, 2014). Feed in the proventriculus undergoes chemical and enzyme digestion by secretions of gastric juice containing mucus, pepsin, certain salts, and hydrochloric acid (Giambrone, 2013).

After mixing with the gastric juice, feed enters the gizzard. The gizzard consists of two pairs of asymmetrical arranged smooth muscles which contracts and provides motions with mixing and grinding effects (Klasing, 1999). The grinding cycle involves not only consequent contractions of the muscles of the gizzard but also peristaltic

contractions in the duodenum. As a result, some of the gastric material is pushed towards the duodenum and some material is pushed back into the proventriculus. The contraction cycle takes place up to four times per minute and grinds material due to rubbing against the koilin layer on the inside of the gizzard and against other particles. The concomitant activity of the proventriculus and gizzard during the grinding cycle physiologically links both organs and regarding digestive function they could be considered as one compartment. In total, the retention time in the proventriculus and gizzard has been estimated to vary between 30 minutes and an hour (Svihus, 2014). Detailed information about gizzard morphology and function is presented in the review article of Svihus (2011). The fine grinding of the feed particles makes them suitable for further digestion and absorption in the small and large intestines (Rodrigues and Choct, 2018).

The small intestine of the chicken is in general similar to mammals but differs markedly in some parts. The length of the small intestine of the chicken is about five to six times the length of the body (Giambrone, 2013). The small intestine is divided into the duodenum, jejunum, and ileum and according to Denbow (2014) by using gross observation they are not distinguishable. However, the duodenal loop clearly divides duodenum and jejunum and the yolk sack residue or so-called Meckel's diverticulum is often used as a landmark to separate the jejunum and ileum (Denbow, 2014). The duodenum, which does not have glands of Brunner, presents a loop supporting the pancreas, and is generally considered to terminate at the entrance of the bile and pancreatic ducts. The duodenum and upper jejunum are the principal sites of reabsorption of secreted bile acids and of absorption of lipids (Sklan *et al.,* 1975). The jejunum and ileum are supported by a mesentery. The small intestine contains a considerable amount of lymphoid tissue and lymph nodules (Giambrone, 2013). The jejunum has a key role since all major nutrients, to a large extent, are digested and absorbed in it. The weight of this segment, even empty, is usually up to 20-50 % higher than the ileum. Digesta retention time in jejunum is usually 40-60 minutes, which is approximately half of the retention time in the ileum. The ileum is the last segment of the small intestine and ends at the ileo-ceco-colic junction. The ileum is partly responsible for digestion and absorption of fat, protein, and starch but its main function is water and mineral absorption (Svihus, 2014).

Kiarie *et al.* (2013) defined four micro-habitats in the gut: (1) the intestinal lumen; (2) the unstirred mucus layer or layer that covers the mucosal epithelium; (3) the deep mucus layer found in the crypts; and (4) the surface of the intestinal epithelial cells. The diversity of bacterial populations and specific metabolic processes within each micro-habitat of the GIT highly depend on digesta flow rate, pH value, anaerobic conditions, types of available substrates and inhibitory factors such as bacteriocins and short-chain fatty acids (SCFAs).

The most distinct characteristics of the small intestine epithelium is the presence of villi and intestinal crypts (Figure 2.14). Epithelial cells of the villi have about 10^5 microvilli per square millimetre on their apical surface, increasing 15-fold the absorbing surface area (Klasing, 1999). Each villus contains a capillary rich bed, which absorbs nutrients and transfers them to the portal blood vessels. Crypts of Lieberkühn open into the lumen of the gut between the bases of adjacent villi. Goblet cells located on the intestinal epithelium secrete copious mucous which protect intestinal epithelium from digestive enzymes and abrasion by the digesta. The mucous is particularly thick along the anterior duodenum, where it protects the villi from excessive acidity of the digesta which leaves the gizzard. The intestine is surrounded by two muscle layers, the inner circular and outer longitudinal, which are responsible for mixing the digesta and propelling it through the tract (Klasing, 1999). In different gut compartments villi have different lengths. As an example, the length of the duodenal villi reaches 1.5 mm but, in the ileum, and rectum their length decreases to 0.4–0.6 mm. The number of villi also decreases from 1 to 10 days of bird age, but after that remain constant (Ferrer *et al.*, 1995; Denbow, 2014).
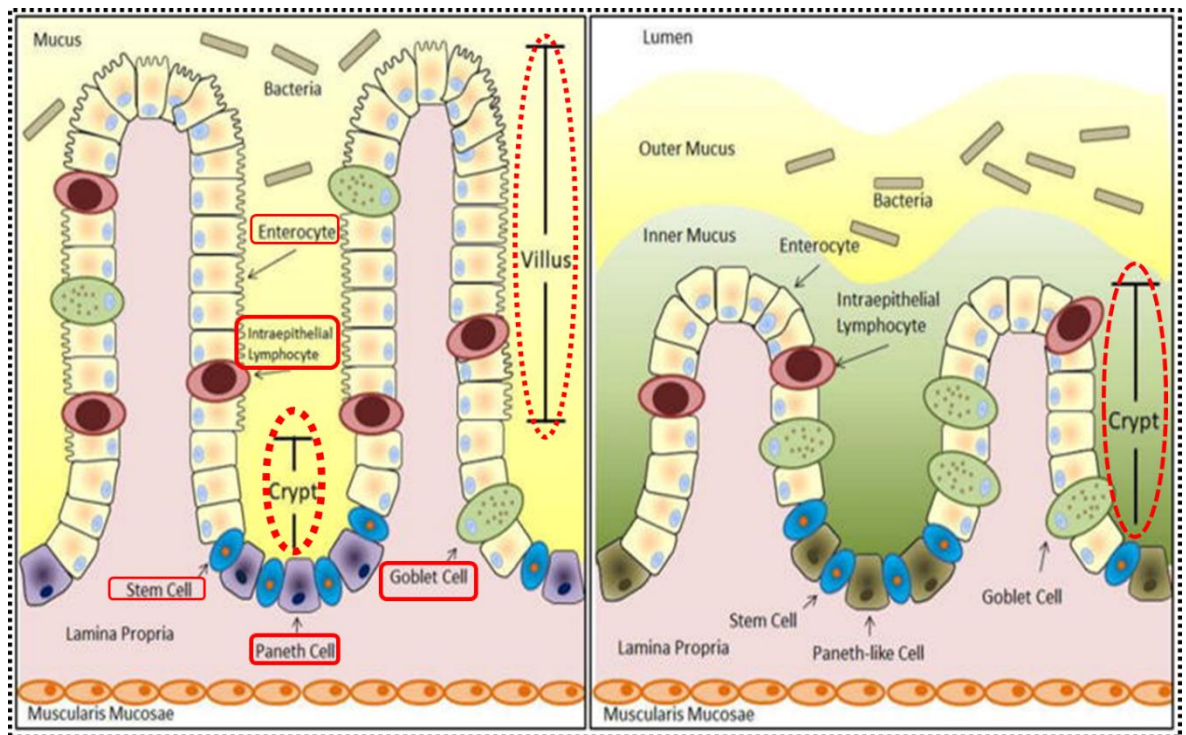
Figure 2.14: Simplified schematic representation of intestinal villus and crypt.

Beyond the small intestine, the GIT of bird continues with the caeca and the colon. The cecum arises at the junction between the ileum and colon (Clench and Mathias, 1995). The paired caeca are blind-ended sacs which extend parallel to the ileum and are loosely attached to it by mesentery and the ileocaecal ligament. The caeca opening has a network of long transversely orientated villi which act as a sieve, allowing only finely ground particles or soluble, low-molecular weight, non-viscous molecules of ileal and renal origin to enter the caeca (Svihus, 2014). The caeca villi are shorter and broader in the mid-portions and in the blind-end the villi are low and blunt with poorly developed intestinal crypts. More lymphoid tissues could be observed in older birds (Giambrone, 2013). The selective passage of colonic contents in the caeca depends on retrograde waves of colonic muscle contraction but at the same time the flow of the bowel`s material is prevented from moving up into the ileum by the contracted ileal sphincter. On the contrary to the relatively rapid movement of digesta through the intestine, caeca morphology facilitates the retention of digesta for longer periods. A mixing action, which is achieved by caecal wall contractions is another important feature of the caeca which not only keeps the contents in general motion and contributes to filling and evacuating the organ but

also facilitate absorption of fluids and molecules in solution (Svihus *et al.,* 2013). The caeca and the lower part of the digestive tract are responsible for the absorption of almost 36 % of the water and 75 % of the sodium of renal origin. Despite that the quantitative scale of this absorption is not certain, it has been suggested that the caeca can also play a role in renal nitrogen recycling (Svihus, 2014). All these specific features of the caeca make it the most appropriate site for fermentation and breakdown of selected feed materials which have escaped the digestion in the upper parts of the GIT (Clench and Mathias, 1995; Svihus *et al.,* 2013).

Some early experiments supported a single function of the caeca but now it is clear that the cecum has the potential to act in many ways. The most important activities are related to (1) utilisation and absorption of water and nitrogenous components; (2) microbial activity of either beneficial or pathogenic organisms, (3) production of immunoglobulins and antibodies. The caecal morphology, feed form and rearing conditions make caecal functions important not only for bird metabolism and health but also for coping with stressors. Taking into consideration the size of the caeca and its fermentation rates, it is obvious that the organ operates in a highly efficient manner (Clench and Mathias, 1995).

The bird`s colon is a straight section of the bowel between the caeca openings and the beginning of the cloaca. The structure of the colon wall resembles that of the small intestine but with fewer and smaller intestinal villi and crypts. The termination of the colon is marked with a slight constriction. The cloaca wall has a structure similar to the colon and small intestine and consists of three portions - the coprodaeum, the urodaeum, and the proctodaeum (Giambrone, 2013).

2.10.   Prebiotics

Historically, prebiotics are "*a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health*" (Gibson and Roberfroid, 1995). The most important characteristics of prebiotics considered for their assessment are presented in Figure 2.15.

Should be palatable as food ingredient and large scale processing must be easy

Should be neither hydrolyzed nor absorbed in the upper part of the gastrointestinal tract

Be a selective substrate for one or limited number of bacteria commensal to caecum/colon which are stimulated to grow or metabolically activated

Prebiotics

Should have known structure, which can be documented

Induce systemic effects that are beneficial to the host`s health

Able to alter the colonic flora in the favour of a healthier composition

(Source: adapted from Gibson and Roberfroid, 1995)

Figure 2.15: Criteria for categorize a compound as prebiotic

In 2007, Roberfroid published a revised definition, which explained prebiotic as "*a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host wellbeing and health*" (Roberfroid, 2007). Later, The International Scientific Association for Probiotics and Prebiotics (ISAPP) has published the expert consensus document which reviewed the definition and scope of prebiotics (Gibson *et al.,* 2017). According to the consensus, the concept of prebiotics is not limited to non-digestible carbohydrates (oligo- and polysaccharides) but also includes conjugated linoleic acid, polyunsaturated fatty acid, phenolics and phytochemicals along with some other candidate prebiotics such as human milk oligosaccharides. The group of non-prebiotic substances is comprised of antibiotics, vitamins, probiotics, proteins and fats (Gibson *et al.,* 2017). Hume (2011) listed inulin, fructooligosaccharides (FOS), galactooligosaccharides (GOS), soy-oligosaccharides, XOS, pyrodextrins, mannan-oligosaccharides (MOS), and lactulose as the most used prebiotics. The alternative term "*colonic foods*" is also

applicable and it is referred to the substances which reach the lower parts of GIT and serve as substrates for the endogenous colonic bacteria and thus indirectly can provide the host with energy and essential micronutrients (Hajati and Rezaei, 2010). Recently, a review article by Markowiak and Slizevska (2018) presents a very detailed review of the development of the definition and concept of prebiotics.

Gibson *et al.* (2017) acknowledged that it is incorrect that all dietary fibres are delineated as prebiotics. Despite that in Figure 2.16 the cellulose is part of non-selectively utilized substances, it could be a successful prebiotic for ruminants, however, for monogastric animals such as humans or chickens it could be associated with excessive gas formation or some other negative effects in the gut. Defining dietary fibres as prebiotics will depend on some important additional factors such as host and specific site of prebiotic activity (Gibson *et al.*, 2017).
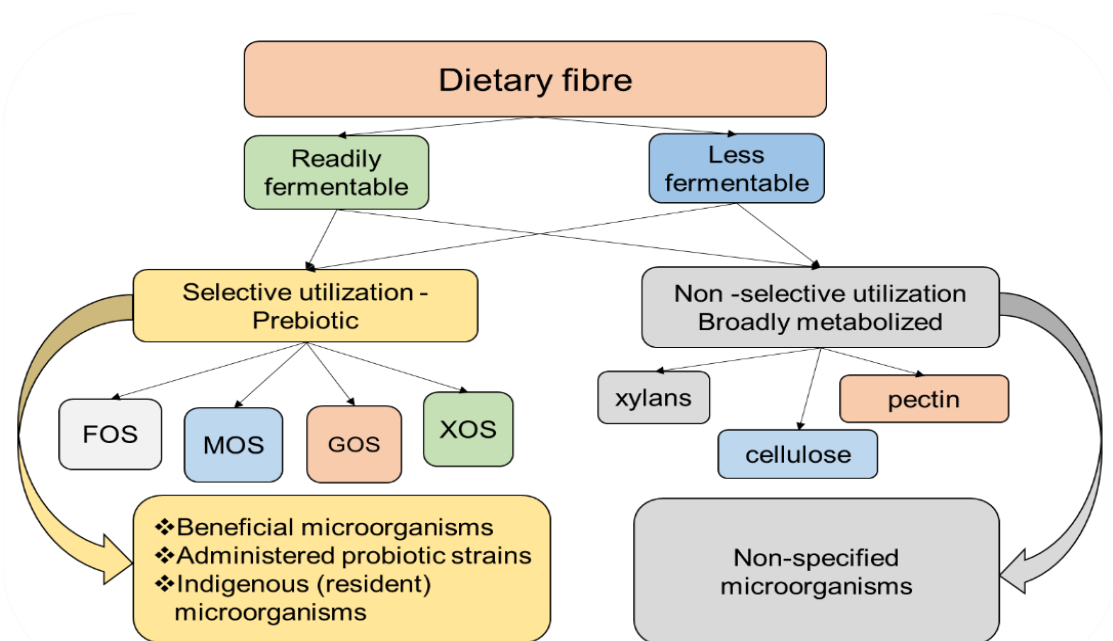
(Source: adapted from Gibson *et al.,* 2017)

Figure 2.16: Relationship between dietary fibres and prebiotics (FOS – fructooligosaccharides, MOS – mannan-oligosaccharides, GOS – galactooligosaccharides, XOS – xylooligosaccharides).

It is worth mentioning that substances which are currently used in animal feed but do not completely satisfy all the criteria for prebiotics by the definition of Roberfroid (2007) are described as "*prebiotic-like substances*" (Roto *et al.,* 2015). The use of prebiotic-like substances is cautioned when there is limited information about their

chemical structure, the optimal dose for inclusion or the mode of action (Jiang *et al.,* 2006). Some of the currently used substances with a potential prebiotic effect in poultry are presented in Table 2.7. It has been suggested that maintaining gut health might not be possible by using a single product and would require a combination of products which could exhibit both pro- and prebiotic effects on the GIT (Adedokun and Olojede, 2019).

Table 2.7. Some substances with a potential prebiotic effect for poultry

Substance	Effect	Animals/breed	References
Mannan-oligosaccharide	Mitigate the effect of stress and microbial dysbiosis in the gut	Arbor Acres broilers	Kridtayopas *et al.,* 2019
Galactooligosaccharides (FOS), Fructooligosaccharides (FOS) and plum fibres	Increase body weight	Cobb 500 (Cornish White rock cross fast-growing)03/05/2019	Hanning *et al.,* 2012
Phosphorylated mannan-oligosaccharides (MOS)	Increase body weight and feed efficiency	Arbor Acres broilers	Abdel-Hafeez *et al.,* 2017
Isomalto-oligosaccharides (IMO)	Improve weight gain, increase the caecal population of lactobacilli and bifidobacteria	Ross 308 broiler chickens	Mookiah *et al.,* 2014
Bio-Mos®; a mannan oligosaccharide derived from the cell walls of the yeast *Saccharomyces cerevisiae*	Improvements in feed conversion ratios	Broiler chicks	Midilli *et al.,* 2008
Fructooligosaccharide (FOS)	Enhanced growth of lactobacilli and bifidobacteria and inhibit *E.coli* in the small intestine and in a caeca digesta	Avian Farms broiler chickens	Xu *et al.,* 2003
Mannan oligosaccharide (MOS, Bio-Mos®); and dextran oligosaccharide (DOS, MHF-Y®)	Increase body weight and improvement in feed conversion (MOS diet)	Ross 308	Bozkurt *et al.,* 2008
Galactooligosaccharides (GOS)	Increased lactobacilli and bifidobacteria count	Broiler chickens	Jung *et al.,* 2008
Lactulose	Improvement in body weight gain and feed conversion ratio	Ross 308	Calik and Ergun, 2015

Currently, some benefits of xylanase have been related to the generation of oligosaccharides with a potential prebiotic effect (Courtin *et al.,* 2008b; Craig *et al.,* 2020). Figure 2.17 presents the main steps in plant material digestion triggered by xylanase activity. According to Karlsson *et al.* (2018) endoxylanase is an excellent tool for the generation of prebiotic oligosaccharides which can stimulate various types of intestinal bacteria. Xylooligosaccharides with different substitutions fermented by the gut microbiota produce mainly short-chain fatty acids (e.g. acetate, propionate, and butyrate, lactate), CO_2 and H_2. The relative amount of these products varies depending on the type of substituent(s) in the oligosaccharide (Karlsson *et al.,* 2018).
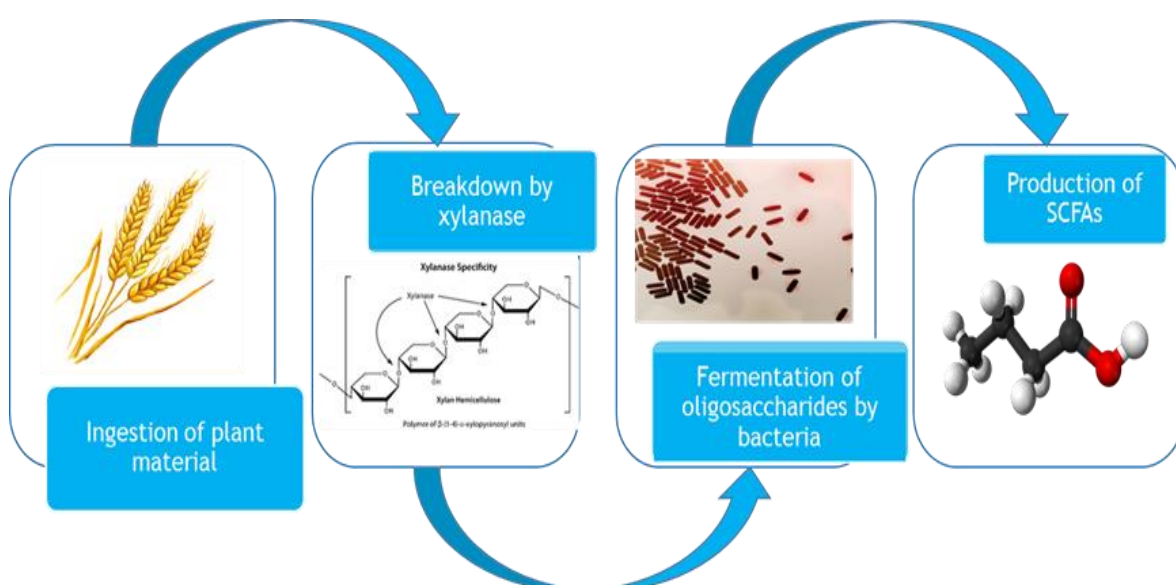
Figure 2.17: Schematic view of the main steps of plant material digestion triggered by xylanase activity.

2.11.   Xylooligosaccharides

Xylooligosaccharides are sugar oligomers made up of xylose units which are produced at an industrial scale manufactured from lignocellulosic materials (LCMs). The extraction of XOS from LCMs rich in xylan is possible by chemical and enzymatic methods and the latter is preferred because of the lack of undesirable side reactions or products (Vazquez *et al.,* 2000). As a food and feed supplement, XOS are a promising prebiotic molecule due to their nutritional benefits in various

animal species, including poultry. However, available data do not provide an exact explanation about the bioactive effects of XOS and numerous questions about the molecular mechanisms of action of XOS remain unanswered (Aachary and Prapulla, 2011).

Xylooligosaccharides have been studied for their beneficial effects on the performance, weight gain and shift in gut microbial populations (Ribeiro *et al.,* 2018). Some research data did not show a positive effect of XOS on chicken body weight during the first week or body weight gain and feed intake during 13-26 days of age (Courtin *et al.* 2008b, Akter, M. and Akter, 2021). However, De Maesschalck *et al.* (2015) reported better feed conversion ratio for chickens fed 0.5 % XOS, although there was no significant body weight response. Similar are the results of Ganapathy *et al.* (2019) who reported better feed conversion ratio and higher body weight for chickens fed either 0.50 % or 0.75 % XOS when compared to the control group. In a study of 59 days duration, Zhenping *et al.* (2013) reported that 10g XOS/kg improved body weight gain and feed conversion ratio.

Analysis of caecal bacteria after one and two weeks also revealed no significant effect of age or XOS on aerobic *Enterobacteriaceae* and aerobic *Lactobacilli*. However, at day 7, the level of *Bifidobacteria* was higher in the caeca of XOS-fed chickens and later the same group showed a marked increase in the level of *Bifidobacteria* in comparison to the control group (Courtin *et al.,* 2008b). In a dose dependent experiment, Cobb 500 broilers that received a diet containing 7.5 g/kg XOS showed lower bacterial total viable count (TVC) in both ileum and caeca and the chickens that received feed supplemented with either 2.5 or 5.0 g/kg XOS showed TVC similar to the control group (Akter, M. and Akter, 2021). Samanta *et al.* (2017) did not observed a positive influence on either live weight or feed conversion efficiency, but 0.5 % XOS supplementation resulted in selective stimulation of *Bifidobacteria* coupled with a reduction in the population of *Streptococci* and *Esherichia coli* in the caecum of broiler chickens. The authors found also that the beneficial changes in the caecal microflora resulted in changes in blood biochemical parameters - lower cholesterol, triglycerides and glucose concentration. Makelainen *et al.* (2010) also noticed that the available XOS did not selectively support the growth of *Lactobacilli* but positively influenced *Bifidobacteria* growth. The experiment conducted by Kabel *et al.* (2002) revealed that fermentation of different

XOS derivatives was a two-phase process. During the first phase, the acetate and lactate were more abundant in comparison with propionate and butyrate. The authors suggested that the fermentation process during this phase was not driven by lactic acid bacteria but most likely by different groups of non-specified intestinal bacteria. During the second phase of the fermentation, propionate and butyrate were the major end products.

Zhenping *et al.* (2013) found that XOS can positively affect the thyroid function and the levels of thyroid hormones which participate in poultry growth and metabolism. The authors also reported a higher serum level of antibody against the AI H5N1 vaccine virus which implies a strengthen humoral immunity in poultry fed XOS supplemented diets. Some other experiments showed the positive effect of XOS on intestinal characteristics, gut microbiota, caecal short-chain fatty acids and plasma immune parameters of broilers (De Maesschalck *et al.,* 2015) and laying hens (Ding *et al.,* 2018). However, the beneficial effect of XOS tended to be dose-dependent and the higher level of inclusion does not necessarily exert a better effect (Zhengping, 2013). Available data about XOS was recently reviewed by Adkihari and Kim (2017) who stressed that there were some inconsistencies in the experimental results and warrant further research.

2.12. GIT microorganisms

Kogut (2013) explained the gut is a highly specific ecosystem and defined its three interrelated elements: (1) the intestinal epithelium with its neuroendocrine signals, (2) the immune system and (3) the commensal microbiota. According to Apajalahti *et al.* (2004) interactions between the host and commensal microbiota are far more complicated than the simple tolerance to the beneficial bacteria and suppression or elimination of the intestinal pathogens. O`Hara and Shahanan (2006) referred to the microorganism entity as the "forgotten" organ and some others such as Turnbaugh *et al.* (2007) used "supraorganism" as a collective term for both microorganisms and the host. The interactions which exist between the host and its microbiome create a true symbiosis which is fundamental for the well-being and health of the host.

Table 2.8 presents information about the main genera which inhabit the main compartments of the chicken GIT. The caeca is the compartment with the most abundant microbiota both in quantity and diversity.

Table 2.8: Chicken intestinal compartments and the principal genera of the microbiota

Chicken intestinal compartment	Quantity, CFU/g	Bacterial genera
Crop	$10^8 - 10^9$	*Lactobacillus, Bifidobacterium, Micrococcus, Staphylococcus* and *Escherichia*
Proventriculus	$10^4 - 10^6$	*Lactobacillus*
Gizzard	$10^7 - 10^8$	*Lactobacillus, Escherichia, Enterococcus,* enterobacteria and *Campylobacter*
Small intestine	$10^8 - 10^9$	*Lactobacillus, Streptococcus, Eubacterium, Escherichia, Clostridium, lachnospiraceae, Enterococcus,* enterobacteria, staphylococci and *Bacteroides*
Caeca	$10^{10} - 10^{11}$	*Clostridium, Ruminococcus, Bacteroides, Eubacterium, Faecalibacterium, Blautia, Bacillus, Alistipes, Enterococcus, Lactobacillus, Streptococcus,* peptostreptococci, *Bifidobacterium, Propionibacterium, Gemmiger, Escherichia, Sporomusa, Actinomyces Pseudomonas, Fusobacterium, Eubacterium, Salmonella, Butyrivibrio, Roseburia, Ethanoligenens, Hespillia, Megamonas, Veillonella, Anaerostipes, Proteus*

(Source: Duggett, 2015)

The poultry intestinal microbiome (the entity of microorganisms and their genome) is shaped by the specific environment of bird`s GIT (Vispo and Karasov, 1997; Pan and Yu, 2014) and responds to additives in the diet (Svihus, 2014). The source and nature of dietary ingredients and the physical structure of feed are considered the major factors for the shift in the microbiome (Rehman *et al.,* 2007; Thomas *et al.,* 2019). Gut microbe-microbe interactions are as important as the host-microbiome interactions (Pan and Yu, 2014, Ajuwon, 2016).

Adil and Magrey (2012) did not find bacteria in any of the gastrointestinal compartments at hatching (day 1) but significant numbers of faecal streptococci and coliforms were detected after three days. However, Pedroso *et al.* (2005) found that even in 1-day old chicks the microbiota was presented with 21 amplicons and each amplicon presented different genotype. Apajalahti *et al.* (2004) reported in the

chicken GIT 640 species from 140 different genera. The authors concluded that approximately 90 % of the bacteria which inhabited the chicken GIT were previously unknown species or even genera. The researchers also suggested that corn-based diets increased proliferation of bacteria with a low %G+C ratio (*Clostridia* and/or *Lactobacilli*) and wheat-based diet increased the number of bacteria with a higher %G+C ratio (*Bifidobacteria*).

The bacterial density in the small intestine increases with age according to Rehman *et al.* (2007) but some others found a decrease in the microorganisms (Jamroz *et al.,* 2009). Munyaka *et al.* (2016) found that in the ileum lactobacilli were the predominant bacteria and the caeca was inhabited mostly with anaerobes from family *Lachnospiraceae* and some other clostridium-related bacteria. The data also revealed that bacterial diversity was greater in ileal digesta in comparison to the caeca digesta. The main groups of microorganisms were lactobacilli, streptococci, enterobacteria, fusobacteria and eubacteria but some moulds and yeast were also detected. Strict anaerobes (anaerobic gram-positive cocci, *Eubacterium spp.*, *Clostridium spp.*, *Lactobacillus spp.*, *Fusobacterium spp.* and *Bacteroides*) were the predominant caecal bacteria in young broilers. Data from molecular studies showed that lactobacilli were about 25 % of the total bacteria examined in 4-day old chick's caeca while bifidobacteria was not detected. However, both lactobacilli and bifidobacteria reach 40 % of the total caeca population in 14-day old broilers (Amit-Romach *et al.,* 2004). Gene sequencing of caecal DNA extracts showed that the majority of bacteria belonged to *Clostridiaceae*

Bjerrum *et al.* (2006) studied the microbiota of conventional and organic farmed chickens and showed the caeca had the highest number of total anaerobic bacteria (10^{10} CFU/g of intestinal content) and lactobacilli were from 5 to 8 % of the microbial community. The authors found that lactobacilli were the dominant species in the ileum, whereas the cecum was characterised by a more diverse microbial community. Additionally, some uncultured bacteria (that can reach 60 %) and some other closely related to *Faecalibacterium prausnitzii* were detected in caeca. Similarly, Wise and Siragusa (2007) observed time-dependant changes in the caeca microflora, with the family *Enterobacteriaceae* dominating microbiome at day 7 but sequentially (by day 14) obligate anaerobes were more abundant.

One of the most important concepts is that through the diet and additives it is possible to modulate not only the weight gain (Angelakis, 2017) and immune status (Kogut, 2009) but also the microbial community in the chicken gut (Adil and Magray, 2012; Hanning and Diaz Sanchez, 2015; Park *et al.*, 2017). The data of Dusel *et al.* (1998) suggested a detectable improvement in the intestinal microflora in response to xylanase supplementation, even with the fully developed microbial population of mature birds. Other researchers found that the caecal microbial community was affected by diet, age and treatment but the ileum microorganisms were affected only by diet and age (Kumar *et al.,* 2018).

Some researchers explained impaired performance of birds fed wheat and barley diets by a reduced population of gram-positive bacteria including lactobacilli and bifidobacteria (Yaghobfar and Kalantar, 2017). Jamroz *et al.* (2009) observed a decrease in the number of lactobacilli in the small intestine and colon of older birds but also acknowledged difficulties explaining the differences among the observed groups of microorganisms and the changes as a result of the feed additives. Kidd (2004) tried to explain existing inconsistencies by suggesting that it was very unlikely that broiler requirements for optimal immunity would completely coincide with those for optimal growth or breast meat accretion. It is possible that intestinal microbiota acts through mechanisms such as: (1) nutrient exchange, (2) immune system modulation, (3) effect on digestive system physiology, and (4) pathogens exclusion (Rubio, 2019). The intestinal microbiota of broilers influences the host by more than a single process and the existing mechanisms can overlap and are interrelated.

2.13.   Production of short-chain fatty acids (SCFAs)

By definition SCFAs are organic fatty acids with 1 to 6 carbons, either with straight or branched-chain conformation produced by bacterial fermentation of undigested dietary carbohydrates and to a lesser extent, dietary and endogenous proteins such as discarded epithelial cells and mucous (Topping and Clifton, 2001). The primary (90 – 95 %) SCFAs present in the gut are acetic acid (C2), propionic acid (C3), and butyric acid (C4). The main sources of SCFAs are carbohydrates but the amino acids valine, leucine, and isoleucine obtained from protein breakdown can be converted into isobutyrate, isovalerate, and 2-methyl butyrate, known as branched-chain SCFAs, which contribute to no more than 5 % of total SCFAs production (Rios-

Covian *et al.,* 2016). Very often lactic acid (a non SCFAs) is analysed along with extracted SCFAs since it is also produced during a fermentation of undigested carbohydrates (Adebowale *et al.,* 2019). Despite being the least abundant of the three major SCFAs, butyrate is the most important metabolite for the colonic epithelial cells because 90 % of butyrate is metabolised by colonocytes (Hamer *et al.,* 2008). Unabsorbed SCFAs are transported through the peripheral circulation. The fractions not absorbed in the gut are distributed to the other organs and tissues for metabolism with the liver as the major site of SCFAs metabolism (Topping and Clifton, 2001; Jozefiak *et al.,* 2007). Short-chain fatty acids can act on different organs and peripheral tissues and could enter diverse carbohydrates and lipid metabolic routes. As an example, propionate is incorporated mainly into gluconeogenesis whilst acetate and butyrate are mostly introduced into the lipid biosynthesis (Rios-Covian, 2016).

According to Hamer *et al.* (2008), DF are important source for colonic fermentation and particularly for butyrate synthesis. Furthermore, soluble NSPs interact with the intestinal microbiota in a different manner and are a mediator in the interplay between potentially harmful bacteria and the gut epithelium (Simpson and Campbell, 2015). The inclusion of fibre in poultry diets also has a significant effect on the fermentative ability of caecal microorganisms. The difficulties of using DF (i.e. NSP) is to balance their anti-nutritive properties and the benefits as a result of their fermentation in the caeca. Additionally, understanding the interactions between DF and SCFAs within the caeca may provide important information about additional energy sources for the bird, inhibition of pathogenic bacteria, effects on blood lipids and cholesterol level in poultry products (Jozefiak *et al.*, 2004).

The quantity of caecal SCFAs depends on diet, microflora, age and caeca development (Hamer *et al.,* 2008). The study of Van der Wielen *et al.* (2000) presents the changes of pH, lactate, and volatile fatty acids according to birds' age. Authors reported caecal pH values in the range of 5.5 to 6.0 during growth of broilers. The earliest detected SCFAs (3-day-old broilers) was acetate and its concentration continued to increase until the 15th day. Later - in 12- to 15-day-old broilers, propionate and butyrate were detected. After the 15th day, the concentration of acetate, propionate and butyrate remained stable. On the contrary, lactate was present only during the first 15 days and thereafter it was not detected.

Exogenous enzymes in poultry feed can also affect SCFAs production. According to Bedford (2000b), exogenous enzymes acted through changes in the availability of fermentable sugars in the caeca which in turn shaped the microbial population. It has been suggested that exogenous enzymes were more active in the upper parts of the GIT but it is very likely that they were active to some extent also in the foregut. Enzymes can provide soluble, poorly absorbed sugars from plant cell wall material which feed beneficial bacteria. Yacoubi *et al.* (2016) found that a multicomponent enzyme preparation (MEP) increased the proportion of wheat water-soluble arabinoxylan (AX) and reduced its molecular weight. The MEP-treated fractions stimulated the production of SCFAs and particularly acetate and butyrate to the same level as prebiotics such as FOS an XOS when incubated with caecal microbiota *in vitro*. Authors suggested that the beneficial action of endoxylanase on broiler performance was achieved by the solubilising and depolymerising of wheat cell wall arabinoxylan.

The study of Calik and Ergun (2015) showed that even in 7-day-old birds, dietary prebiotic (lactulose) increase the concentrations of acetate, propionate, butyrate and total SCFAs. However, the authors did not find apparent differences on day 21 but on day 42 the increased concentration was again significant. Ding *et al.* (2018) also have found a positive effect of XOS on the contents of acetic and butyric acid. However, Walugembe *et al.* (2015) reported that increasing level of dietary supplemented fibre did not influence the concentrations of some of the examined SCFAs but resulted in a significant decrease in the concentration of butyric acid. Other data also suggested that not all dietary formulations have a positive effect on the production of SCFAs. Results of several experiments revealed that the use of whole wheat, whole sorghum, or whole barley did not have effect of caecal pH and SCFAs concentration in two breeds of male broiler chickens (Biggs and Parsons, 2009).

In theory, the perfect antibiotic growth promoter substitute would be a component that could eliminate pathogens, reduces the risk of diseases, improves bird health and at the same time is cheap to produce, stable during storage and easy to be supplemented in the feed (Alloui *et al.*, 2013; Bedford and Gong, 2018). There are at least two approaches towards utilisation of SCFAs. The first one is the direct use of a single or multiple SCFAs as additives to poultry feed (Van Immerseel *et al.,*

2004; Adil *et al.,* 2011; Gonzalez-Ortiz *et al.,* 2019). The second approach relies on physiological mechanisms, mainly through poultry caeca fermentation to stimulate SCFAs synthesis. In any case, SCFAs either as feed supplements or metabolically generated through the inclusion of feed materials seems promising and safe alternatives (Dibner and Buttin, 2002; Oviedo-Rondon, 2019). However, there is insufficient information about the exact mechanism of how SCFAs beneficially effect poultry.

2.14. Conclusions/research gap

Currently, there is a shift in the interest of exogenous enzymes and prebiotics towards their use as alternatives to antibiotic growth promoters and as successful growth and health promoters in animal nutrition. Despite some promising data on improved growth performance, better feed conversion ratio and increased fermentation in the caca, the available information about xylanase, XOS or their combination on growth parameters, gut development and caeca fermentation is very limited. Research will provide important information about the contribution of xylanase, XOS and NSP as prebiotic sources for improved GIT fermentation and their role in broiler chicken production and health. Data revealing the possible direct or indirect mechanisms (including interactions) through which supplemented XOS, alone or in combination with xylanase, influences the important parameters in poultry performance and health will enhance their use as additives and also explain limitations in broiler response indicated by the literature.

Chapter 3. General materials and methods

3.1.   Animals

All experimental procedures were approved by Harper Adams University Research Ethics Committee and were conducted according to UK regulations (Animal Welfare Act 2006). Birds were observed at least twice a day.

Day-old male Ross-308 chicks were obtained from a commercial hatchery (Cyril Bason Ltd, Craven Arms, UK). On arrival, birds were weighed and relocated into floor pens, with 20 birds in each (excluding ill or malformed birds). Floor pens have a solid floor with an area of 2.1 m^2 covered with clean wood shavings. Access to food and water was *ad libitum*. A standard lighting programme for broilers was used decreasing the light: dark ratio from 23h: 1h from one- day-old to 18h: 6h at 7 days of age which was maintained until the end of each study. Mortality was recorded daily, and weight of dead chickens or culled were recorded at the time of removal. The feed provided and the birds (considered as a group per pen basis) were weighed at delivery and after that at the specific day until the end of each experiment - day 35. The average bird weight (BW) was recorded. Feed intake (FI), body weight gain (WG) and mortality corrected feed conversion ratio (FCR) were calculated.

During the last three days of each rearing phase, either 5 (for grower phase) or 3 birds (for finisher phase) chosen at random from each pen, were placed in raised-floor pens situated in the same room. The raised-floor pens had a wire mesh bottom and were equipped with feeders, nipple drinkers and clean dropping trays under each cage. Excreta samples, visibly free of feed and feathers, were collected twice (following 36-h periods) from the trays beneath each pen and then kept in the freezer (– 20°C). At the end of the study, all samples were thawed at room temperature, oven-dried (65°C), ground (0.5-mm screen) and stored for analysis. After the excreta collection, one bird per cage was culled by cervical dislocation and the following organs were weighed: liver, proventriculus and gizzard, pancreas, duodenum, jejunum, ileum, caeca and spleen. All other birds were returned to their respective floor pens at the end of the collection period.

3.2. Diets

All diets were isonitrogenous and isocaloric, with formulations following Aviagen recommendations (Aviagen Ltd, Edinburgh, UK, 2018).

3.3. Laboratory analysis

3.3.1. Dry matter (DM)

Fresh excreta were dried for 72 h at 62 °C and after that was left at room temperature for 24 h to cool. Dry matter (DM) in feed and excreta samples was determined by drying of samples in a forced draft oven at 105 °C to a constant weight (AOAC 2000; method 934.01).

3.3.2. Gross energy

Gross energy (GE) of the diets and excreta was measured using an adiabatic bomb calorimeter (Parr 6200 Instrument Company, Moline, IL, 61,265, United States). Benzoic acid was used as the standard.

3.3.3. Ether extract

Fat (as ether extract) was analysed according to AOAC 2000, method 945.16 using a Soxtec system (Foss Ltd., Warrington, UK) and following FOSS (2008) procedure. Petroleum ether 40-60ºC was used as a solvent to extract the oil.

3.3.4. Nitrogen

Crude protein (6.25×N) in samples was determined using the DUMAS method by combustion (AOAC 2000; method 990.03) using a LECO FP-528 N (Leco Corp., St. Joseph, MI). In short, 0.1g sample is placed into a tin foil, this sample is dropped into a furnace at 950 °C in a pure atmosphere of helium and oxygen. The sample is burnt and the nitrogen released is measured against a standard of EDTA.

3.3.5. Acid insoluble ash determination

Acid insoluble ash (AIA) in feed and excreta was determined according to Van Keulen and Young (1977). The use of AIA as an indigestible marker was proven to provide consistent results in the bioassay of apparent metabolisable energy in experimental diets with or without exogenous enzyme and undigested NSP and fiber

fractions when fed to broiler chickens (Scott and Hall, 1998). The AIA analysis in brief consists of weighing (4-5g) of dried milled sample in porcelain crucible and then in muffle furnace to burn at 550ºC for 4 hours for ash determination. The formulas used for calculation are as follow:

Weight of ash= weight of crucible plus ashed sample – weight of crucible

Percentage ash in sample (% ash) = $\frac{ash\ weight\ (g)}{sample\ weight\ (g)} x\ 100$

Ash in sample (d ash/kg sample) = $\frac{ash\ weight\ (g)}{sample\ weight\ (g)} x\ 1000$

After ash determinantion the content of the crucible is put in a Kjeldal tube with 100 ml of 2M HCl and digested for 10 min at approximately 175 ºC. The hot digest is filter through ash free filter paper and the tube and filter are washed with hot distilled water. The filter paper and digested sample are folded and placed in the crucible again and the crucible is placed in a muffle furnace for a further 4 hours at 550 ºC. Calculation of AIA is done according to the following equation:

AIA (g/kg) = $\frac{weight\ of\ crucible\ with ashed\ acid\ sample-weight\ of\ crucible}{initial\ weight\ of\ sample} x\ 1000$

3.4.   Relative development of GIT

The relative development of organs was expressed as a percentage, according to the procedure described by Amerah and Ravindran (2008). Samples of different segments from the GIT were taken at the end of each rearing period following the withholding of feed for several hours before sampling. One bird per pen was weighed (BW) and killed by cervical dislocation. The GIT and organs were carefully excised, and any content left was squized out gently by palpation. The empty weight and length of duodenum (pancreatic loop), jejunum (from the pancreatic loop to Meckel's diverticulum), ileum (from Meckel's diverticulum to ileocaecal junction), and caeca (left and right) were recorded. Empty weight of the crop with proventriculus (weighed althogether) and empty weight of gizzard were recorded. Additionally, the weights of pancreas, liver and spleen were also recorded. The relative organs weight (g/kg of bird BW) were then calculated.

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3.5.   Histomorphometry

Histomorphometry was completed in collaboration with the Faculty of Veterinary Medicine, Trakia University, Stara Zagora, Bulgaria. The morphometric variables being examined were villus height and crypt depth. The villus height was measured from the tip of the villus to the base, whereas the crypt depth was measured as the depth of invagination between adjacent villi. Ileal segments were fixed in 10 % aqueous formaldehyde solution and after that, were rinsed with water, dehydrated with ethanol, rinsed with xylene and embedded in paraffin. Serial histological sections of 5 to 7 µm thickness were cut from the waxed tissues on a microtome YD-335A (J.Y.M.A. Ltd., China) and mounted on slides. The slides were stained with haematoxylin, counter-stained with eosin and examined under a light microscope VDN-200M (LUMENLAB, China) coupled with a camera CMOS to a computer. Villus height, villus width, crypt depthwere recorded. Measurements of villus height and crypt depth were taken only from sections where the section plane ran vertically from the tip of the villus to the base of an adjacent crypt. All measurements were done using ScopeImage Advanced Micro-image Process Software, with analysis following the procedure described by Yovchev *et al.* (2019).

3.6.   Enzyme activity

Dietary enzyme activity was analysed by a product specific validated ELISA method, using a Quantiplate Kit for Econase XT, supplied by Envirologix (AB Vista Laboratories, Innovation & Technology Centre, Ystrad Mynach, UK). In the study, Econase®-XT and SIGNIS® were used. The active agent of Econase®-XT and SIGNIS® is endo-1,4- -xylanase produced by a strain *Trichoderma reesei* (CBS 114044). The enzymatic activity is expressed in xylanase units (BXU) where 1 BXU is the amount of endo-1,4- -xylanase that liberates 1 nmol xylose from birchwood xylan per second at pH 5.3 and 50 °C.

3.7.   SCFAs analysis

Short-chain fatty acid concentrations (SCFAs) of caecal content were analysed by Alimetrics Diagnostics Ltd (Espoo, Finland) as free acid by gas chromatography, following the method and procedures described by Apajalahty *et al.* (2019).

3.8. Calculations

- Calculation of mortality corrected feed conversion ratio (FCR) used the following equiation:

$$FCR = \frac{FI}{WG + BW\ dead\ and\ culled\ birds}$$

Where WG is weight gain per pen and BW is recorded body weight of dead or culled for sampling purposes birds

- Calculation of AME was according Scott and Hall (1998):

$$AME = GE_{feed} - \left(\frac{GE_{excreta} \times AIA_{feed}}{AIA_{excreta}}\right)$$

Where GE_{feed} is the gross energy in the feed (MJ/kg), $GE_{excreta}$ is the gross energy in the excreta (MJ/kg), AIA_{feed} is the concentration of AIA in the feed (g/kg) and $AIA_{excreta}$ is the concentration of AIA in the excreta (g/kg).

- The AMEn value of the experimental diets was determined with the following formula:

$$AMEn = GEfeed - (GEfeed\ x\ AIA\ excreta)/AIAexcreta - \frac{(34.39\ x\ N\ retained)}{1000}$$

where AMEn (MJ/kg) = N-corrected apparent metabolisable energy content of the diet, GE_{feed} is the gross energy in the feed (MJ/kg), $GE_{excreta}$ is the gross energy in the excreta (MJ/kg), AIA_{feed} is the concentration of AIA in the feed (g/kg) and $AIA_{excreta}$ is the concentration of AIA in the excreta (g/kg). 34.39 (MJ/kg) = energy value of uric acid; and *N retained* (g/kg) is the N retained by the birds per kilogram of diet consumed. The retained N was calculated as:

$$N\ Retained = Nfeed - (\frac{Nexcreta\ X\ AIAfeed}{AIAexcreta})$$

Where N_{feed} is the nitrogen in the feed (g/kg), $N_{excreta}$ is the nitrogen in the excreta (g/kg), AIA_{feed} is the concentration of AIA in the feed (g/kg) and $AIA_{excreta}$ is the concentration of AIA in the excreta (g/kg).

- Dietary nutrient retention coefficients (DM, nitrogen, fat) were calculated using the following equation:

$$Nutrient\ retention\ coefficients = \frac{\frac{Nfeed}{AIAfeed} - \frac{Nexcreta}{AIAexcreta}}{\frac{Nfeed}{AIAfeed}}$$

Where N_{feed} is the corresponding nutrient in the feed (g/kg), $N_{excreta}$ is the corresponding nutrient in the excreta (g/kg), AIA_{feed} is the concentration of AIA in the feed (g/kg) and $AIA_{excreta}$ is concentration of AIA in the excreta (g/kg).

3.9. Statistical analysis

Data handling and calculations were performed in Excel 2013 (Microsoft Corporation) and statistical analysis performed using GenStat 18th edition (VSN International Ltd, Oxford, UK) by analysis of variance (ANOVA). Summary statistics were used to check data for outliers (<> 3 SD) and normality. Significance was set at ≤ 0.05 and tendencies were discussed at 0.05 < p < 0.1. Duncan's multiple range test was used to separate means when significant main effects (P<0.05) existed.

Chapter 4. Effect of xylanase and SIGNIS® on growth performance, dietary available energy, nutrient retention, GIT development and caecal short-chain fatty acids of broiler chickens fed wheat-maize-based diets

4.1. Introduction

The primarily commercial application of enzymes as feed additives aims to enhance nutrient digestibility of feed by removing the anti-nutritive effects of NSP, such as arabinoxylans and *β*-glucans from broiler diets based on viscous grains like wheat, rye, barley or triticale (Bedford and Morgan, 1996). The application of enzymes extends further than NSP, enhancing the digestibility of other nutrients such as phytate, alleviating the impact on the environment by reducing phosphorus excretion (Selle and Ravindran, 2007). However, the industry is not only in search of highly efficacious enzymes for non-viscous cereal grains but also for new areas of application, for example as an alternative to in-feed antibiotics (Cowieson and Kluenter, 2019). According to Choct (2006), feed enzymes can be used against anti-nutrients other than NSP and phytate, to degrade non-conventional feed resources to yield metabolizable energy and *in vivo* to generate specific low weight carbohydrates, which can produce specific health outcomes in birds. The improvement of enzyme technology depends on better characterisation of substrate structures, the gut microflora, and the immune system (Choct, 2006).

Xylanase, as part of the carboxylase enzyme family, acts upon xylan, one of the most abundant NSP found in poultry feed. Feed supplemented with exogenous xylanase not only improves utilisation of plant materials but also has been suggested to produce xylooligosaccharides with prebiotic properties (Masey O`Nielll *et al.,* 2014b; Karlsson, 2018). It has been suggested that enzymes and prebiotics, either synthesized in the gut or exogenous, could act synergistically and have a positive effect on bird performance (Ribeiro *et al.,* 2018).

4.2.   Objectives of the study

To determine if supplementing a combination of xylanase and XOS in wheat-maze based diets have a synergistic effect on broiler production performance, gut health and dietary nutritional value.

4.3.   Materials and methods

4.3.1.   Animals

Five hundred and fourty, day-old male Ross-308 chicks used in the study were alocated into 27 floor pens with 20 birds in each (excluding ill or malformed birds). All procedures applied are described in detail in Chapter 3 – General materials and methods.

4.3.2.   Diet formulation

Chicks were fed one of three diets in two phases named starter (0–21 day) and finisher (22–35 day). The main ingredients in the basal diets were wheat, maize and soybean meal (Table 4.1). The basal diet for each phase was split into three batches: (1) control diet – **CTR**, without supplements (activity of xylanase <2000 BXU/kg and phytase - 500 FTU/kg), (2) **XT diet** - the basal diet supplemented with 100 g/t Econase®-XT (xylanase enzyme, producing strain *Trichoderma reesei*) (AB Vista, Marlborough, UK, providing 16 000 BXU/kg units of xylanase). The third batch – diet (3) **SIGN**, the basal diet was supplemented with 100 g/t SIGNIS® (a blend of xylanase providing 16 000 BXU/kg and xylooligosaccharides – 50 g/t). The diets were fed in mash form and did not contain any coccidiostat, antimicrobial growth promoters or prophylactics.

Table 4.1. Composition of the experimental basal diets

Ingredients g/kg	Starter diet 0–21 day	Finisher diet 22–35 day
Wheat	290.0	390.0
Maize	312.7	354.0
Soybean meal	333.0	197.0
Soya oil	15.2	11.0
Salt	2.8	3.0
Limestone	11.0	9.5
Monocalcium Phosphate,	6.2	4.5
Lysine HCl	1.5	2.9
DL-Methionine	3.1	2.6
Threonine	0.3	1.0
Valine	0.00	0.5
[1]Vitamin & Mineral premix	4.0	4.0
[2]Quantum Blue 5G	0.1	0.1
Acid insoluble ash	20.0	20.0
TOTAL, %	100.00	100.00
Calculated values		
AME MJ/kg	12.35	12.67
DM, g/kg	888.1	889.1
Crude protein, g/kg	225.0	175.0
Calcium %	0.90	0.76
Phosphorus %	0.72	0.60
Analysed values		
GE[3], MJ/kg	15.75	15.83
DM, g/kg	891.8	892.1
CP, g/kg	223.13	166.88
Crude fat, g/kg	30.33	33.33
NSP, g/100g		
Soluble	1.8	1.8
Insoluble	7.8	6.7
Total	9.6	8.5

[1] The vitamin/mineral premix provided the following content per kg of diet: vitamin A, 10,000 IU; vitamin D3, 2,500 IU; vitamin E, 25 IU; vitamin E, 50 mg; vitamin K3, 1.5 mg; vitamin B1, 2 mg; vitamin B2, 7.5 mg; vitamin B6, 3.5 mg; vitamin B12, 20 µg; niacin, 35mg; pantothenic acid, 12 mg; choline chloride, 460 mg; folic acid, 1.0 mg; biotin, 0.2 mg; Fe as iron sulphate, 265 mg; Cu as copper sulphate, 48 mg; Mn as manganese oxide, 140 mg; Zn as zinc sulphate, 165 mg; I as potassium iodide, 1.2 mg; and Se as sodium selenite, 0.33 mg.; [2]Quantum Blue 5G, AB Vista, Marlborough, UK; 500 FTU/kg; [3]GE – gross energy

4.3.3.   Statistical analysis

Data handling and calculations were performed in Excel 2013 (Microsoft Corporation) and staistical analysis was performed using GenStat 18th edition (VSN International Ltd, Oxford, UK) statistical software. Experimental data were analysed as a randomised block design by two-way ANOVA, consisting of nine replicates (pens of twenty animals) per dietary treatment. The main factor being diet supplements. Summary statistics were used to check data for outliers (<> 3 SD) and normality. Means were separated only when the treatment p-value was significant, and Duncan's multiple range test was used to determine differences between treatment groups. Significance was set at p value ≤ 0.05 and tendencies were discussed at $0.05 < p < 0.1$.

4.4.   Results

4.4.1.   Analysis of phytase and xylanase activity

Expected and analysed activity of phytase and xylanase in experimental diets are presented in Table 4.2. Activity of the enzymes in the starter feed corresponded to the expected values. In the finisher diet analysed activity was slightly higher - phytase activity in XT diet and xylanase activity in SIGN diet, respectively.

Table 4.2: Analysis of phytase and xylanase activity in control (CTR) and supplemented with xylanase (XT) or SIGNIS® (SIGN) diets

Feed/ additive	Expected		Analysed	
	Phytase, FTU/kg	Xylanase, BXU/kg	Phytase, FTU/kg	Xylanase, BXU/kg
Starter diet				
CTR	500	0	658	<2000
XT	500	16000	482	16000
SIGN	500	16000	625	15800
Finisher diet				
CTR	500	0	853	<2000
XT	500	16000	939	17100
SIGN	500	16000	651	18600

4.4.2. Bird growth performance

There was no difference (p = 0.768) in the body weight (BW) of birds when they were allocated to the different treatments and the average BW was 42.46 g, 42.22 g, 42.29 g (SEM = 0.231, SD±0.674) respectively for control, xylanase and SIGNIS® diets.

Performance variables such as body weight (BW), weight gain (WG), feed intake (FI) and calculated mortality corrected feed conversion ratio (FCR) were not affected (p>0.05) by inclusion of xylanase or SIGNIS® (Table 4.3).

Table 4.3: Effect of dietary supplemented xylanase and SIGNIS® on body weight (BW), weight gain (WG), feed intake (FI) and feed conversion ratio (FCR) of broiler chickens fed wheat-maize based diet

Outcome	Diet supplements			Statistics	
	Control	**Xylanase**	**SIGNIS®**	**SEM**	*p-value*
21 d-old					
BW, g	633.02	637.69	641.49	13.99	0.913
WG, g/b/d	24.58	24.68	24.82	0.816	0.959
FI, g/b/d	45.81	45.66	45.55	0.668	0.963
FCR, g/g	1.71	1.71	1.67	0.035	0.713
35 d-old					
BW, g	1645.64	1653.49	1666.11	28.63	0.879
WG g/b/d	59.70	60.07	60.97	1.539	0.835
FI g/b/d	127.34	128.98	130.74	2.938	0.719
FCR, g/g	1.95	1.92	1.92	0.066	0.951
Overall, d 0 to 35					
WG, g/b/d	37.41	37.65	38.00	0.723	0.854
FI, g/b/d	75.20	76.17	76.58	1.247	0.727
FCR, g/g	1.90	1.89	1.89	0.046	0.964

4.4.3. Apparent metabolisable energy (AME) and nutrient retention

Metabolisable energy and retention coefficients (Table 4.4) were not affected by xylanase and SIGNIS® at the end of the starter phase but at the end of the finisher phase both supplements had a significant positive effect on AME (p=0.011), AMEn (p=0.006) and DMR (p=0.014).

Table 4.4: Effect of dietary supplemented xylanase and SIGNIS® on apparent metabolisable energy (AME), metabolisable energy corrected for nitrogen (AMEn) and nutrient retention (dry matter retention - DMR, nitrogen retention – NR and fat retention – FR) in 21 and 35 day-old broiler chickens

Outcome	Diet supplements			Statistics	
	Control	Xylanase	SIGNIS®	SEM	p- value
21 d-old					
AME	11.61	11.76	11.59	0.079	0.279
AMEn	10.80	10.94	10.80	0.073	0.335
DMR	0.720	0.727	0.720	0.0037	0.321
NR	0.657	0.669	0.655	0.0089	0.481
FR	0.744	0.770	0.761	0.0131	0.404
35 d-old					
AME	12.58[a]	12.88[b]	12.94[b]	0.080	0.011
AMEn	12.14[a]	12.41[b]	12.41[b]	0.822	0.006
DMR	0.776[a]	0.791[b]	0.794[b]	0.0043	0.014
NR	0.689	0.706	0.690	0.0063	0.123
FR	0.821	0.827	0.841	0.0107	0.437

[1]Different superscripts in the rows indicate significant differences between treatments ($p < 0.05$)

4.4.4. Gastrointestinal tract development

Both xylanase and SIGNIS® decreased the relative weight of the pancreas in 21-day-old birds (Table 4.5), and xylanase alone increased the weight of the small intestine and GIT in 35-day-old birds. The feed supplements did not have significant effects (P>0.05) on any other compartment of the GIT of broiler chickens.

Table 4.5: Effect xylanase and SIGNIS® on gastrointestinal tract development of broiler chickens fed wheat-maize based diet

Outcome	Diet supplements			Statistics	
	Control	**Xylanase**	**SIGNIS®**	**SEM**	**_p_- value**
21-day-old					
[1]**PG**	3.91	3.65	3.77	0.115	0.294
Duodenum	1.38	1.40	1.38	0.065	0.957
Jejunum	2.32	2.44	2.34	0.096	0.643
Ileum	1.87	1.87	1.86	0.086	0.995
Caeca	0.55	0.53	0.58	0.030	0.527
[2]**Small intestine**	5.57	5.70	5.57	0.189	0.842
[3]**GIT**	10.02	9.89	9.92	0.221	0.900
Pancreas	0.49[a]	0.43[b]	0.43[b]	0.018	0.045
Spleen	0.08	0.10	0.08	0.010	0.441
Liver	2.89	2.90	2.87	0.096	0.979
35-day-old					
[1]**PG**	2.11	2.38	2.27	0.114	0.259
[2]**Small intestine**	2.67[a]	3.09[b]	2.64[a]	0.092	0.003
Caeca	0.36	0.42	0.36	0.024	0.120
[3]**GIT**	5.22[a]	5.90[b]	5.41[ab]	0.184	0.041
Pancreas	0.25	0.28	0.22	0.019	0.123
Spleen	0.10	0.10	0.11	0.007	0.356
Liver	2.49	2.55	2.72	0.076	0.114

[1]PG = proventriculus and gizzard
[2]Small intestine = duodenum, jejunum and ileum
[3]Gastrointestinal tract (GIT) = PG, duodenum, jejunum, ileum and caeca
[4]Different letter superscripts in the rows indicate significant differences between treatments ($p < 0.05$)

4.4.5. Effect of dietary treatment on caeca content of SCFAs

SIGNIS® supplemented diets increased the concentration of SCFAs (p=0.024) and butyric acid (p=0.002) in 21-day-old birds. In the caeca of older birds both xylanase and SIGNIS® increased the levels of SCFAs (p=0.005) in comparison to the control diet and additionally xylanase alone had a significant effect on acetic acid (p=0.011), butyric acid (p=0.036) and volatile fatty acids (p=0.038) in comparison to control diet.

Table 4.6: The effect of experimental diets on caecal content of SCFAs (mM)

Outcome	Diet supplements			Statistics	
	Control	**Xylanase**	**SIGNIS®**	**SEM**	**p- value**
21 d-old					
SCFAs	90.85[a]	91.77[a]	123.11[b]	8.538	0.024
AA	69.70	71.52	85.58	7.33	0.269
PA	2.39	2.25	2.53	0.583	0.947
BA	12.05[a]	12.48[a]	21.70[b]	1.811	0.002
VFAs	89.42	85.66	109.81	8.320	0.113
LA	11.71	13.08	14.27	2.973	0.696
VA	0.21	0.09	0	0.128	0.277
BCFs	1.68	1.71	1.70	0.748	0.966
35 d-old					
SCFAs	59.71[a]	101.75[b]	86.30[b]	8.084	0.005
AA	49.39[a]	77.70[b]	66.08[ab]	6.005	0.011
PA	2.77	3.46	2.82	0.405	0.418
BA	6.16[a]	12.97[b]	8.51[ab]	1.766	0.036
VFAs	62.40[a]	95.44[b]	76.51[ab]	8.540	0.038
LA	5.11	6.32	4.31	1.984	0.773
VA	0.11	0.18	0.16	0.058	0.646
BCFs	0.94	0.98	1.42	0.281	0.427

[1]SCFAs – short-chain fatty acids, AA – acetic acid, PA – propionic acid, BA – butyric acid, VFAs – volatile fatty acids, LA – lactic acid, VA – valeric acid, BCFs – branch-chain fatty acids
[2]Different superscripts in the rows indicate significant differences between treatments ($p < 0.05$)

4.5. Discussion:

4.5.1. Bird growth performance and effect on AME

Mortality levels recorded during the experiment were very low (1.23 % for the first 7 days, 0.5 % mortality from 7 to 21 days, and 0.6 % mortality from 22 to 35 days, 2.34 % mortality during the whole experiment) and was not affected by the diets.

Studies have shown that feed supplemented with xylanase, alone or in combination with other enzymes, can have a positive effect on apparent metabolisable energy (AME) or feed conversion ratio (FCR) (Peng *et al.*, 2003; Masey O`Neill *et al.*, 2012a, b, Williams *et al.*, 2014; Yuan *et al.*, 2017). Stefanello (2016) found that the inclusion of 100 FXU/kg of xylanase was sufficient to improve AMEn and to increase energy utilisation and digestibility of crude protein in a corn/soy-based diet. Masey O'Neill *et al.* (2014a) found that enzyme addition improved FCR. The data of Amerah *et al.* (2017) showed that a combination of xylanase, amylase and protease along with phytase (1000 FTU/kg) can improve AMEn. Similar results were obtained by Sanchez *et al. (*2019) and Cozannet *et al. (*2017) who found that multi-enzyme supplement/ multi-carbohydrase complex have a positive effect both on BWG and FCR and improved the digestibility of all nutrients examined.

In the current study, xalanase and SIGNIS® did not change WG, FI or FCR but improved feed utilisation and increased AME and AMEn in older birds. It is possible that the magnitude of this positive change was not so prominent to affect FCR but it also should not be underestimated as a positive trend. It has been suggested that the changes in AME only would not be sufficient to predict chicken performance (Bedford, 1996; Aggrey *et al.*, 2010). Nian *et al.* (2011) also did not find the effect of xylanase supplementation on the WG and FI but FCR was improved by 4.3 %. According to Gao *et al.* (2008) the measurement of AME along with the changes in the GIT development of birds could increase the understanding of the effects of the diet and supplements on bird performance.

There was a lack of effect of xylanase on chicken performance reported by Garcia *et al.* (2008) and Abdulla *et al.* (2018). Schramm (2017) found that effects of xylanase can be substantial but may depend on the characteristics of the diet. The

study of Gonzalez-Ortiz *et al.* (2016), which used eight wheat samples found that xylanase can increase ileal utilisation of energy (IDE) measured as MJ/kg, regardless of wheat but the improvement of retention of dry matter and nitrogen as well as AME and AMEn was significant only for two of all diets studied. Jung *et al.* (2008) did not find a difference in BW, feed intake and feed conversion and Khadem *et al. (*2016b) reported that apparent digestibility and AMEn was not affected by xylanase but apparent digestibility of crude fat tended to be higher in the presence of xylanase, which was not supported by the data in the current experiment.

Yang *et al.* (2008) reported that the addition of xylanase did not have an effect on the growth of birds during the first 7 days but later on (from day 8 to day 21) the BWG of birds increased and the FCR was improved by xylanase. Other data showed that the benefit of xylanase supplementation was more pronounced during the finisher phase (days 21–35) than in the starter period (Dusel *et al.,* 1998; De Keyser *et al.* 2018). On the contrary, Amerah *et al.* (2017) found that xylanase alone or with complementary enzymes (amylase and protease) can improve WG during the starter phase (1–21d) but xylanase alone did not affect the overall WG and FI. Sorbara *et al.* (2009) found an improvement in bird performance only during the grower phase when xylanase and amylase were provided in the diet. Smeets *et al.* (2018) found positive effects of an enzyme mixture during the starter and grower period.

Dusel *et al.* (1998) suggested that the effect of xylanase was more pronounced in mature birds because the gut microflora was more mature and thus its ability to respond to dietary ingredients increased. Choct *et al.* (2006) suggested that birds can produce endogenous fibre-degrading enzymes and the interpretation of the results of exogenous enzyme supplementation could face difficulties because each bird can produce different amounts of these enzymes, even though they were fed identical diets and were reared in the same environment. Kaczmarek *et al.* (2014) suggested that it is very likely that GIT enzyme deficiency in young chickens may not be as pronounced as originally thought.

4.5.2. Effect of dietary treatment on relative weight of gastrointestinal organs

Results of the current study about the development of the GIT are presented in Table 4.5. Supplementation of xylanase, alone or in combination with XOS resulted

in lighter pancreas. It could be related either to its secretory rate of enzymes or to its response to hormonal stimuli, thus requiring further research. The other analysed intestinal sections were not affected significantly during the starter phase, which was expected, since the diets did not contain excessive amounts of viscous grains. Similar results are reported by Rehman *et al.* (2008), Wang (2016) and Roofchaei *et al.* (2019).

Gonzales-Ortiz *et al.* (2019) observed that xylanase did not influence the relative weights of any intestinal sections, except the crop, which was smaller in xylanase supplemented birds. The authors assumed that xylanase could improve performance without a pronounced effect on the broiler intestine. The relative weight and length of the bird's GI compartments were unaffected by xylanase supplementation in the experiment of Figueiredo *et al.* (2012). Mirzaie *et al.* (2012) also observed that the relative weight of organs and length of the duodenum, jejunum and ileum were not affected by NSP content of wheat and exogenous enzyme supplementation. Gonzalez-Ortiz *et al.* (2017) found that xylanase increases only the relative gizzard weight but did not affect the relative weights of heart, liver, and total GIT.

4.5.3.   Effect of dietary treatment on caecal content of SCFAs

The increased concentration of SCFAs in the ceacal content was related to enhanced fermentation (Bedford, 2000b). It has been suggested that there is a link between the availability of dietary ingredients and the increase in the number of beneficial bacteria such as *Feacalibacterium prausnitzii* - butyric producers (Meimandipour *et al.*, 2010). The SCFAs production depends on the nature of polysaccharides which are provided in the diet (McCafferty *et al.*, 2019; Sanchez *et al.*, 2019). The detailed data of Macfarlane and Macfarlane (2003) showed that the inclusion of pectin and xylan resulted in acetate production; inclusion of arabinogalactan resulted in large amounts of acetate and propionate and only the inclusion of starch released substantial amount of butyrate. However, Engberg *et al.* (2002) observed that in mash fed birds, microbial fermentation in the caeca and the quantity of the volatile fatty acids (VFAs) were lower. Gonzalez-Ortiz *et al.* (2020) reported no effect of xylanase supplementation of the SCFAs in broilers or turkeys.

It was very likely that the diet formulation in the current experiment did not provide enough quantity of metabolisable substrates in order to increase microbial fermentation or the production of prebiotics was not sufficient and thus the difference between the dietary treatments at the end of the starter phase was not found. However, at the end of the grower phase xylanase and SIGNIS® significantly increased the total concentration of SCFAs when compared to the control diet. In comparison to the control diet, both supplements increased the concentration of acetic acid, butyric acid and volatile fatty acids, although the effect of xylanase was statistically significant and the effect of SIGNIS® was only numerical. Similar results were obtained by Kareem *et al.* (2017) who reported that the concentration of acetic acid was higher in birds fed diets supplemented with prebiotics and probiotics when compared to birds fed non-supplemented diets. The results of Singh *et al.* (2012) revealed that xylanase supplementation to a maize-based-diet could increase the caecal content of SCFAs but failed to improve digestibility and had no effect on the weight of GIT organs. Masey O'Neill *et al.* (2014a) found a link between the addition of the enzyme and increases in total caecal VFA concentration, suggesting this was due to the production of prebiotic oligosaccharides. The study of Jung *et al.* (2008) also suggested that prebiotics (galactoolygosaccharides, GOS) could directly and selectively influence the faecal microflora of broiler chickens.

4.6.   Conclusions:

Dietary supplemented xylanase and SIGNIS® did not affect WG, FI and FCR but positively affected AME and AMEn in older birds, which is an indication of better feed utilisation. There was a decrease in the relative weight (%) of the pancreas of 21-day-old birds fed xylanase and SIGNIS® supplemented diets which can be related to its secretory function or could be a result of hormonal stimuli. Such hormonal regulation could be governed by prebiotics but the mechanism of action needs to be elucidated. Xylanase alone increases the relative weight of the small intestine and the GIT of 35-day-old birds. This could be related to a well developed and actively functioning GIT with better absorbtion capacity. SIGNIS® increased the concentration of SCFAs in the caeca of both 21 day-old and 35 day-old birds, which is a benefit of supplementing XOS. Xylanase alone significantly increased acetic acid, butyric acid and volatile fatty acids in 35 day-old birds in comparison to the

control diet. This could be related to the generation of different oligosaccharides which stimulate more than one species of beneficial bacteria in the caeca, resulting in a variety of fermentation end products detectable in the caeca content. Suplementation of SIGNIS® in younger birds seems advisory in order to establish more competent caeca microflora capable of utilising dietary fibres. However, xylanase supplementation could provide diversity in the degree of polymerasation of oligosaccharides, providing suitable substrates for more species of beneficial bacteria not only in the caeca but also in other sections of the poultry GIT.

Chapter 5. Effect of xylanase and SIGNIS® on growth performance, dietary available energy, nutrient retention and caecal short-chain fatty acid concentration of broiler chickens fed maize-based diet with wheat bran (2.5 %)

5.1.   Introduction

Low-cost ingredients containing high fibre content are commonly utilised by the feed industry as by-products from the agricultural and food processing industries (Audren *et al.*, 2002; Slominski *et al.*, 2004). Wheat bran (WB) is a by-product of wheat flour processing through a procedure of sequential and controlled removal of grain layers prior to milling (Hemery *et al.,* 2007). A variety of compounds have been identified in wheat bran and summarised by Apprich *et al.* (2014) into several major groups: (1) soluble and insoluble dietary fibre (mainly arabinoxylan and *β*-glucan); (2) sugars and their derivatives (starch, glucose and succinic acid); (3) secondary plant metabolites (e.g. ferulic acid); (4) proteins and (5) minerals and salts.

Incorporation of WB in poultry feed could reduce production cost and is not associated with increase mortality of broilers or laying pullets (Donkoh *et al.*, 1999; Martinez *et al.*, 2015). Further studies on the inclusion of WB and derived arabinoxylooligosaccharides revealed an improvement in the feed conversion ratio and nutrient utilisation efficiency (Courtin *et al.*, 2008a; Taheri *et al.*, 2016). According to Shang *et al.* (2020), WB may have a role in replacing antibiotics through improved intestinal immunity, barrier function, and microbial composition in broilers.

Currently, the research on WB as a source of dietary fibres such as arabinoxylan (AX), arabinoxylooligosaccharides (AXOS) and xylooligosaccharides (XOS) focuses on their prebiotic effects on the gut microflora. According to Yacoubi *et al.* (2018) despite AX, AXOS and XOS not being recognised yet as prebiotics, they are regarded as promising prebiotic-like compounds. The prebiotic effect of WB derived AXOS was related to enhanced natural immunity in broilers and protection against pathogens such as *Salmonella enteritidis* and *Eimeria* species (Eekhaut *et al.*, 2008; Akhtar *et al.*, 2012). Vermeulen *et al.* (2017) supposed a link between WB and

decreased *Salmonella* invasion due to production of short-chain fatty acids (SCFAs), in particular butyric and propionic acid. However, considering wheat bran is a promising prebiotic-like component, it warrants further research.

5.2.   Objective of the study:

To evaluate the effect of dietary supplementation of xylanase alone or in combination with XOS (SIGNIS®) and wheat bran on gut health and bird performance.

5.3. Materials and methods

General materials and methods relating to this section can be found in Chapter 3.

5.3.1. Animals

In this study, seven-hundred-and-twenty-day-old male Ross-308 broiler chickens were used. On arrival birds were weighed and allocated into thirty-six floor pens with twenty birds in each.

5.3.2. Diets

The main ingredients of the basal diet (Table 5.1) were maize, soybean meal and soya oil. The basal diet (xylanase activity <2000 BXU/kg) was designated as diet without wheat bran (PC) and with wheat bran (NC) when maize was partially substituted with 2.5 % wheat bran. Each basal diet (PC and NC) was split into three batches - one was control and the others were supplemented either with xylanase or with a combination of xylanase and xylooligosaccharides (SIGNIS®) which resulted in six diets per phase (three dietary phases) or 18 diets in total (2 x 3 factorial design). Xylanase was provided as Econase®-XT (ABVista, Marlborough, UK) at 100 g/t (xylanase activity 17100 BXU/kg, producing strain *Trichoderma reesei*). The combination of xylanase and xylooligosaccharides was provided as SIGNIS® (ABVista, Marlborough, UK) at 100 g/t (xylanase activity 18 600 BXU/kg, 50 g/t xylooligosacharides with degree of polymerization between 2 to 7). Birds were fed diets in three phases - starter, grower and finisher. The PC and NC diet were similar in apparent metabolisable energy (AME) - 12.55/12.38, 12.76/12.58 and 12.87/12.72 MJ/kg for the starter, grower and finisher phases, respectively. Starter diets were fed in crumb form, whereas grower and finisher diets were fed in pelleted form. Coccidiostat, antimicrobial growth promoters, prophylactics, or other additives were not used.

Table 5.1: Ingredients in the experimental diets for the three rearing periods (starter 0–10 day, grower – 11–24 day and finisher – 25–35 day)

Ingredients g/kg	Starter phase 0–10 day		Grower phase 11–24 day		Finisher phase 25–35 day	
	(positive control – PC)	(negative control – NC)	(positive control – PC)	(negative control – NC)	(positive control – PC)	(negative control – NC)
Wheat Bran		25.0		25.0		25.0
Maize	564.4	539.4	659.6	634.6	696.4	671.4
Soybean meal	359.5	359.5	268.6	268.6	237.2	237.2
Soya oil	26.6	26.6	21.3	21.3	19.9	19.9
Salt	2.8	2.8	2.9	2.9	2.9	2.9
Limestone	11.3	11.3	10.7	10.7	9.4	9.4
Monocalcium phosphate	7.4	7.4	6.7	6.7	0.53	0.53
L-Tryptophan					0.01	0.01
Lysine HCl	0.9	0.9	2.4	2.4	1.9	1.9
DL-Methionine	3.1	3.1	2.9	2.9	2.6	2.6
Threonine			0.5	0.5	0.4	0.4
Valine			0.4	0.4		
Vitamin & Mineral premix[1]	4.00	4.00	4.00	4.00	4.00	4.00
Quantum Blue 10G	0.05	0.05	0.05	0.05	0.05	0.05
AIA[2]	2.00	2.00	2.00	2.00	2.00	2.00
TOTAL, %	100	100	100	100	100	100
Calculated values						
AME MJ/kg	12.55	12.38	12.76	12.58	12.87	12.72
DM, g/kg	861.6	861.6	859.5	859.5	858.3	858.3
CP[3], g/kg	225.0	226.8	190.7	192.4	177.5	179.3
CF[4], g/kg	22.6	24.9	21.7	23.9	21.4	23.7
Calcium %	0.90	0.90	0.84	0.84	0.76	0.76
Phosphorus %	0.74	0.76	0.68	0.71	0.63	0.66
Analysed values						
GE[6], MJ/kg	16.54	16.72	16.35	16.57	16.51	16.61
DM, g/kg	935.4	936.2	940.4	940.4	938.5	936.7
CP[3], g/kg	228.0	236.0	208.2	197.1	172.9	184.5
Crude fat, g/kg	44.64	41.66	40.46	41.87	39.86	40.22
NSP[5] g/100g						
Soluble NSP	1.4	1.6	0.8	1.7	1.1	1.2
Insoluble NSP	7.1	7.6	7.2	7.0	6.6	7.1
Total	8.5	9.3	8.0	8.7	7.7	8.3

[1] The vitamin/mineral premix provided the following content per kg of diet: vitamin A, 10,000 IU; vitamin D3, 2,500 IU; vitamin E, 25 IU; vitamin E, 50 mg; vitamin K3, 1.5 mg; vitamin B1, 2 mg; vitamin B2, 7.5 mg; vitamin B6, 3.5 mg; vitamin B12, 20 µg; niacin, 35mg; pantothenic acid, 12 mg; choline chloride, 460 mg; folic acid, 1.0 mg; biotin, 0.2 mg; Fe as iron sulphate, 265 mg; Cu as copper sulphate, 48 mg; Mn as manganese oxide, 140 mg; Zn as zinc sulphate, 165 mg; I as potassium iodide, 1.2 mg; and Se as sodium selenite, 0.33 mg.; AIA[2] – acid insoluble ash; CP[3] – crude protein, CF[4] – crude fibre, NSP[5] – non-starch polysaccharides, GE[6] – gross energy

5.3.3.   Statistical analysis

Data handling and calculations were performed in Excel 2013 (Microsoft Corporation) and staistical analysis were performed using GenStat 18th edition (VSN International Ltd, Oxford, UK) statistical software. The experiment was analysed as a randomised block 2 x 3 factorial design (two levels of wheat bran and three levels of supplements) by two-way ANOVA, consisting of six replicates (pens of twenty animals) per dietary treatment. Summary statistics were used to check data for outliers (<> 3 SD) and normality. Means were separated only when the treatment p-value was significant, and Duncan's multiple range test was used to determine differences between treatment groups. Significance was set at $p \leq 0.05$ and tendencies were discussed at $0.05 > p < 0.1$.

5.4. Results

5.4.1. Bird growth performance

The analysis confirmed the values of expected activity of phytase and xylanase in the feed (Table 5.2). There was only one slightly higher value for xylanase activity in the finisher diet with WB and SIGNIS®.

Table 5.2: Expected and analyzed phytase and xylanase activity in maize-based diet

Diet	Expected Phytase FTU/kg	Expected Xylanase BXU/kg	Analysed Phytase FTU/kg	Analysed Xylanase BXU/kg
Starter diet				
WB				
No	500	0	379	< 2000
Yes	500	0	428	< 2000
Additive				
No + XT	500	16000	408	15900
Yes + XT	500	16000	689	15900
No + SIGN	500	16000	470	16700
Yes +SIGN	500	16000	413	16400
Grower diet				
WB				
no	500	0	248	< 2000
yes	500	0	330	< 2000
Product				
No + XT	500	16000	525	15800
Yes + XT	500	16000	418	15200
No + SIGN	500	16000	367	16300
Yes + SIGN	500	16000	433	16600
Finisher diet				
WB				
No	500	0	349	< 2000
Yes	500	0	286	< 2000
Product				
No + XT	500	16000	358	16200
Yes + XT	500	16000	286	16300
No + SIGN	500	16000	284	16700
Yes + SIGN	500	16000	635	19700

The mean value of bird body weight (BW), weighed on a pen basis at their arrival, was 41.81g (SEM 0.376, SD±0.861). There was no difference (p=0.990) in the BW of birds allocated to the different diets. The growth rate of the broiler chickens during the rearing period was good and at the end of the starter phase BW reached 84 % of Aviagen performance objectives (Aviagen, 2018) and at the end of the grower and finisher phase – 96 % and 99 %, respectively. There was no significant effect (p> 0.05) on BW due to WB or additives supplementation. The weight gain and feed intake for all the intermittent and final periods did not significantly differ (p>0.05) across any of the used additives. Birds fed supplemented diets had numerically greater BW compared to the control fed birds (P>0.05). However, this trend was not accompanied with significant differences in weight gain and feed intake neither in birds fed the WB diet nor in birds fed the diet with additives.

Wheat bran had a significant negative effect on FCR in the starter period (p=0.022). The effect of additives was significant only in the finisher period – from 25 to 35 days (p=0.038), since xynanase improved the FCR in comparison to SIGNIS® but this effect was similar to the control diet. As an overall effect (0-35 days) on FCR, the additives showed only a trend (p=0.126) and analysis showed an interaction (p=0.037) for WB and additives.

Table 5.3: Effect of maize-based diets with or without wheat bran (WB), xylanase (XT) and SIGNIS (SIGN) on broiler`s performance parameters – body weight (BW), weight gain (WG), feed intake (FI) and feed conversion ratio (FCR) corrected for mortality

Treatment	BW (g)			WG (g/b/d)					FI (g/b/d)					FCR (g/g)				
Day	10	24	35	0-10	11-24	0-24	25-35	0-35	0-10	11-24	0-24	25-35	0-35	0-10	11-24	0-24	25-35	0-35
WB																		
No	273	1216	2317	18.72	57.81	39.50	100.15	55.99	24.75	94.85	62.02	165.28	90.12	1.182	1.408	1.360	1.661	1.491
Yes	269	1232	2358	18.30	58.13	39.79	101.70	56.18	24.79	96.36	62.74	169.63	91.50	1.203	1.418	1.373	1.667	1.501
SEM	2.2	10.771	20.8	0.193	0.750	0.371	1.289	0.601	0.190	0.741	0.463	1.631	0.711	0.0060	0.0062	0.0050	0.0072	0.0036
Additive																		
Control	268	1207	2321	18.33	57.50	39.13	101.23	56.00	24.56	95.65	62.31	166.73	90.70	1.196	1.423	1.375	1.657[ab]	1.499
XT	272	1231	2357	18.51	57.57	39.24	101.38	55.91	24.90	95.26	62.24	167.96	90.60	1.198	1.408	1.363	1.652[a]	1.489
SIGN	273	1233	2335	18.69	58.84	40.57	100.16	56.35	24.86	95.90	62.59	167.67	91.13	1.185	1.408	1.360	1.684[b]	1.501
SEM	2.6	13.192	25.5	0.236	0.919	0.455	1.579	0.736	0.233	0.908	0.567	1.998	0.871	0.0074	0.0076	0.0061	0.0088	0.0044
WBxAdditive																		
No	274	1203	2307	18.68	56.91	39.00	100.35	55.66	24.79	94.64	61.91	166.46	90.33	1.184	1.418	1.367	1.670	1.500[ab]
Yes	263	1211	2335	17.97	58.08	39.26	102.11	56.34	24.34	96.66	62.71	167.00	91.08	1.207	1.429	1.382	1.645	1.498[ab]
No+XT	271	1199	2297	18.68	56.37	38.77	99.82	55.33	24.79	93.40	61.36	163.02	88.95	1.192	1.414	1.366	1.644	1.487[a]
Yes+XT	273	1262	2417	18.35	58.77	39.71	102.93	56.49	25.00	97.13	63.12	172.90	92.25	1.203	1.403	1.361	1.660	1.490[a]
No+SIGN	274	1245	2348	18.80	60.15	40.74	100.27	56.99	24.67	96.51	62.78	166.35	91.07	1.171	1.392	1.345	1.671	1.486[a]
Yes+SIGN	271	1221	2322	18.59	57.53	40.40	100.06	55.71	25.04	95.28	62.40	168.99	91.19	1.203	1.423	1.375	1.697	1.515[b]
SEM	3.7	18.656	31.6	0.236	1.299	0.643	2.233	1.041	0.330	1.284	0.802	2.826	1.231	0.0105	0.0108	0.0086	0.0125	0.0062
p-values																		
WB	0.273	0.305	0.183	0.137	0.766	0.588	0.402	0.825	0.879	0.162	0.280	0.071	0.180	**0.022**	0.264	0.077	0.559	**0.046**
Additive	0.473	0.317	0.607	0.558	0.519	0.063	0.840	0.907	0.554	0.883	0.901	0.902	0.902	0.466	0.283	0.226	**0.038**	0.126
WBxAdditive	0.288	0.080	0.146	0.739	0.153	0.619	0.759	0.475	0.431	0.167	0.423	0.242	0.403	0.661	0.169	0.146	0.112	**0.037**

[1]Different superscripts in the columns indicate significant differences between treatments ($p < 0.05$)

5.4.2. Effect of dietary treatment on AME, AMEn and nutrient retention

Wheat bran (WB) and additives did not have an effect on AME and AMEn for the starter period but WB significant decreased AME and AMEn in the grower (p=0.002) and finisher periods (p < 0.001) (Table 5.4). In the finisher period, both xylanase and SIGNIS® increased AME and AMEn in comparison to the control diet and for SIGNIS® this increase was statistically significant (p=0.023). No interaction between WB and additives was observed (p>0.05).

Table 5.4: Effect of maize-based diets with or without wheat bran, (WB), xylanase (XT) and SIGNIS® (SIGN) on AME and AMEn in broiler chickens

Treatment	AME			AMEn		
	starter	grower	finisher	starter	grower	finisher
WB						
No	12.95	13.75	13.93	12.01	12.88	13.19
Yes	12.98	13.59	13.80	12.05	12.73	13.07
SEM	0.048	0.033	0.021	0.045	0.043	0.019
Additive						
Control	12.90	13.67	13.82^a	11.97	12.80	13.09^a
XT	12.97	13.64	13.86^{ab}	12.04	12.78	13.13^{ab}
SIGN	13.03	13.70	13.92^b	12.10	12.84	13.18^b
SEM	0.083	0.040	0.026	0.055	0.052	0.023
WB x Additive						
No	12.93	13.71	13.90	11.99	12.84	13.16
Yes	12.86	13.62	13.74	11.95	12.76	13.01
No + XT	12.98	13.72	13.91	12.05	12.85	13.18
Yes + XT	12.95	13.56	13.81	12.03	12.70	13.08
No + SIGN	12.94	13.82	13.98	12.01	12.95	13.25
Yes + SIGN	13.13	13.58	13.86	12.19	12.72	13.13
SEM	0.117	0.057	0.036	0.078	0.074	0.033
p-values						
WB	0.684	0.002	<0.001	0.525	<0.001	<0.001
Additive	0.265	0.536	0.035	0.252	0.512	0.023
WB x Additive	0.242	0.390	0.703	0.302	0.372	0.627

[1]Different superscripts in the columns indicate significant differences between treatments ($p < 0.05$)

Wheat bran had a negative effect on DMR in starter (p=0.012) and grower (p=0.03) periods (Table 5.5). The WB also had a significant negative effect on FR for the grower (p=0.027) and finisher period (p=0.004), respectively.

Table 5.5: Effect of maize-based diets with or without wheat bran (WB), xylanase (XT) and SIGNIS® (SIGN) on nutrient retention (dry matter - DMR, nitrogen - NR and fat - FR retention) in broiler chickens

Treatment	DMR			NR			FR		
Day	10	24	35	10	24	35	10	24	35
WB									
No	0.761	0.803	0.809	0.735	0.778	0.746	0.865	0.921	0.958
Yes	0.752	0.794	0.806	0.725	0.772	0.742	0.870	0.900	0.941
SEM	0.0025	0.0018	0.0022	0.0037	0.0031	0.0036	0.0094	0.0086	0.0038
Additive									
Control	0.753	0.798	0.804	0.729	0.773	0.744	0.864	0.905	0.949
XT	0.756	0.797	0.807	0.728	0.775	0.741	0.863	0.908	0.949
SIGN	0.760	0.801	0.811	0.734	0.776	0.746	0.876	0.919	0.950
SEM	0.0030	0.0022	0.0027	0.0045	0.0038	0.0044	0.0115	0.0106	0.0066
WBxAdditive									
No	0.760	0.800	0.808	0.743[b]	0.774	0.746	0.874	0.915	0.960
Yes	0.746	0.796	0.801	0.714[a]	0.773	0.743	0.854	0.895	0.937
No + XT	0.762	0.801	0.807	0.732[ab]	0.779	0.745	0.865	0.918	0.959
Yes + XT	0.750	0.793	0.808	0.723[ab]	0.770	0.738	0.861	0.898	0.938
No + SIGN	0.761	0.808	0.814	0.730[ab]	0.781	0.749	0.855	0.930	0.954
Yes + SIGN	0.759	0.794	0.808	0.737[b]	0.772	0.744	0.896	0.908	0.947
SEM	0.0043	0.0031	0.0039	0.0064	0.0053	0.0062	0.0163	0.0149	0.0093
p-values									
WB	0.012	0.003	0.104	0.061	0.157	0.359	0.671	0.027	0.004
Additive	0.285	0.474	0.096	0.631	0.850	0.746	0.685	0.399	0.946
WBxAdditive	0.329	0.345	0.346	0.034	0.771	0.960	0.174	0.995	0.437

[1]Different superscripts in the columns indicate significant differences between treatments ($p < 0.05$)

5.4.3. Effect of dietary treatment on GIT

Wheat bran and additives affected some organs of the GIT of broiler chickens (Table 5.6). Proventriculus and gizzard (PG), despite being statistically non-significant (p=0.108) were heavier in young chicks (10-day-old) fed WB than those fed a diet without WB (5.222 vs. 4.861). In 35-day-old birds WB tended to increasethe relative weight of the caeca - 0.305 vs. 0.358 (p=0.075) and ileum - 0.962 vs.1.063 (p=0.063). Wheat bran significantly (p=0.038) increased the relative weight of the pancreas - 0.201 vs .0.179. In the finisher phase, the small intestine of birds (35-day-old) fed WB was also significantly heavier (p=0.038) than the relative weight of the small intestine in birds fed diets without WB (2.824 vs.2.610) and the same trend (p=0.104) was true for the jejunum (1.225 vs. 1.144). There was only one interaction (p=0.044) between WB and the additives for jejunum in 35-day-old birds.

Table 5.6: Effect of maize-based diets with or without wheat bran (WB), xylanase (XT) and SIGNIS® (SIGN) on relative weight (%) of gastrointestinal tract of broiler chickens

Treatment	Caeca %			Duodenum %			Ileum %		
Day	10	24	35	10	24	35	10	24	35
WB									
No	0.917	0.505	0.305	0.463	0.918	0.504	2.123	1.340	0.962
Yes	0.891	0.531	0.358	0.473	0.982	0.536	1.854	1.373	1.063
SEM	0.0426	0.0282	0.0198	0.0164	0.0343	0.0182	0.1182	0.0495	0.0367
Additive									
Control	0.881	0.563	0.331	0.492	0.965	0.524	2.139	1.443	1.004
XT	0.902	0.468	0.318	0.459	0.932	0.500	1.986	1.286	1.016
SIGN	0.927	0.522	0.345	0.453	0.953	0.537	1.840	1.340	1.016
SEM	0.0522	0.0346	0.0243	0.0200	0.0420	0.0223	0.0965	0.0606	0.0449
WBxAdditive									
No + No	0.923	0.514	0.310	0.465	0.965	0.509	2.394	1.422	0.885
Yes + No	0.839	0.613	0.353	0.520	0.965	0.539	1.883	1.464	1.123
No + XT	0.944	0.474	0.292	0.438	0.880	0.488	2.074	1.284	1.020
Yes + XT	0.911	0.463	0.345	0.480	0.985	0.512	1.898	1.288	1.012
No + SIGN	0.882	0.526	0.315	0.487	0.908	0.515	1.901	1.313	0.980
Yes + SIGN	0.922	0.518	0.375	0.418	0.998	0.559	1.780	1.367	1.053
SEM	0.0738	0.0489	0.0344	0.0283	0.0593	0.0315	0.1671	0.0857	0.0899
p-values									
WB	0.670	0.523	0.075	0.687	0.195	0.218	0.060	0.641	0.063
Additive	0.820	0.169	0.749	0.339	0.860	0.511	0.223	0.196	0.976
WBxAdditive	0.705	0.452	0.970	0.073	0.639	0.947	0.461	0.955	0.163

[1]Different superscripts in the columns indicate significant differences between treatments ($p < 0.05$)

Table 5.6: (continued) Effect of maized-based diets with or without wheat bran (WB), xylanase (XT) and SIGNIS® (SIGN) on relative weight (%) of gastrointestinal tract of broiler chickens

Treatment		Jejunum %			Liver %			Pancreas %		
	Day	10	24	35	10	24	35	10	24	35
WB										
	No	2.285	1.623	1.144	1.679	2.568	2.221	3.113	0.315	0.179
	Yes	2.243	1.698	1.225	1.764	2.594	2.255	3.262	0.309	0.201
SEM		0.0914	0.0527	0.0340	0.0781	0.0577	0.0701	0.0651	0.0125	0.0072
Additive										
	Control	2.229	1.690	1.177	1.769	2.670	2.093	3.152	0.337	0.177
	XT	2.473	1.608	1149	1.783	2.528	2.263	3.313	0.299	0.192
	SIGN	2.360	1.683	1.228	1.613	2.546	2.358	3.097	0.300	0.202
SEM		0.1119	0.0646	0.0416	0.0956	0.0707	0.0858	0.0798	0.0153	0.0088
WBxAdditive										
No + No		1.959	1.660	1.050[a]	1.743	2.649	2.154	3.173[a]	0.344	0.165
Yes + No		2.498	1.721	1.303[b]	1.795	2.690	2.033	3.131[a]	0.330	0.188
No + XT		2.498	1.581	1.176[ab]	1.611	2.469	2.336	3.056[a]	0.298	0.187
Yes + XT		2.448	1.636	1.122[ab]	1.955	2.586	2.190	3.570[b]	0.300	0.197
No + SIGN		2.397	1.627	1.206[ab]	1.683	2.586	2.174	3.110[a]	0.301	0.185
Yes + SIGN		2.323	1.739	1.250[b]	1.543	2.506	2.543	3.085[a]	0.298	0.218
SEM		0.1582	0.0913	0.0589	0.1352	0.0999	0.1214	0.1128	0.0216	0.0125
	p-values									
WB		0.294	0.319	0.104	0.477	0.752	0.736	0.119	0.768	0.038
Additive		0.319	0.617	0.409	0.392	0.320	0.108	0.161	0.159	0.151
WB x Additive		0.111	0.942	0.044	0.217	0.613	0.076	0.033	0.927	0.679

[1]Different superscripts in the columns indicate significant differences between treatments ($p < 0.05$)

Table 5.6: (continued) Effect of maized-based diets with or without fibre (wheat bran, WB), xylanase (XT) and SIGNIS® (SIGN) on relative weight (%) of gastrointestinal tract of broiler chickens

Treatment		PG[1] %			Small intestine[2] %			Spleen %		
day	10	24	35	10	24	35	10	24	35
WB										
No	4.861	2.592	1.557	4.871	3.963	2.610	0.081	0.073	0.119
Yes	5.222	2.551	1.611	4.750	4.054	2.824	0.082	0.077	0.111
SEM	0.1529	0.0821	0.0565	0.1372	0.0991	0.0695	0.0064	0.0050	0.0047
Additive										
Control	5.135	2.568	1.526	4.860	4.222	2.704	0.074	0.073	0.109
XT	4.965	2.526	1.651	4.918	3.826	2.665	0.082	0.079	0.116
SIGN	5.025	2.621	1.574	4.654	3.976	2.781	0.088	0.073	0.120
SEM	0.1872	0.1006	0.0692	0.1681	0.1214	0.0851	0.0079	0.0061	0.0057
WB x Additive										
No + No	5.072	2.568	1.517	4.818	4.295	2.443	0.075	0.075	0.107
Yes + No	5.198	2.568	1.536	4.901	4.149	2.965	0.074	0.070	0.110
No + XT	4.620	2.491	1.659	5.010	3.745	2.684	0.080	0.074	0.124
Yes + XT	5.310	2.560	1.644	4.827	3.908	2.646	0.084	0.084	0.108
No + SIGN	4.893	2.716	1.495	4.785	3.849	2.701	0.088	0.070	0.125
Yes + SIGN	5.157	2.256	1.654	4.522	4.104	2.861	0.089	0.076	0.115
SEM	0.265	0.1422	0.0978	0.2377	0.1717	0.1203	0.0111	0.0086	0.081
p-values										
WB	0.108	0.732	0.506	0.538	0.523	0.038	0.878	0.636	0.242
Additive	0.811	0.801	0.448	0.513	0.087	0.624	0.467	0.713	0.386
WB x Additive	0.548	0.647	0.643	0.750	0.483	0.081	0.975	0.654	0.478

[1]PG – provetriculus and gizzard; [2]Small intestine = duodenum +jejunum +ileum; [3]Different superscripts in the columns indicate significant differences between treatments ($p < 0.05$)

5.4.4. Histomorphology

In the current study, statistical analysis revealed an interaction (p<0.001) between WB and additives for all ileal histomorphology parameters (Table 5.7). Excluding villus width, feed supplemented with WB increased the mean value for all other variables - CD, VH and VH:CD. The control diet had the highest mean values for crypt width (225.23), villus height (1253.08) and VH:CD ratio (22.82). In comparison to SIGNIS®, xylanase had a more pronounce effect on the crypt depth (66.99 vs. 65.47) and villus width (100.42 vs. 96.55). On the contrary, SIGNIS® resulted in higher values for crypt width and villus height.

Table 5.7: Effect of maize-based diets with or without wheat bran (WB), xylanase (XT) and SIGNIS® (SIGN) on ileal histomorphometry of broilers

Treatment	CD[1]	CW[2]	VW[3]	VH[4]	VH:CD[5]
WB					
No	58.19	173.97	98.87	927.34	16.13
Yes	67.03	250.84	80.89	1511.57	23.07
SEM	0.307	2.947	0.223	5.918	0.174
Additive					
Control	55.37	225.23	72.81	1253.08	22.82
XT	66.99	199.41	100.42	1180.68	17.37
SIGN	65.47	212.57	96.55	1224.61	18.60
SEM	0.375	3.609	0.274	7.240	0.213
WBxAdditive					
No + No	55.01[a]	193.43[b]	90.07[d]	883.91[a]	16.52[b]
Yes + No	55.74[a]	257.04[d]	55.54[a]	1622.25[e]	29.12[e]
No+XT	61.56[c]	169.47[a]	126.06[f]	878.22[a]	14.27[a]
Yes+XT	72.43[d]	229.34[c]	74.78[b]	1483.14[d]	20.48[d]
No+SIGN	58.02[b]	159.01[a]	80.77[c]	1019.89[b]	17.60[c]
Yes+SIGN	72.91[d]	266.13[d]	112.34[e]	1429.32[c]	19.60[d]
SEM	0.531	5.104	0.387	10.239	0.301
p-values					
WB	<0.001	<0.001	<0.001	<0.001	<0.001
Additive	<0.001	<0.001	<0.001	<0.001	<0.001
WBxAdditive	<0.001	<0.001	<0.001	<0.001	<0.001

[1]CD - crypt depth; [2]CW – crypt width; [3]VW – villus width; [4]VH – villus high; [5]VH:CD - villus high to crypt depth ratio; [6]Different superscripts in the columns indicate significant differences between treatments ($p < 0.05$)

5.4.5. Effect of dietary treatment on caeca content of SCFAs

In 35-day-old birds, WB cause a significant decrease in the total content of SCFAs (p=0.048) – 95.82 vs. 80.39 mM and propionic acid (PA) (p=0.024) – 8.32 vs. 5.09

mM (Table 5.8). Additionally, in older birds (35- day-old), WB diet reduced the values for volatile fatty acids (VFAs) (p=0.064) and acetic acid (AA) (p=0.097) - 80.09 vs. 94.33 mM and 64.76 vs.73.77 mM, respectively. In 10-day-old birds, WB tended (p=0.102) to decrease the content of butyric acid– 12.74 vs. 9.11 mM. However, in young birds (24-day-old), WB significantly increased (p=0.033) the content of lactic acid (p=0.048). Additionally, in 24-day-old birds the non-supplemented diet showed the higher mean value for butyric acid – 12.19 mM in comparison to the the additives (p=0.029). The additives had the same effect on VFAs (p=0.045) in 24-day-old birds since control diets had a higher value – 94.16 mM in comparison to xylanase (76.65 mM) or SIGNIS® (73.08 mM) supplemented diets.

There was an interaction between WB and additives for lactic acid (p=0.034) in 10-day-old birds as diets without fibre but with SIGNIS® had the greatest content of lactic acid in the ceaca– 12.57 mM. Analysis also showed that diets supplemented with SIGNIS® tended (p=0.084) to increase the total bacteria count at 35-day-old since the estimated value was the highest - 12.31 mM and there was also an interaction (p=0.002) between WB and additives.

Table 5.8: Effect of maize-based diets with or without wheat bran (WB), xylanase (XT) and SIGNIS® (SIGN) on ceacal content of SCFAs (mM) in broiler chickens

Treatment	SCFAs			AA			PA		
day	10	24	35	10	24	35	10	24	35
WB									
No	93.26	86.96	95.82	71.28	71.04	73.77	0.55	2.40	8.32
Yes	94.48	86.56	80.39	71.69	67.77	64.76	0.37	1.70	5.09
SEM	4.987	5.267	5.152	4.408	3.757	3.695	0.185	0.390	0.947
Additive									
Control	93.69	99.50	96.00	67.71	78.65	75.94	0.53	2.80	6.85
XT	91.43	82.67	85.93	71.19	64.71	68.01	0.21	1.58	6.48
SIGN	97.48	78.11	81.93	75.55	64.85	63.85	0.65	1.78	6.79
SEM	6.108	9.123	6.310	5.399	4.601	4.525	0.226	0.477	1.160
WBxAdditive									
No + No	84.67	105.59	93.81	65.85	85.54	73.07	0.74	3.11	7.90
Yes + No	100.71	93.41	98.19	69.56	71.77	78.82	0.33	2.49	5.81
No + XT	102.32	77.25	93.77	81.22	61.14	72.56	0.28	1.89	8.84
Yes + XT	80.53	88.08	78.10	61.16	68.28	63.45	0.14	1.27	4.11
No + SIGN	92.78	78.03	98.98	66.74	66.44	75.68	0.63	2.19	8.22
Yes + SIGN	102.19	78.19	64.89	84.35	63.26	52.03	0.66	1.36	5.36
SEM	8.638	9.123	8.924	7.635	6.507	6.400	0.320	0.675	1.641
p-values									
WB	0.864	0.958	0.048	0.947	0.544	0.097	0.499	0.220	0.024
Additive	0.763	0.065	0.286	0.595	0.066	0.179	0.379	0.173	0.970
WBxAdditive	0.084	0.462	0.119	0.062	0.293	0.091	0.786	0.984	0.713

[1]SCFAs – short-chain fatty acids, AA – acetic acid, PA – propionic acid, BA – butyric acid, VFAs – volatile fatty acids, LA – lactic acid, VA – valeric acid, BCFs – branch-chain fatty acids; [2]Different superscripts in the columns indicate significant differences between treatments ($p < 0.05$)

Table 5.8: (continued) Effect of maize-based diets with or without wheat bran (WB), xylanase (XT) and SIGNIS® (SIGN) on ceacal content of SCFAs (mM) in broiler chickens

Treatment		BA			VA			LA		
day	10	24	35	10	24	35	10	24	35
WB									
No	12.74	8.54	11.42	0.07	0.21	0.42	8.46	4.55[a]	0.41
Yes	9.11	9.76	9.42	0.04	0.13	0.36	7.35	6.84[b]	0.30
SEM	1.512	1.212	1.131	0.031	0.062	0.071	1.051	0.717	0.157
Additive									
Control	9.73	12.19[b]	11.93	0.06	0.23	0.51	6.30	5.34	0.40
XT	11.57	9.13[ab]	10.07	0.06	0.20	0.31	7.75	6.02	0.40
SIGN	11.49	6.18[a]	9.26	0.05	0.08	0.34	9.67	5.73	0.27
SEM	1.852	1.484	1.386	0.037	0.075	0.087	1.288	0.878	0.192
WB x Additive									
No + No	10.78	11.74	11.58	0.11	0.26	0.51	6.90[a]	4.65	0.45
Yes + No	8.68	12.63	12.28	0.00	0.20	0.51	5.70[a]	6.03	0.34
No + XT	15.00	9.28	11.26	0.07	0.28	0.26	5.73[a]	4.51	0.34
Yes + XT	8.14	8.98	8.88	0.06	0.12	0.36	9.77[ab]	7.52	0.46
No + SIGN	12.45	4.64	11.40	0.04	0.07	0.49	12.75[b]	4.48	0.44
Yes + SIGN	10.52	7.75	7.11	0.06	0.08	0.20	6.59[a]	6.98	0.10
SEM	2.619	2.099	1.960	0.053	0.107	0.123	1.821	1.241	0.272
p-values									
WB	0.102	0.474	0.225	0.425	0.424	0.528	0.465	0.033	0.630
Additive	0.733	0.029	0.389	0.959	0.329	0.232	0.200	0.862	0.863
WBxAdditive	0.573	0.710	0.450	0.411	0.725	0.280	0.034	0.800	0.706

[1]SCFAs – short-chain fatty acids, AA – acetic acid, PA – propionic acid, BA – butyric acid, VFAs – volatile fatty acids, LA – lactic acid, VA – valeric acid, BCFs – branch-chain fatty acids; [2]Different superscripts in the columns indicate significant differences between treatments ($p < 0.05$)

Table 5.8: (continued) Effect of maize-based diets with or without wheat bran (WB), xylanase (XT) and SIGNIS® (SIGN) on ceacal content of SCFAs (mM) in broiler chickens

Treatment		BCFs			VFAs			Total bacteria count		
day		*10*	*24*	*35*	*10*	*24*	*35*	*10*	*24*	*35*
WB										
No		0.10	0.22	0.34	84.80	82.41	94.33	11.92	12.10	12.27
Yes		0.07	0.24	0.46	81.70	80.18	80.09	11.91	12.19	12.23
SEM		0.048	0.089	0.110	5.436	4.919	5.199	0.036	0.037	0.033
Additive										
Control		0.13	0.29	0.36	78.25	94.16[b]	95.60	11.88	12.10	12.18
XT		0.02	0.21	0.26	83.68	76.65[a]	85.54	11.92	12.16	12.25
SIGN		0.09	0.19	0.58	87.81	73.08[a]	80.50	11.95	12.17	12.31
SEM		0.058	0.109	0.135	6.658	6.025	6.367	0.044	0.045	0.041
WB x Additive										
No		0.09	0.28	0.29	77.77	100.93	93.36	11.86	12.02	12.32[ac]
Yes		0.17	0.29	0.43	78.74	87.38	97.85	11.91	12.18	12.04[b]
No + XT		0.02	0.15	0.31	95.59	72.74	93.43	11.92	12.15	12.26[ac]
Yes + XT		0.03	0.28	0.85	70.77	80.56	77.65	11.91	12.18	12.24[ac]
No + SIGN		0.17	0.24	0.43	80.03	73.55	96.21	11.99	12.13	12.22[a]
Yes + SIGN		0.00	0.15	0.10	95.06	72.60	64.79	11.91	12.22	12.41[c]
SEM		0.083	0.155	0.191	9.416	8.520	9.005	0.063	0.064	0.057
p-values										
WB		0.670	0.880	0.457	0.690	0.752	0.064	0.858	0.081	0.045
Additive		0.420	0.821	0.255	0.602	0.045	0.252	0.534	0.457	0.084
WB x Additive		0.309	0.783	0.094	0.104	0.462	0.157	0.561	0.616	0.002

[1]SCFAs – short-chain fatty acids, AA – acetic acid, PA – propionic acid, BA – butyric acid, VFAs – volatile fatty acids, LA – lactic acid, VA – valeric acid, BCFs – branch-chain fatty acids. [2]Different superscripts in the columns indicate significant differences between treatments ($p < 0.05$); 3 total bacteria count as log10 CFU/ml

5.5. Discussion

5.5.1. Bird growth performance

In the current experiment wheat bran and additives did not affect BW. Weight gain and feed intake for all the intermittent and for the final periods also did not significantly differ with any of the used additives. Similar results were reported by Ali *et al.* (2008) and Salami *et al.* (2018) who found that WB did not have a significant effect on weight gain or FCR. The study of Courtin *et al.* (2008b) also revealed that neither XOS nor AXOS provided significant negative or positive effects on chicken growth after two weeks of feeding. Similar results were reported from Wang *et al.* (2017) who did not observe a difference in WG and FI throughout a 35-day study. No significant differences were found between the control and supplemented groups in overall feed intake, feed conversion ratio, and mortality of birds fed fructo-oligosaccharide (FOS) and mannan-oligosaccharide (MOS) prebiotics in the experiment of Kim *et al.* (2011). Dos Santos *et al.* (2019) also did not find an effect of dietary supplemented fibre on the FI and FCR of broilers.

Cozannet (2017) found that the use of a multi-carbohydrase complex rich in xylanase and arabinofuranosidase, reduced deleterious effect of fiber and improved the overall nutrient digestibility in broiler diets. On the contrary, Sacranie *et al.* (2012) did not observe any improvement in weight gain and the gain: feed ratio. They suggested an increased starch digestibility which prevent negative effects when the feed was diluted with coarse hulls. Animal studies revealed that regardless of feed form the addition of moderate amounts of structural insoluble fibre in the diet can improve the growth performance of young broilers (Jimenez-Moreno *et al.*, 2010; Jimenez-Moreno *et al.*, 2016). The fibre supplemented diet (25 and 50 g pea hulls /kg) not only improved growth performance (Jimenez-Moreno *et al.,* 2011) but from 1 to 21 d of age can also improve the total tract apparent retention for most nutrients (Gonzalez-Alvarado *et al.*, 2007). Hetland *et al.* (2003) and Jimenez-Moreno *et al.* (2019) also suggested an increased starch digestion after fibre supplementation. Courtin *et al.* (2008a) found that supplementation of bran AXOS at either 0.5 % (w/w) to wheat-based diets or at 0.25 % (w/w) to maize-based diets significantly improved the feed conversion ratio but it was not accompanied with the increase in the BW of the animals. The authors assumed that the reason was an improvement in nutrient utilisation since feed utilisation was as efficient in diets with bran and

AXOS as in feed containing xylanase. An improved body weight gain and FCR over the 42 day grow out period in maize or wheat-based/wheat bran diets was reported after the addition of xylanase (Kiarie *et al.*, 2012).

On the contrary, Yacoubi *et al.* (2018) found that supplementation of water-soluble short-chain AX obtained by the enzymatic treatment of wheat improved the FI and daily weight gain but had no effect on FCR in the starter period of broilers. The authors concluded that any beneficial effects which were observed were clearly attributable to the effect of carbohydrate-degrading multi-enzyme preparations (MEP).

In the current study, wheat bran had a significant negative effect on FCR (p=0.022) in the starter period and as an overall effect, there was an interaction between WB and additives (p=0.037). This result is in line with the study which used 30 g/kg of either sugar beet pulp, rice hulls or a combination of them, with the result being impaired daily weight gain in the growing period and a negative effect on FCR across the entire rearing period (Sadeghi *et al.*, 2015). In the experiment with rice bran, Farrel and Martin (1998) also reported depressed chick performance and a significant decline in growth rate and food intake, which worsened with the increasing inclusion level of bran (0, 200, 400 g). The same researchers did not find any positive effect of either of the enzymes supplemented.

Annison (1993) provided a possible explanation for the lack of the effect in simultaneous addition of fibre and enzymes. The researcher supposed that the digestive enzymes, either exogenous or endogenous, could affect the structure of the cell wall matrix in such a way that some cell wall non-starch polysaccharide (NSP) could be released. If these NSP are water-insoluble when they are still attached to the cell matrix and it is likely that they can dissolve under the enzyme action resulting in excessive amounts of NSP released during passage through the GIT. This could lead to a depressed growth performance and energy utilisation.

5.5.2. Effect on AME and AMEn

The positive effect of fibre inclusion on improved nutrient retention was reported by Jimenez-Moreno *et al.* (2019) who found oat hulls have a more pronounced effect on retention than sunflower or rice hulls. The AMEn of the diets increased by 2.5 % after fibre inclusion but no additional benefit was obtained with a further increase of

fibre content up to 5.0 %. Kimiaeitalab *et al.* (2018) found that the dilution of the diet with 30 g of sunflower hulls per kg did not affect chick performance but improved the AMEn of the diet in chickens at 21 d of age, which was not confirmed by the data for AME and AMEn in the current study since the diet without WB showed similar or higher values for AME and AMEn for all three rearing phases.

Amerah *et al.* (2009) found that the calculations showed an improvement in AMEn by dietary treatment with cellulose or wood shavings only after some corrections for energy contribution. The results of Jimenez-Moreno *et al.* (2009) were even more convincing since they observed that the inclusion of oat hulls or soy hulls improved total tract apparent retention of nutrients. The researchers also noticed that there were beneficial effects of fibre inclusion on fat retention and for younger birds, AMEn was higher. The results in the current experiment showed quite the opposite since there was no effect on AME or AMEn in younger birds but in the grower and finisher phase, fibre decreased both AME and AMEn significantly. Additionally, DMR, NR and FR values were, in general, higher for diets without WB, which showed a consistent impediment on these parameters due to WB. In line with these observations are the data of Kras *et al.* (2013) who used high-fibre diets and have found that independent of breed lines, chickens had lower energy retention coupled with lower performance. The researchers also examined the assumption that broilers fed fibre-diluted diets would increase the feed intake in order to compensate the lower energy intake, but it was not confirmed either.

Mathlouthi *et al.* (2002) found that the total metabolisable energy value of WB was not changed by xylanase supplementation. However, in the current study, despite only during the starter phase, SIGNIS® showed a significant positive effect in WB supplemented feed through increased NR. In young birds (7 day-old), Wils-Plotz and Dilger (2013) also reported similar results for increased retained nitrogen in cellulose-fed birds but observed a reduced DM digestibility for pectin-fed birds in comparison to control and cellulose supplemented diets.

5.5.3.   Gastrointestinal tract development

The addition of fibre could dilute the diet and as a result, diminish nutrient digestibility and performance but at the same time a moderate inclusion of insoluble fibres (2 to 3 %) may stimulate gizzard development and nutrient utilisation and chick growth can be improved (Donadelli *et al.,* 2019). Jimenez-Moreno *et al.* (2009) concluded

that young broilers might need at least a minimal amount of fibre in the diet for optimal performance because they observed that fibre addition reduced gizzard pH and improved nutrient retention. It is worth mentioning that different intestinal segments showed a specific pattern of changes due to fibre inclusion (Sklan *et al.,* 2003). According to Jimenez-Moreno *et al.* (2019) the effect of dietary fibre is not consistant. As an example, Amerah *et al.* (2009) found that wood shavings increased the relative gizzard weight and improved ileal starch digestibility, but in the same experiment all gut compartments were shorter in birds given diets containing insoluble fibre (cellulose and wood shavings) compared to the control and whole wheat diets. In the current experiment, in older birds (35-day-old) fed WB the caeca (p=0.075), ileum (p=0.063) and jejunum (p=0.104) tended to be heavier. Feeding wheat bran resulted in significantly (p=0.038) heavier small intestine and pancreas in older birds (35-day-old) in comparison to diet without WB.

Jimenez-Moreno *et al.* (2010) reported that the relative weight of the gizzard was higher with oat hulls and sugar beet pulp than with cellulose or the control diet. These results were similar to the current experiment since the proventriculus and gizzard in young chicks (10 day- old) fed WB were heavier, despite non-statistically significant, in comparison to the non-supplemented diet. In other experiments, fibre increased the absolute and relative weight of the empty gizzard with more pronounced effects with 5 % than with 2.5 % fibre inclusion and with oat hulls than with the other two fibre sources - rice or sunflower hulls (Jimenez-Moreno *et al.,* 2019). Gonzalez-Alvarado *et al.* (2007) found that fibre inclusion increased the relative weight not only of the gizzard but also the relative weight of the caeca and GIT. Similar results were reported by Jimenez-Moreno *et al.* (2011) who found that the relative weight of proventriculus, gizzard and caeca increased linearly as the level of pea hulls in the diet increased. Gizzard size increased with inclusion of oat hulls, whole wheat, wood shavings and grit (Hetland *et al.,* 2003).

Wickramasuriya *et al.* (2019) noticed that the large intestine was not affected nor by dietary energy level or by multi-carbohydrase supplementation, but the diet formulation (energy sufficient vs. energy deficient diet) increased the caeca weight by 12.5 %. Rezaei *et al.* (2011) suggested that the inclusion of fibre in broiler diets have a positive effect on BW and FCR but did not find any effect on relative weight of gizzard, ceaca and intestine of the birds.

5.5.4. Histomorphometry

The architecture of the intestinal mucosa is an important source of information about gut health. Antinutritional factors in the feed interact with the mucosal surface and relatively quickly can disturb its homeostasis. Shorter villi and deeper crypts have been associated with negative changes in intestinal morphology (Xu *et al.,* 2003). The ratio between the length of the villus and the depth of its corresponding crypts is also an important parameter for their optimal functionality. A high ratio indicates a long villus with sufficiently maturated epithelium coupled with a shallow crypt which indicates a constant but well-balanced cell renewal without exhaustion of the mucosa (Caspary, 1992; Star *et al.,* 2010). Shortened villi and deeper crypts indicate faster tissue turnover and the effect on birds could be poor nutrient absorption, increased secretion in the GIT and lower performance (Xu *et al.,* 2003). In the current experiment, WB increased the mean value for crypt depth and width, villus high and width and VH:CD. Due to the interaction of WB and additives it is difficult to make a clear statement about the effect of WB. The histomorphology data could be interpretate both as indicative for undesirable changes in the crypts due to increased cell turnover and as a sign for the formation of excessively long and fragile villi, which coud be sensitive to digesta flow and more prone to abrasion and destruction. However, the VH:CD reflected the changes in VH and CD and this along with the heavier GIT compartments could be related to better absorption.

Yamauchi and Isshiki (1991) suggested that the surface area as determined by the size of the individual villus could be important parameters for the absorptive ability of the intestine. The authors found that food intake regulation and the capacity of the GIT in broiler chickens were related and it was accompanied by increased absorptive surface in the small intestine. When the authors compared broiler and layer chickens, they found that the former had larger villi in all intestinal segments thus a higher absorptive rate, which was considered as the main reason for the faster growth of broiler chickens. The study of Kimiaeitalab *et al.* (2018) also showed that broilers had better growth performance and nutrient retention at 9 d of age and better ileum absorptive capacity at 21 d than pullets. Jacquier *et al.* (2019) concluded that higher feed efficiency was correlated with a significant increase in intestinal microvilli length.

Histomorphometry data in some experiments were contradictory to the general view. Burkholder *et al.* (2008), as an example, found that birds subjected to heat stress had reduced CD but unchanged VH and VH:CD when compared to the birds at normal temperature. Wils-Plotz *et al.* (2013) suggested that VH can be negatively affected by infection but in fact they have measured the greatest VH in infected birds fed a diet with pectin and low quantity of threonine. As a conclusion, the authors suggested that numerically longer villi were associated with deeper crypts, but CD was essentially unchanged in infected chicks.

Histomorphology data in the current study also showed some contradictions. Xylanase had a more pronounce effect on CD and villus width and on the contrary, SIGNIS® supplementation resulted in greater crypt width and villus hight. The observed effect of SIGNIS® was similar to the results obtained by Xu *et al.* (2003) who reported significantly longer ileal microvillus in birds fed diets with 2.0 and 4.0 g/kg prebiotics (fructooligosaccharides) versus control. However, the authors merely associated the changes of the intestinal tissue with a direct action of the prebiotics but suggested an indirect effect and more favourable intestinal microbial environment.

Rahmatnejad and Saki (2015) have observed that intestinal histomorphology was unaffected by cellulose, but carboxymethyl cellulose led to an increase in parameters such as CD, VH, VH:CD, villus width and villus surface area (VSA) in comparison to control or cellulose supplemented diets. The different effect on histomorphology resulting from different fibres and dietary supplements have been reported also from Wils-Plotz and Dilger (2013). The researcher did not find an effect of dietary treatment on VH or VH:CD, but CD was affected by purified fibre source, with cellulose- and pectin-containing diets having deeper crypts compared with the control diet. They also observed a trend for an interaction between fibre source and supplemental threonine concentration for crypt depth, where the cellulose diet elicited the deepest crypts when supplemented with adequate threonine level. The results in the current study also showed an interaction between the fibre and additives for all the parameter examined.

It has been suggested a relationship between increased secretion of amylase and epithelial cell proliferation in broilers results in increased VH and ileal thickness (Salim *et al.*, 2013). Yamauchi *et al.* (1995) assume that the ileum could be less

active than the other gut compartments but in turn might have another specific function in addition to the conventional absorptive function. All these data warrant further research regarding the relationship between histomorphology parameters and other factors involved.

Some positive effects on gut health related exclusively to the enzyme in the broiler feed are also possible. Costa *et al.* (2008) presumed that NSP-degrading enzymes can indirectly act as a prebiotic provider at the caecal level. The altered histomorphometry was related to the beneficial shift in the bacterial composition in the gut (Missotten *et al.,* 2013). Despite that the current study did not examine the composition of the gut bacteria, the fact that xylanase and SIGNIS® increased the mean value for VH and VH:CD in fibre supplemented diets in comparison to non-supplemented could be considered as a beneficial effect on chicken health (Chichlowski *et al.,* 2007). Similarly, Yasar and Forbes (2000) estimated that enzyme addition significantly increased VH and VH:CD and other researchers found that diets rich in fibre can reduce the villus length, width and surface but the addition of the enzyme could alleviate some of these negative effects (Moharrery and Mohammadpour, 2005).

On the contrary to the current study, Zulkifli *et al.* (2009) did not find a significant effect on the measurement of villi in chicks when diets contained either 0 % or 25 % palm kernel meal. Kimiaeitalab *et al.* (2018) also did not find an effect of sunflower hulls supplementation on VH and CD and the same lack of effect was reported by Alshelmani *et al.* (2016) for three segments of the small intestine – duodenum, jejunum and ileum – in the starter or finisher phase. Ileal histomorphology in the study of Gonzalez-Ortiz *et al.* (2019) also did not show any interactions of the ileal histomorphology parameters measured at 42 days of age. Xylanase supplementation did not affect VH or CD and birds fed diet supplemented with sodium butyrate had higher VH:CD compared to control birds, but this effect was due to the numerically lower CD observed in those treatments.

5.5.5. SCFAs content

Short-chain fatty acid content is an important parameter used for preliminary accessment of bacterial activity in the GIT and particully, the caeca. Quantitative and qualitative analysis of SCFAs make assumptions about the link between

particular species of microorganisms and the most probable outcomes for animal performance and gut health (Mroz *et al.,* 2006).

Jozefiak *et al.* (2007) reported an increase in lactic acid affected by both cereal type and xylanase supplementation and this data agreed with the current study - in younger birds (24-day-old) WB significantly increased the content of lactic acid. In 35-day-old birds, on the contrary, WB inclusion significantly decreased the quantity of SCFAs and propionic acid. Similar results were reported by Chu *et al.* (2017) who observed that despite acetic and butyric acid not being affected by WB (fermented, 10 %), the content of propionic acid was significantly reduced in comparison to the control diet.

In the current study, butyric acid content in 24-day-old birds was higher when fed the control diet in comparison to xylanase and statistically significant in comparison to SIGNIS®. The same significant effect was true for VFAs for the control diet in comparison to either xylanase or SIGNIS®. Similar results were reported by Juskiewicz *et al.* (2010) who noticed that the addition of NSP-degrading enzymes to diets with a different content of high-fibrous sunflower meal significantly decreased caecal SCFAs, disregarding the increased bacterial glycolytic activity. The authors also reported that the enzyme caused a significant decrease in the total VFAs and butyric acid as well as an increase in isovaleric acid concentration. The caecal acetic acid concentration in the control group was significantly higher than in the birds fed the enzyme supplemented diets. Explanation of these results is challenging but they are not unusual. Bedford and Apajalahti (2001) explained that in some experiments with the exogenous enzymes and other additives it is highly possible that control diets could present themselves unexpectedly well in comparison to the experimental diets. However, Wils-Plotz *et al.* (2013) studied infected birds fed pectin and threonine supplemented diets and found that compared with all other treatments (regardless of dietary threonin concentration) the total SCFAs production was lower in the control uninfected birds and total SCFAs, acetate, propionate, and butyrate all exhibited 3-way interactions between fibre, threonine, and infection treatment. The same authors found that numerically the caeca with the heaviest weights also had the greatest total SCFAs concentration. In the current study, despite the tendency (p=0.075) of heavier caeca in WB fed birds the quantity of SCFAs were significantly lower (p=0.048).

On the contrary, Yacoubi *et al.* (2018) found a positive effect of carbohydrate-degrading multi-enzyme preparations (MEP) on wheat AX due to the water extractable fraction which increased acetate and butyrate content in the caeca. Similarly, Jozefiak *et al.* (2004) showed that the type of cereal as well as supplementation with exogenous enzymes significantly increased acetate and SCFAs in the caeca. Furthermore, supplemented enzyme also increased the butyrate concentration in comparison with unsupplemented groups but at the same time it was not related to any significant effect on liveweight of the birds. In conclusion authors indicated that a high concentration of total dietary fibre in the diet was not necessarily connected with increased fermentation in the caeca.

In a later study, Jozefiak *et al.* (2007) found that xylanase inclusion had no effect on the caecal acetic, propionic, butyric and total quantity of acids. Additionally, Lazaro *et al.* (2003) did not find that enzyme supplementation to rye based diets modify volatile fatty acid concentration and Kimiaeitalab *et al.* (2018) did not find effects of fibre (sunflower hulls) either on the pH of the GIT digesta or the concentration of SCFAs in the caeca. Similarly, Hou *et al.* (2020) studied three levels of fibre and two rearing systems and despite the differences in the microorganisms, they did not find any interactions between dietary fibre and the rearing system. The main effects on SCFAs of the factors studied were also non-significant.

Bedford and Apajalahti, (2001) assumed that addition of the enzyme could increase the rate at which starch and protein are removed from the small intestine and thus provide more energy for the birds and minimize the quantity of the possible substrates for the microbial fermentation. Another possible reason for the controversial results in the published data could be the use of different fibres and/or due to xylanase activity and specificity in the experiments. It has been found that xylanases from families GH 10 and GH 11, as an example, act differently on the soluble arabinoxylan from WB and the end products differ in their degree of polymerisation. These products could be metabolised by different bacteria and the quantity of SCFAs could vary (Beaugrand *et al.*, 2004). Zdunczyk *et al.* (2015) warrant that because the literature data are often contradictory the effect of NSP-degrading enzymes in the caeca should be further investigated.

A study where xylanase hydrolysis of WB released AXOS resulted in increased number of butyrate producing bacteria could be considered as a prebiotic effect with

a beneficial nutritional outcome (Ravn *et al.*, 2017). However, the effect of wheat bran AXOS or xylanase is not always additive or straightforward. Some data showed that wheat bran AXOS significantly increased the level of bifidobacteria but not the total bacteria in the caeca of the chickens and this effect was not related neither with xylanase nor with fructo-oligosaccharide (Courtin *et al.,* 2008a). In the current study WB significantly (p=0.045) decreased total bacteria count and there was an interaction between fibres and additives. However, in 35-day-old birds SIGNIS[®] tended (p=0.084) to increase the total bacteria count which is in line with the data of Kim *et al.* (2011) who used 0.25 % FOS and 0.05 % MOS, reporting an increase in total bacteria count. On the contrary, Yang *et al.* (2008) have found that up to day seven, xylanase increased the total anaerobic bacteria (TAB) in the caeca and by day 21 TAB were lower in birds given the xylanase-supplemented diet than in those fed the control diet. However, the simple estimation of total bacterial count does not provide any specific information on the genera and species which are affected by the WB or additives used. There is also a lack of information of some possible shifts in the caeca microorganism diversity which make any conclusions highly speculative and prone to misinterpretation.

5.6.   Conclusions:

The inclusion of 2.5 % WB, xylanase alone or in combination with XOS (SIGNIS[®]) did not significantly affect bird performance, including feed intake. It was confirmed that the inclusion of WB would have a negative effect on AME and AMEn, however, in 35-day-old birds, SIGNIS[®] improved dietary metabolisable energy. Young birds were more susceptible to the negative effects of high dietary WB inclusion, resulting in an increased FCR. As expected, the inclusion of WB reduced DMR and FR. The addition of dietary WB likely impacts gut health with evidence of better gut histomorpology (on average increased villus height and VH:CD) however, reduced caecal SCFA content, particularly PA but increased concentrations of caecal LA. The use of dietary additives improved gut histomorphology in diets supplemented with WB but not to the level of WB alone. Maize based diets benefit from low inclusion levels of WB (2.5 %) to enhance broiler chicken gut health, while xylanase and SIGNIS[®] may still benefit gut health but to a lesser extent.

Chapter 6. General discussion and conclusions on the effect of xylanase and its combination with xylooligosaccharides for broiler chickens

6.1.   The benefits of using exogenous enzymes and xylanase

The objectives of this thesis were to evaluate the best strategy for using xylanase for broiler chicken production. Enzymes, along with plant secondary metabolites, acidifiers, pro- and pre - biotics are extensively studied because of their potential as alternatives to the AGPs. Exogenous enzymes as feed supplements are associated with a reduction in maintenance requirements during bird rearing, alleviation of antinutritive effects of dietary components (such as fibre and phytate), improvements in utilisation of starch, amino acids, fat, Ca and P (Cowieson, 2010). Exogenous enzymes are multifactorial in their effect because they not only increase the content of available nutrients but also provide some substrates for specific bacteria in the gut. Understanding these interactions will increase profitability of using exogenous enzymes and in particular xylanase (Bedford and Cowieson, 2012).

6.2.   The effect on broiler growth performance and AME

In both experiments conducted there was no difference in the BW of birds which were allocated to the different diets and mortality was low. However, during the first experiment, the body weight of the birds was relatively low in comparison to the Aviagen performance objectives (Aviagen, 2018). On the contrary, during the second experiment the growth rate of broiler chickens was very good and at the end of the starter phase BW reached 84% of Aviagen performance objectives and at the end of the grower and finisher phase – 96 % and 99 %, respectively (Aviagen, 2018). Such performance indicated that the birds were approaching their genetic potential. According to Bedford (2002) if the FCR of the birds fed a control diet is excellent there is almost nothing that can be achieved by dietary modification and further improvement in performance or benefit from an exogenous enzyme could not be expected. Considering these facts, a direct comparison of body weight of the birds from the two experiments is not only inconsistent but could be misleading. However, if the experiments are considered independently some possible conclusions can be made.

In wheat-maize based diet, xylanase or SIGNIS® (Table 4.3) did not affect performance variables such as body weight (BW), weight gain (WG), feed intake (FI) and calculated feed conversion ratio mortality corrected (FCR). Similarly, there was no significant effect (p> 0.05) on BW due to WB or additives supplementation. The weight gain and feed intake for all the intermittent and final periods also did not significantly differ (p>0.05) across any of the used additives. The assumption that the response of birds towards the WB diluted diet would be increased feed intake to compensate for the energy and nutrient demand was not confirmed by the current study. Similarly, Sacranie *et al.* (2012) did not observe an improvement in weight gain or feed efficiency. They suggested an increased starch digestibility which prevented the negative effects when the feed was diluted with coarse hulls.

In the current study, the positive effect of xylanase and SIGNIS® resulted in an increased AME (p=0.011), AMEn (p=0.006) and DMR (p=0.014), and this effect was more pronounced at the end of the finisher phase. In line with the results from the first experiment, in the second experiment WB, xylanase and SIGNIS® did not show an effect on AME and AMEn for the starter period. However, WB significantly decrease AME and AMEn in the grower (p=0.002) and finisher periods (p < 0.001) (Table 5.4). Again, both xylanase and SIGNIS® increased AME and AMEn in comparison to the control diet, and for SIGNIS® this increase was statistically significant in the finisher period. Stefanello (2016) also found that xylanase can improve AMEn through increase energy utilisation and digestibility.

Wheat bran had a significant negative effect on FCR (p=0.022) in the starter period and an overall effect (p=0.046) which means that the inclusion of the WB in the diet of young broiler chickens could overload their immature digestive tract and prevent optimal utilisation of nutrients. Wheat bran also has a negative effect by decreasing DMR but only in the starter (p=0.012) and grower (p=0.03) periods (Table 5.5). The WB had the same negative effect on FR for the grower and finisher periods. In 35-day-old birds, xylanase and SIGNIS® showed a consistent effect increasing DMR which in the wheat-maze based diet was statistically significant (p=0.014) and in maze-soy diet was a tendency (p=0.096). These findings are in line with other research data which implied that the chickens benefited more from the enzyme addition at a younger age but are contradictory of the view that the contribution of the enzymes towards nutrient retention decreased with the age of chickens (Olukosi *et al.,* 2007). Kaczmarek *et al.* (2014) suggested that it is very likely that endogenous

enzyme deficiency in young chickens may not be as pronounced as originally thought but the effect of xylanase and SIGNIS® on DMR, FR and NR during the rearing period should not be underestimated.

The effects of additives were significant only in the finisher period – from 25 to 35 days (p=0.038), since xylanase improved the FCR in comparison to SIGNIS® but this effect was similar to the control diet. As an overall effect (0-35 days) on FCR the additives showed only a trend (p=0.126) and analysis showed an interaction (p=0.037) for WB and additives.

Cowieson and Kluenter (2019) explained that the performance of the animals under observation could have a dictating effect on the magnitude and consistency of the response to the additives. As a result, they introduced five types of response to feed additives. Briefly summarised these effects could be associated with low growth of control animals with possible outcome of poor response to additives or on the contrary - increased response. At average growth of control animals, substantial responses to additives are associated with reduced variation and insignificant impact of environmental factors. In high performing flocks, the possible outcomes are diminishing responses to the additive due to approaching genetic potential and there is little further benefit from the additives. The results of the current study indicated that the presence of xylanase and SIGNIS® in the starter diet was important for nutrient retention and their continuing effect through the rearing period resulted in increased AME and AMEn, and alleviation of WB negative effects. Omitting the fibre from the starter diet to avoid some negative effects on young birds could improve their performance but also can slow down the process of gut development diminish gut microbial diversity and negatively affect gut health (Hou *et al.*, 2020).

6.3.   The effect on GIT and histomorphometry

Wheat-maze based diets and maize-soy diets, used in the experiments did not contain excessive amounts of soluble fibre with a viscous nature that could significantly alter the gut viscosity and motility and affect the weight and length of the broiler chicken GIT. The choice of the diet formulation in the current experiments allows for some more detailed observation on gut development and some discreet changes which in other cases could be ignored or left unnoticed. One of the most important observations from both experiments was the effect of the additives and

WB on the relative weight of the pancreas. In wheat-maze diet, supplementation of xylanase and SIGNIS® resulted in decreased weight of the pancreas which implies better control over secreted enzymes and low energy expenditure for their synthesis. The pancreas is responsible for the secretion of at least five different enzymes with a paramount role in digestion (Denbow, 2000). On the contrary, WB resulted in a heavier pancreas, but the concomitant presence of additives did not have an effect. The xylanase and SIGNIS® diet (Table 4.5) also increased the relative weight of the small intestine and GIT at 35 day-old. It has been found that high digesta viscosity may negatively affect the GIT development in young chickens, but enzyme supplementation alleviated the disturbances in digestive tract development (Smulikowska *et al.*, 2002). However, because the diets in the current study were not supposed to increase the gut viscosity it is very likely that the increase relative weight of the small intestine could indicate well-developed mucosa, better absorption and a healthy gut.

Insoluble fibres have an effect on the development of the PG (Svihus, 2011). In the current study, dietary WB addition numerically increased PG in 10-day old chickens. Increased fermentation activity in the ceaca also was related to increased PG weight (Masey-O`Neill *et al.*, 2014a). In the current experiment, in 35-day-old birds, WB numerically increased the relative weight of the caeca and also the ileum. The small intestine was also affected by WB inclusion and the effect was significant (p=0.038) in older birds (35 day-old) as the relative weight of small intestine in birds fed diets without fibre was lower (2.610) than in birds fed diet with fibre (2.824) and the same tendency was true for the jejunum. There was only one interaction (p=0.044) between wheat bran and product for jejunum in 35-old birds. Observed interactions between WB, xylanase and SIGNIS®, along with some occasional effects on particular gut compartments could obscure the role of the additives. The GIT is directly involved not only in chicken performance but also is the largest immune organ in the body (Kraehenbuhl and Neutra, 1992). Whether the changes in GIT development are simple indicators for increased weight of the tissue or are connected with some changes in the physiology of the compartments and their absorptive capacity or immune function warrant further research. Without additional data it is difficult to make an unbiased conclusion how these changes affect functionality of the gut and which of these changes could be considered as indicators for a healthy gut.

To overcome some of the limitation of the data for the GIT development, an ileal histomorphometry analysis was applied in the second experiment. Since maintenance or enhancement of gut health is far more complex than the modulation of the gut microflora through probiotics or prebiotics, some variables such as histomorphology can be useful in obtaining information about gut status (Choct, 2009). In the current study, statistical analysis revealed an interaction (p<0.001) between WB and additives for all ileal histomorphology parameters (Table 5.7). However, excluding villus width, feed containing WB increased the mean value for all other variables - CD, VH and VH:CD. The control diet had the highest mean value for crypt width, VH and VH:CD ratio. In comparison, xylanase had a more pronounce effect on the CD and villus width and SIGNIS® resulted in higher values for crypt width and VH.

The data from the histomorphometry analysis in the current study did not allow for any explicit conclusions. An increased crypt width observed in birds fed the control feed could mean a wider space between villi, a reduced number of villi on the surface area and higher gut flow, accompanied with an increased loss of nutrients (Yamauchi, 2002). However, this suggestion has not been tested yet, but there is some new method which can add valuable information on the subject (Wilson *et al.*, 2018).

6.4.   The effect on SCFAs

In a wheat-maze based diet, SIGNIS® increased the caecal concentration of SCFAs and butyric acid, and numerically also VFAs in 21-day-old birds. In the caeca of older birds both xylanase and SIGNIS® increased the level of SCFAs in comparison to the control diet and additionally xylanase alone had a significant effect on acetic, butyric and volatile fatty acids (p=0.038) in comparison to the control diet. For the same acids, at least numerically, SIGNIS® also showed higher values in comparison to the control diet. The increased content of SCFAs and especially butyric acid which is considered as the main nutrient source for colonocytes as a result of xylanase and SIGNIS® activity could be taken as a good indicator for their effect on gut health. The variety of SCFAs for which content increased in older birds could mean indicate oligosaccharides with different degrees of polymerisation and possible prebiotic effect, boost of caecal bacteria or increased diversity of caeca microorganisms. Ribeiro *et al.* (2018) suggested that enzymes and generated or supplemented

prebiotics can act synergistically, thus positively affecting bird performance. Dusel *et al.* (1998) also suggested that the effect of xylanase is probably more pronounced in mature birds because the gut microflora is well established and more mature and its ability to respond to dietary ingredients is significantly higher. An *in vitro* study revealed that xylan can generate XOS with different DP independently of their structure. Comparative assessment of the result shows that the composition of xylan, xylanase and reaction time determine the yield of each oligosaccharide in the hydrolysate mixture (Akpinar *et al.*, 2009).

Dietary fibre is considered an antinutritional factor due to the negative impacts on nutrient utilisation (Mateos *et al.*, 2012) and in the second experiment WB inclusion also had a detrimental effect on the concentration of caecal SCFAs. In 10-day-old birds, WB tended ($p=0.102$) to decrease the content of butyric acid and in 35-day-old bird WB cause a significant decrease of the total content of SCFAs and propionic acid (Table 5.8). Additionally, in older birds (35- day-old) WB diet reduced the values for volatile fatty acids (VFAs) and acetic acid. However, in young birds (10-day-old) there was an interaction between WB and additives but the content of lactic acid in the ceaca of birds fed SIGNIS® had the highest value – 12.57 mM. Additionally, in 24-day-old birds the non-supplemented diet showed the higher mean value for butyric acid in comparison to the additives. Xylanase and SIGNIS® had the same effect on VFAs in 24-day-old birds since control diets had a higher value in comparison to xylanase or SIGNIS® supplemented diets. Kiarie *et al.* (2013) suggested that production of fermentable oligomers may well be a large part of the total response to feed enzyme supplementation, but it is also possible that the xylanase have not released significant quantities of oligosaccharides *in situ*.

Overall, the lactic acid content of diets with WB and additives revealed a very clear trend of diminishing values throughout the rearing periods. The value of LA in general was higher in younger birds and decreased in older ones. In the opposite direction was the changes of propionic acid – its content was lower in younger birds and increased in older ones. It is worth studying the changes of SCFAs coupled with consecutive changes of gut microorganisms which are very likely directly involved in these processes. It is very likely that some of these changes are age-related, feed dependant, hormone regulated or quintessential for the normal gut physiology and therefore do not provide room for the activities of the additives or just conceal their

effect. The study of Bautil *et al* (2019) indicated that the capacity of the intestinal microbiota to degrade AX in the hindgut increases as the broiler ages. The researcher suggested that the benefits of xylanase supplementation of broiler feeds depend on the interaction with the intestinal microbiota and AX presence in the GIT at specific broiler age. Oakley *et al.* (2014) also suggested that evaluation of a feed additive is determined through natural successional changes in the GIT microbiome. These changes in bacterial community composition and function occur naturally as birds mature and are highly significant and consistent across treatments. Proper understanding and management of temporal changes in the GIT microbiome will be important for maintaining bird health and improvement in productivity. Still, there is very little information about the specific effect of each fatty acid in the chicken gut and some suggestions are simply based on the fact that they are monogastric animals or just extrapolating information obtained from other species (Jha *et al.,* 2019).

The general trend of TBC also showed an increased number as birds grow. Analysis showed that there was an interaction between WB and additives for TBC in 35-day-old birds, significant effect of WB (p=0.045) and trend in the effect of additives (p=0.084). However, at the end of the finisher phase, in diets supplemented with SIGNIS® or its combination with WB, the mean values for the TBC were highest – log 10 CFU/ml - 12.31 and 12.41, respectively. This increase in the number of bacteria could be the result of a prebiotic effect. The prebiotic nature of XOS was suggested by *in vitro* fermentation carried out using known probiotic strains of bifidobacteria in the study of Kallel *et al.* (2015). It has been suggested that some bacterial species are related to more efficient feed utilisation in broiler chickens (Stanley *et al.*, 2013). However, the general overview on the TBC analysis done in the current study does not provide such insight in the ceacal microorganisms or abundance of particular species.

6.5.   General conclusions

This thesis evaluated the strategy of using xylanase and XOS for broiler chicken production. In low fibre diets (wheat-maze based), both xylanase and SIGNIS® provide beneficial effects through better utilisation of nutrients and increased dietary available energy. However, this does not always translate into improved growth performance in rapidly growing modern broilers. Supplementing diets with xylanase

and SIGNIS® also increases caecal SCFAs concentration, suggesting microbial proliferation associated with a positive impact on gut health. Starter diets supplemented with xylanase or SIGNIS® have thus been demonstrated in this thesis to be beneficial for the utilsation of nutrients in the latter phase of broiler production. Furthermore, xylanase and SIGNIS® can alleviate detrimental effects on bird performance associated with the inclusion of dietary wheat bran. However, the type of fibre may also impact the effectiveness of these products and the fermentive ability of caecal microbiota, due to substrate availability. This is an area for further research for practical poultry diet formulation. In conclusion, xylanase and SIGNIS® fed at the industry recommended rate are important feed additives for commercial chick starter diets, ensuring optimised bird performance, especially in the later growing phases.

6.6.　　Areas for further research

The study results imply that the effect of xylanase and SIGNIS® is dependent on the dietary formulation and the level and type of supplemented fibre. Further research exploring different dietary formulations and the inclusion of different types of fibres is waranted.

Beneficial effects associated with the prebiotic activity of XOS, either supplied directly in the diet or generated from substrates contained in dietary ingredients through the action of xylanase, are a new area for study. Further reserach may show how XOS can be combined with other prebiotics to optimise gut health. Another possibility is to explore the effect of prebiotics (including XOS) on selected gut hormones.

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