Universitat Autònoma de Barcelona

FEED-ASSOCIATED FACTORS TO XYLANASE RESPONSE IN CORN-BASED POULTRY DIETS

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Summary

Poultry production faces several challenges related to the use of natural resources, with feed efficiency being the factor that can be influenced through the use of different nutritional strategies. Proper use of exogenous enzymes, such as xylanase, could improve growth performance, feed conversion, gut health, and improve environmental problems as fewer undigested nutrients are excreted. In this way, the present work seeks to increase the knowledge about the suggested action mechanisms of this enzyme as well as to investigate the relevance of some dietary factors that may be affecting the efficiency of xylanase in the production of broiler chickens. Four studies were designed with the aim of testing the following hypotheses.

The carbohydrases, specially xylanase, could be a part of a multidisciplinary antibiotic's displacement strategy. Therefore, the **Article I** is a literature review performed in order to compare the xylanase suggested mechanisms (nutrient digestibility, microbiota modulation and improvement of intestinal health) and the described antibiotic effects when are used as growth promotors in animal diets. The available literature showed that it is difficult to consider that enzymes per se can replace antibiotics in efficient animal production, but it is plausible to think that they can be part of a holistic program that reduces the negative impact of antinutritional components as challenging compounds for intestinal health and efficiency.

The variability in the physicochemical composition of corn could affects the xylanase response. Therefore, **Article II** is an "in vitro" test that was carried out using the genetic background of corn and the position of the ear as a source of nutrient variability and where the xylanase response was evaluated. The results showed that the xylanase supplementation increase the soluble components of the corn independently of the

physicochemical variation, however, the xylotriose production interact for both the position of the kernels in the cob and genotype, showing that the corn physicochemical composition can affect the response of the enzyme at least in the production of XOS.

The nutrient variation associated to the genotype affects the broiler performance and nutrient utilization. Therefore, in **Article III** an in vivo trial was development in order to study how the variability of physicochemical composition of corn, due to genetic background, could produce negative interactions among the different physicochemical components, and consequently changes in broiler chicken's growth performance and nutrient digestibility. The results showed that the variation in the content and nature of the non-starch polysaccharides in corn can reduce the performance and digestibility in broilers mainly due to the capture of nutrients.

The corn particle size distribution affects the xylanase response influencing the performance and intestinal health of broiler chickens fed with corn-based pelleted diets. Therefore, in the **Article IV** the xylanase response was evaluated in different particle size distributions of corn-based pelleted diets. The results showed that the xylanase prebiotic mechanism was affected by particle size in corn-based pelleted diets. The effectiveness of the enzyme and gizzard development could be improved by considering using coarse particle size distribution in pelleted diets.

Resumen

La producción avícola enfrenta varios desafíos relacionados con el uso de los recursos naturales, siendo la eficiencia alimenticia el factor que puede ser influenciado mediante el uso de diferentes estrategias nutricionales. El uso adecuado de enzimas exógenas, como la xilanasa, puede mejorar el rendimiento productivo, la conversión alimenticia, la salud intestinal y mejorar los problemas ambientales, debido a la menor excreción de nutrientes no digeridos. De esta forma, el presente trabajo busca incrementar el conocimiento sobre los mecanismos de acción sugeridos de esta enzima así como investigar la relevancia de algunos factores asociados al alimento que pueden estar afectando la eficiencia de la xilanasa en la producción de pollos de engorde. En la presente tesis doctoral se diseñaron cuatro estudios con el objetivo de probar las siguientes hipótesis.

Las carbohidrasas, especialmente la xilanasa, podrían ser parte de una estrategia multidisciplinaria para el remplazo de los antibióticos en la producción animal. Así el Artículo I es una revisión de la literatura realizada con el fin de comparar los mecanismos sugeridos por la xilanasa en las dietas a base de maíz (digestibilidad de nutrientes, modulación de la microbiota y mejora de la salud intestinal) y de los antibióticos cuando se utilizan como promotores del crecimiento en dietas animales. La literatura disponible mostró que es difícil concluir que las enzimas per se puedan reemplazar a los antibióticos en la producción animal intensiva, pero es plausible pensar que pueden ser parte de un programa holístico que reduzca el impacto negativo de los componentes fibrosos de la dieta que tienen efectos antinutritivos que desafían la salud intestinal y la eficiencia.

La variación de la composición fisicoquímica del maíz podría afectar la respuesta de la xilanasa. En este sentido, el Artículo II es un ensayo "in vitro" que se llevó a cabo

utilizando el componente genético del maíz y la posición de los granos en la mazorca como fuente de variabilidad de nutrientes para evaluar la respuesta de la xilanasa. Los resultados mostraron que la suplementación con xilanasa aumenta los componentes solubles del maíz independientemente de la variación fisicoquímica, sin embargo, la producción de xilotriosa interactuó tanto con la posición de los granos en la mazorca como con el genotipo, mostrando que la composición del maíz puede afectar la respuesta de la enzima en la producción de XOS.

La variación de nutrientes asociada al genotipo afecta el rendimiento y la utilización de nutrientes de los pollos de engorde. En este aspecto, en el artículo III se desarrolló un ensayo in vivo para estudiar cómo la variabilidad de la composición fisicoquímica del maíz, debido a la genética, podría producir interacciones negativas entre los diferentes componentes fisicoquímicos y, en consecuencia, cambios en el rendimiento productivo y la digestibilidad de nutrientes de los pollos de engorde. Los resultados mostraron que la variación en el contenido y la naturaleza de los polisacáridos no amiláceos del maíz pueden reducir el rendimiento y la digestibilidad en los pollos de engorde principalmente debido a la disminución en la disponibilidad de nutrientes.

La distribución del tamaño de las partículas del maíz afecta la respuesta de la xilanasa produciendo cambios en el rendimiento y la salud intestinal de los pollos de engorde. Por lo tanto, en el Artículo IV se evaluó la respuesta de la xilanasa en diferentes distribuciones de tamaño de partícula de dietas granuladas a base de maíz. Los resultados mostraron que el mecanismo prebiótico de la xilanasa se vio afectado por el tamaño de las partículas en las dietas granuladas a base de maíz. La eficacia de la enzima y el desarrollo de la molleja podrían mejorarse considerando el uso de una distribución de tamaño de partícula gruesa en las dietas granuladas.

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CHAPTER I

INTRODUCTION

1. First Approach

Estimates predict human population growth to 9.1 billion in 2050, with most of the growth occurring in developing countries (UN, 2017). This increase in the number of people will need a concomitant increase in the food production sector to produce high quality protein. The poultry sector is considered as one of the most prominent sectors among livestock (OECD/FAO, 2019), with the highest sustainability standards due to the poultry products show a low environmental impact compared with other animal protein production (De Vries & De Boer, 2010). Therefore, chicken meat and eggs have the lowest water and carbon footprint per kg of edible protein, and very high land use efficiency per kg of edible protein (Flachowsky et al., 2018). However, the poultry production, like all animal production sectors, has the challenge of improving the efficiency of the use of resources, including feed. Currently, one of the main concerns in this field is obtaining feed formulas that result in greater efficiency, improving the performance of the birds and reducing the excretion of nutrients in the environment. In this way, most of the countries of America and Asia use corn-based diets extensively because it is considered a raw material with little variation in its nutritional composition and with a low presence of fibrous components compared to other cereals (Figure 1). Nevertheless, it is reported that the corn physicochemical composition can vary due to different factors with consequences in the birds performance response (Latham et al., 2016; O'Neill et al., 2012), thus showing still margins to improve the feed efficiency. In this scenario, the use of exogenous enzymes represents one of the key technologies that can help address this challenge. The appropriate use of enzymes, such as carbohydrases, may not only improve the nutrient utilization, but may also have benefits on the intestinal health and microbiota profile. Thus, it is important to know how the response of these

enzymes could be limited by different factors, some of them associated with the feed, in order to get the most benefit from their supplementation.

In this context, the present work attempts to identify and investigate the relevance of some dietary factors that may be affecting the efficiency of xylanase in the production of broiler chickens. Therefore, below we describe the suggested mechanisms and benefits of including xylanase in corn-based poultry diets and the most relevant feed-associated factors that could be affecting its response.

2. Xylanase Supplementation

Exogenous enzymes include larges groups, such as carbohydrases, proteases, lipases, and phytases, and the feed industry has been using these enzymes as zootechnical additives for non-ruminants for the last three decades. The main objective for doing so is the accepted view that they may improve the productive value of diets by increasing nutritive content and boosting animal performance, with the consequent reduction in feed costs and environmental impact. However, the supplementation of these exogenous enzymes needs to consider several factors including the nature of the operation, diet composition, target market weight, stocking density, ambient temperature, age of animals and many other factors (Cowieson & Kluenter, 2019). Thus, the suitable use of these enzymes could have beneficial changes in digestion, physiology, microbiology and immunology due to the variation in the characteristics of the ingesta, the balance and source of nutrients in the diet.

The carbohydrases represent one of the most commonly exogenous enzymes used in animal nutrition; these include all enzymes that catalyze a reduction in the molecular weight of polymeric carbohydrates. Two of all these are the ones that are marketed in the highest proportion: xylanase and glucanase, other commercially available carbohydrases

include α-amylase, β-mannanase, α-galactosidase and pectinase (Castillo & Gatlin, 2015).

The xylanase can break down the NSP β-1, 4-xylan bonds in the plant cell wall to shorter polysaccharides and to oligosaccharides (Faulds & Williamson, 1999). As was mentioned previously the NSP content and nature vary in a large proportion among cereals; the nature of the chains could be water-extractable or water-unextractable (Choct, 1997). The soluble fraction (water-extractable) is greater in cereals such as wheat, barley and oats, and it is responsible for viscosity (Bedford, 2002), and the insoluble fraction (water-unextractable) in the most of cereals (including corn), it has been related with the encapsulation of nutrients (O'Neill et al., 2014). Therefore, the use of xylanase has led to an increase in the dietary levels of those ingredients with anti-nutritional components interfering with the digestive function, for example wheat and barley in poultry diets. Nevertheless, corn has proved enclose very variable of arabinoxylans (AX) content, and thought these mostly have insoluble nature; they could be a useable substrate for this enzyme. Xylanase has showed several benefits in monogastric animals via several mechanisms described below:

a) Disrupting anti-nutritional factors that are contained in some ingredients, e.g. the viscous soluble arabinoxylans in wheat, barley or rye, (Bedford, 2002).
b) Hydrolyzing water insoluble arabinoxylans in cell wall polysaccharides to eliminate nutrient encapsulation, such as starch or protein (O'Neill et al., 2014).
c) Releasing small oligosaccharide molecules for more effective foregut and hindgut fermentation, and the modulation of intestinal microbiota (Bedford & Cowieson, 2012).
d) Reducing the flow and loss of endogenous nutrients, including amino acids, contained in intestinal mucins (Cowieson & Kluenter, 2019).

Thus, excluding the reduction on viscosity effect due to soluble arabinoxylans; it is plausible to obtain benefits of these mechanisms on corn-based poultry diets. Nowadays, the information about the effects of xylanase supplementation in corn-based diets is scarce. Considering that corn is the most common energy source used in animal diets, and by far the most important cereal for all poultry feed (Dei, 2017).

In this context**, we found interesting to define the components and variables that may determine xylanase efficacy in corn-based broiler diets. Additionally, the benefits obtained when xylanase is supplemented are very close to those reported with antibiotics as growth promoters, so a literature review will be necessary to address this particular issue.**

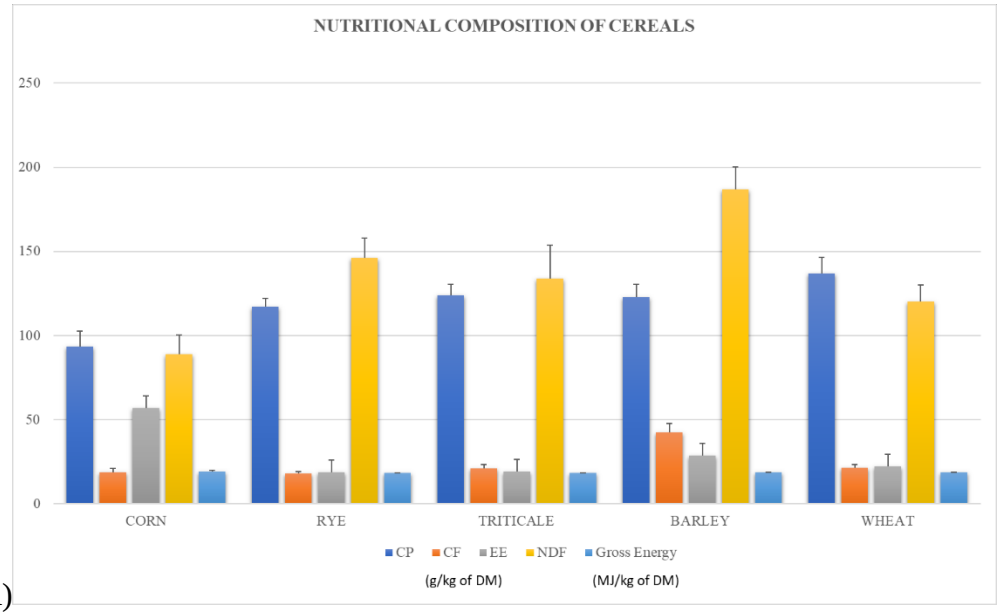
3. Corn Physicochemical Composition

Corn is the most common energy source used in commercial animal diets, especially in the American, Southern Europe and most Asian countries where corn grain is the primary cereal for all poultry feed (Dei, 2017; M Larbier et al., 1994). Due to its high-nutrient content, corn may contribute up to 65% of metabolizable energy and 20% of protein in poultry diets (Gehring, Cowieson, et al., 2013). Usually, the composition and nutritional value of raw materials (including corn) used for animal feeding is shown worldwide in many nutritional feed tables (e.g. FEDNA, 2019; NRC, 2012; Brazilian Tables, 2017; CVB, 2021). However, the variability in nutritional composition of corn cannot be captured due to a large number of factors, including the genetics, agronomic conditions, geographical location, and pre- and post-harvest processing. Thus, it has been reported variation in protein (5 %), starch (2 %), protein solubility index (20 %) among corn samples due to the geographical location (O'Neill et al., 2012). Similarly, the drying process (35 vs. 120, °C) has shown effects on the proportion of amylose within the starch (21 vs. 27, %) and in the starch gelatinization (3 vs 1.3, %) in hard kernel corn (Córdova-

Noboa et al., 2020). As shown, many factors, some of them less studied, could provide variability in macronutrients, but also could affect the anti-or hypo-nutritional components of corn, including non-starch polysaccharides (NSP), phytin inhibitors, lectins, and resistant starches (Cowieson, 2005; Englyst, 1989). Regarding to the NSP, the content in the cereals of these fiber components could represent from 10 % to 30 % of the total composition, and the major NSP are the arabinoxylans, cellulose and B-glucans (Choct, 2015). Despite the fact that corn contains lower AX content and solubility compared to other cereals (figure 1), the variability could be greater than previously thought. The antinutritional effects of AX are viscosity and trapping effect attributed to water-extractable and water-unextractable AX, respectively (Bedford & Classen, 1992). Thus, corn variability in the physicochemical composition could have consequences on the availability of nutrients, due to AX trapping impact, producing a nutritional unbalance and then affecting the performance of the animals. On this subject, the near infrared spectroscopy (NIRS) technology, widely used by the feed industry represent an important tool to account for this variability. Thus, currently most of the feed mills use NIRS to predict nutrient composition, and try to correct the physicochemical variability in the primary feed ingredients, including corn. Nowadays, the NIRS analysis is generally focused on the macronutrients such as moisture, crude protein, starch, crude fiber and crude fat. Nevertheless, the NIRS technology can be also calibrated and used to more complex parameters such as in vivo digestibility and apparent metabolizable energy (AME) values (Landau et al., 2006), or fiber compounds such as NSP content, and also to predict the nature of their chains. Consequently, it becomes of interest to investigate if prediction models develop in cereal samples could be applied to efficiently assess the fiber compounds, such as NSP.

In this sense, **one of the spotlights of the present work will be to study the content and nature of the NSP in different corn samples, in which genetics and kernel size (due to their position on the cob) will be used as sources of variation.**

A)



B)

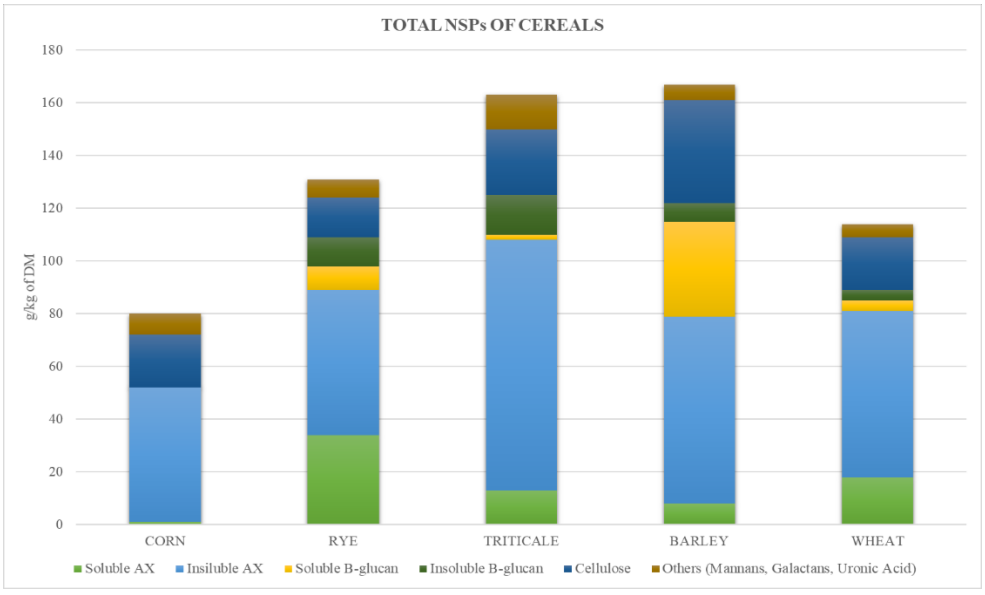


Figure 1. A) Comparison of nutrient composition of main cereals used in animal feeding. CP: Crude protein; CF: Crude fiber; EE: Ethereal extract; NDF; Neutral detergent fiber. Adapted from Rodehutscord et al., 2016. B) Content and nature of the total non-starch polysaccharides of main cereals used in animal feeding. Arabinoxylans: AX. Adapted from Mingan Choct, 1997.

3.1. Corn Genetic Background

Genetics has been shown as an important source of biochemical and nutrient variability in cereals (Rodehutscord et al., 2016). Regarding corn, the phenotypic characteristics such as the grain-filing duration, related with physiological maturity and composition, growth rate and moisture of the kernel are specific for each genotype and could affect the nutrient value (Prado et al., 2014; Seebauer et al., 2010). Additionally, the program hybrids selection is regularly oriented in agronomical issues such as drought tolerance, yield potential, disease resistance, tolerance for different soils conditions and heat stress (Prasanna et al., 2021; Sibiya et al., 2013), leaving aside the nutrient variability and/or nutrient availability, especially for livestock. As was mentioned, Rodehutscord et al. (2016) determined that genotype affect the physicochemical composition in several cereals, including corn. Thus, it was reported for 27 different corn hybrids a range from 78.1 to 112 g/kg DM for protein, from 660 to 783 g/kg DM for starch, from 18.8 to 20.7 MJ/kg MS for gross energy, and from 71 to 110 g/kg DM for neutral detergent fiber. These variations in the physicochemical composition could translate on the one hand into undetected imbalances in the final feed rations, and on the other hand, it is plausible to have negative nutritional interactions due to this nutrient variability, especially between protein and starch with fiber compounds, such as AX.

Therefore, **one the efforts of the present work will be to evaluate the magnitude of the nutrient variation due to the genetic background of corn, and use it as a model that allows detecting the possible negative interactions of some physicochemical components that contribute to limiting the availability of nutrients, with consequences on broiler chickens performance**.

3.2. Kernel Cob Position

The position of the grains on the cob affects by decreasing the grain size from the base to the top, and the size of the grain could affect the nutrient composition, as has been seen with phosphorus (Nadeem et al., 2014). In this way, it is logical to believe that the extreme apical grains could have less nutritional value, affecting the final nutrient composition of animal diets. However, little is known about the magnitude of this nutrient variation and even the proportion of grains that can be categorized under this cluster, due to the mix and merging of cultivars, varieties and qualities, and harvesting maturity in the same grain handling and storage.

Thus, **it is in our interest to study about the magnitude of this nutrient variability as well as the amount of the distribution of these grains in the cob in different corn hybrids**.

4. Feed Manufacturing Aspects

4.1. Particle Size

The hammer and roller grinders have traditionally been used to reduce the particle size of feed grains. Internationally, the type of milling used (hammer vs roller) includes Europe as the main user of hammer mills (Svihus et al., 2004) because diets are usually formulated with multiple cereals, such as barley, oats and wheat, and some cereal husks can cause inconvenience in roller mills (Vukmirović et al., 2017). On the other hand, roller mills are widespread in America and Asia and are the most common mill used in the US corn belt. Hammer grinding has typically created a wider particle size distribution, while roller mill grinding has created a more compact or narrow particle distribution (Nir et al., 1995). Therefore, the type of milling and their configuration will determine the structure, size and shape of the particles of the diets using to feed the broiler chickens. The distribution of the particles in the diets is an important factor as it could influence

several aspects of broiler gastrointestinal functionality and physiology (Celi et al., 2017). Traditionally, fine particles have been associated with larger relative surface area, resulting in greater enzyme activity in the gastrointestinal tract of poultry (Amerah et al., 2007a; Hetland et al., 2002). Consequently, although grinding represents a considerable cost in terms of energy consumption and feed mill capacity, cereals used for poultry are usually ground in a hammer mill fitted with a screen between 3 and 4.5 mm in size (Amerah et al., 2007a). However, the poultry have been reported to prefer larger feed particles (Schiffman, 1968) and to distinguish the differences in feed particle size by mechanical sensors located in the beak (Gentle, 1985). Furthermore, the most notable functional contribution of dietary coarse particles is to improve gizzard development in chickens, which directly influences intestinal motility (Duke et al., 1977; Ferket & Gernat, 2006). Feeding large corn particles has been reported to increase gizzard relative weight in several studies (Dahlke et al., 2003; Pacheco et al., 2013; Zang et al., 2009), and the gizzard has shown to be the key gastric organ for improved digestive efficiency (Xu et al., 2015). Thus, a well-developed gizzard reduced digesta pH and passage rate (Nir et al., 1994), enhanced enzymatic digestion efficiency, and improved energy utilization and nutrient digestibility (Amerah et al., 2007a). Overall, the particle size distribution of the final diets need to be adapted to the physiological capabilities of the birds in order to improve nutrient digestibility and animal performance, facilitate further technological processes, such as mixing, pelleting or extrusion/expansion (Kersten et al., 2005; Vukmirović et al., 2017), and improve the nutritional additives activity. Regarding this last aspect, it has been reported that the response of exogenous enzymes can be affected by PSD (Amerah et al., 2008a); however, the data published on this aspect are limited.

In this context, **it will be necessary to address a study with different PSD of corn-based broiler diets in order to evaluate the xylanase response on performance, digestibility and gut health.**

4.2. Physical Feed Form

Physical feed form has a great influence on feed intake and growth performance in broiler chickens (Dozier et al., 2010; Lv et al., 2015). The presentation of broilers feed include mash, crumble and pellet. Currently, the use of crumble and pellet diets is generalized due to the benefits on performance and feed efficiency (Jafarnejad et al., 2010; Nir et al., 1994). Crumble diets consist in crushing pellets in order to obtain a consistency coarser than mash. The use of crumble feed form is used in the starter period of broiler production since an increase in feed consumption *vs.* pelleted diets in young birds has been reported (Choi et al., 1986).

On the other hand, the pellet form nowadays is the principal feed form used in most stages of broiler production. The objective of pelleting is to agglomerate smaller feed particles into larger particles as pellets to enhance the economics of production by increasing the feed intake, and thus growth performance and feed efficiency (Amerah et al., 2007b). The structure, size and form of the cereal cell wall matrix obtained with different milling configurations may also produce different responses to the hydrothermal and pelleting processes (Amerah et al., 2008b), affecting digestion kinetics (Al-Rabadi et al., 2009; Mahasukhonthachat et al., 2010). The inclusion of coarse particles in poultry diets have showed to increase the size of the proximal gastrointestinal tract, principally gizzard, with a consequence improved growth performance (Zaefarian et al., 2016). It is clearly that pelleting process reduces particle size, however, it seems that beneficial influence of coarse particles could be obtained after pelleting. Additionally, it should be noted that the grain hardness and type of cereal used in the diet might have important

effect, being necessary to evaluate the effect of cereal type, feed form and particle size and their interactions on gut morphology and microbiota profile. On the other hand, it is commonly accepted that the coarse particles diets affects pellet durability and quality, this despite the fact that there are contradictory results in the bibliography (Reece et al., 1986; Thomas et al., 1998). The heat, moisture and mechanical pressure ("second grinding") applied during the pelleting, produced some feed chemical and physical alterations. In this way, the reduction of particles, the gelatinize starch, denaturation of proteins and cell wall breakage are described as possible effects (Amerah et al., 2007b), however, it is not clear how these changes could affect the nutritional additives response.

Therefore, **the use of pelleted diets as a factor affecting the particle size and its implications on the nutritional additives response (such as enzymes) becomes of interest in the present work**.

5. Final Justification Arguments and Hypothesis

The use of xylanase is extended in viscous cereal-based diets (wheat, rye, triticale, etc.), in order to control the viscosity associated to the soluble NSP. This negative effect is not shown in corn-based diets, due to the lower soluble NSP content. However, it exists preliminary evidence that the use of xylanase in corn-based poultry diets could also have benefits. Thus, Latham et al., (2016) reported that the use of xylanase in different corn batches increased early body weight (BW) and had the greatest impact on corn diets with the lowest AME values, including a decrease on feed conversion ratio (FCR) (1.65 vs. 1.62, g/g). Similarly, Kiarie et al., (2014) found an improvement in BW and FCR at 42 days in birds feed diets with xylanase independent of cereal source, thus, showing positive effects also in the corn diets when the xylanase was added (BW: 2113 vs. 2210, g; F:G: 1.81 vs. 1.75, g/g). Some of the suggested mechanisms include the increment of nutrient availability or xylo-oligosaccharides production, bringing an improvement on nutrient

digestibility, intestinal health and performance. In contrast, there are also studies that did not found performance differences when xylanase was supplemented in corn-based poultry diets (Gehring, Bedford, et al., 2013; Nian et al., 2011). These contradictory reports suggest a variable response of this enzyme due to different factors, being the feed-associated factors possibly the most important.

Overall, the present work tries to study the possible effects and interactions due to feeding factors that could affect the response of xylanase, as well as to improve the knowledge of its mechanisms of action in order to maximize the benefits obtained from its supplementation.

In this way, it will be necessary to develop some studies to test the following hypotheses:

- The suggested mechanisms of action of carbohydrases, especially xylanase, not only improve the digestibility of nutrients but also improve intestinal health and modulation of the microbiota, thus allowing them to be considered as part of the replacement scheme for the use of antibiotics such as growth promoters in animal production.
- The variability of the physicochemical composition of corn generates negative interactions between macronutrients and fiber components, and these changes could be one of the main factors affecting the xylanase response.
- The variability in the corn physicochemical composition provided by the genetic background and kernel size could generate lacks in digestibility and performance of broilers.
- The technological process of feed produces changes in the kinetics of nutrients and in the animal digestive tract that affect the efficiency response of xylanase in corn-based poultry diets.

CHAPTER II

OBJECTIVES

The present work aims to evaluate the effect of the feed-related factors, including corn nutrient composition and particle size distribution on the response of xylanase supplementation in performance, nutrient utilization and intestinal health of broilers fed with corn-based diets, as well as to evaluate the postulated mechanisms of xylanase action in corn-based diets.

To achieve this objective, the following specific objectives and the corresponding tests are proposed:

- To explore published reports on the opportunity for feed enzymes, especially carbohydrases, to form part of a multidisciplinary antibiotic's displacement strategy. Thus, the **Article I** is a literature review performed in order to compare the xylanase suggested mechanisms in the corn-based diets (nutrient digestibility, microbiota modulation and improvement of intestinal health) with the antibiotic effects when are used as growth promotors in animal diets (***Melo-Duran et al., 2019***).
- To investigate the magnitude of variability in nutrient composition, physical characteristics and NSP content due to corn genotype and kernel cob position, and how this variability affect the response of xylanase supplementation. Therefore, the **Article II** is an "in vitro" carried out using the corn genetic background and kernel cob position as source of nutrient variability and tested the xylanase response. Considering the mains suggested mechanisms of xylanase action, the release of encapsulated nutrient and xylo-oligosaccharides production will be evaluated after "in vitro" xylanase supplementation (***Melo-Durán et al., 2021a***).
- To determine the effects of nutrient variation associated to the genotype on broiler performance and nutrient utilization. Thus, the **Article III** will be an in vivo trial development in order to study how the variability of physicochemical composition

of corn, due to genetic background, could produce negative interactions among the different physicochemical components, and consequently changes in broiler chicken's growth performance and nutrient digestibility (***Melo-Duran et al., 2021b***).

- To determine the effects of corn particle size distribution and xylanase supplementation in performance and intestinal health of broiler chickens fed with corn-based pelleted diets. In this way, the **Article IV** will involve the manufacturing feed factors; thus, the xylanase response will be evaluated in different particle size distributions of corn-based pelleted diets. The animal performance, nutrient digestibility, microbiota modulation and short chain fatty acids will be evaluated to understand the possible effects of the xylanase and their interaction with the particle size (***Melo-Duran et al., 2020***).

CHAPTER III

PUBLISHED ARTICLES

ARTICLE I

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View of enzymes as an alternative to antibiotics

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Abstract

The animal industry still uses antibiotics to prevent disease and improve the yield of animal production in challenging conditions. However, the emerging public health crisis in relation to antibiotic resistance, due to the possibility of these bacteria being transferred to other animals and humans (farmers, veterinarians and consumers), has stimulated efforts to end the use of prophylactics in feed antibiotics. This review summarizes the current developments and perspectives regarding the use of the enzymes as an alternative to antibiotics, with a specific focus on carbohydrases. It is difficult to conclude that enzymes per se could replace antibiotics in efficient animal production. Nevertheless, it is important to recognize their role as a fundamental part of a multidisciplinary displacement intervention strategy that integrates a mixture of different additives and more efficient management programs. Positive effects of carbohydrase enzymes on performance are normally associated with increases in nutrient digestibility because of the release of encapsulated nutrients, as well as hydrolysis or partial hydrolysis of non-starch polysaccharides. There also seems to be an influence on the environmental physico-chemical conditions in digesta and the composition of the microbiota in the digestive tract.

Keywords: antibiotic resistance, carbohydrases, gut health.

20.1 Introduction

The feed industry has been using enzymes as zootechnical additives for swine and poultry for the last three decades. The main objective for doing so is the accepted view that that they may improve the productive value of diets by increasing nutritive content and boosting animal performance, with the consequent reduction in feed costs and environmental impact. Nowadays, phytase is the most widely used in poultry and pig diets, followed by carbohydrases, with xylanases and glucanases being the two dominant enzymes.

Carbohydrases improve intestinal digestion in monogastric animals via several mechanisms, as described in previous Chapters of this conference: 1.- by disrupting anti-nutritional factors that are contained in some ingredients, e.g. the viscous soluble non-starch polysaccharides (NSP) in wheat, barley or rye, (Bedford, 2002); 2.- by hydrolysing water insoluble NSP in cell wall polysaccharides to eliminate nutrient encapsulation, such as starch or protein in maize (O'Neill *et al.*, 2014); 3.- by releasing small oligosaccharide molecules for more effective foregut and hindgut fermentation, and the modulation of intestinal microbiota (Bedford and Cowieson, 2012); and 4.- by reducing the flow and loss of endogenous nutrients, including amino acids, contained in intestinal mucins (Cowieson and Kluenter, 2019).

Therefore, the use of xylanases has led to an increase in the dietary levels of those ingredients with antinutritional components interfering with the digestive function, for example wheat and barley in poultry diets. Limitations on in-feed incorporation of wheat (established at maximum recommended values of 20-30%) in poultry diets may be removed when xylanase is provided, while barley may be raised from 3-10% to 30-40% in broiler diets with in-feed xylanases and β-glucanases (Fedna, 2010). Moreover, the recent price volatility of traditional feed ingredients and the quest for sustainable agriculture suggest that the swine and poultry industries will also try to use feed ingredient by-products, such as those derived from the biofuel and milling industries. The high NSP and indigestible protein content of these ingredients may also limit their inclusion rate and present new challenges to the monogastric digestive tract and the feed enzymes industry.

Through their actions on the physico-chemical properties of digesta and the availability of substrates for fermentation, feed enzymes may also affect the intestinal microbiota. Carbohydrases may have a positive impact on the gut function under specific conditions, with a likely role being the prevention of enteric infections.

The present Chapter will try to explore published reports on the opportunity for feed enzymes to form part of a multidisciplinary antibiotics displacement strategy, either in experimentally challenging or commercial conditions.

20.2 Antimicrobial uses in the swine and poultry industries

The discovery of antibiotics was one of the most relevant events for human medicine. Their availability from the twentieth century has made it possible to treat and prevent infections in humans and animals. However, massive use of antibiotics is leading to an increasing prevalence of resistance to the same among some pathogens, which poses a risk on their efficacy in the future. In particular, antimicrobials are used for the treatment of infectious diseases in animals, either for curative purposes, metaphylaxis (treatment of healthy animals kept in close contact with diseased animals) and prophylaxis (treatment of healthy animals when the probability of becoming sick is high). Antimicrobials were also used as growth promoters at sub-therapeutic doses from the 1940's and largely contributed to the rapid expansion of the swine and poultry industries worldwide (Dibner and Richards, 2005; Niewold, 2007). The mechanism is still unclear (Lin *et al.*, 2013), but it is accepted that antibiotics may act to modify intestinal microbiota to control gastrointestinal pathogens. They may also provide an optimal equilibrium between the microbiota and the host to improve digestion (Gaskins *et al.*, 2002; Roth and Kirchgessner, 1993), immunity (Costa *et al.*, 2011; Schoevers *et al.*, 1999) and growth (Gaskins *et al.*, 2002). The intestinal microbiome has the potential to promote either beneficial or harmful effects on the host animal, and the use of antibiotics as a growth promoter could improve this equilibrium and thus enhance animal productivity (Cowieson and Kluenter, 2019).

A large number of studies have analysed the beneficial effects of antibiotics on animal performance in challenging and non-challenging conditions (Table 20.1). Results show that growth conditions can alter the impact of antibiotics on microbiota composition and performance. In-feed antibiotics improve animal weight gain and feed efficiency in challenging conditions, and reduce pathogen counts, morbidity and mortality (Gaskins *et al.*, 2002).

Table 20.1. Effects of antibiotics in performance and gut health parameters of chickens and piglets.

Species (study length, d)	Diet	Challenge	Antibiotic	Performance[1]	Gut health parameters[2]	Reference
Chicken (28 d)	Wheat-sorghum	*Eimeria* spp. + *C. perfringens*	Zn-bacitracin, 845 ppm + Monensin, 100 ppm	↑BW ↓FCR ↓Mortality	↓Intestinal lesion scores ↓*C. perfringens*.	(Geier *et al.*, 2010)
Chicken (38 d)	Corn	E. *coli*	Virgamicin, 11 ppm	↑FCR (35d)	↓*Lactobacilli* ↓*Bifidobacterium* (cecum)	(Baurhoo *et al.*, 2007)
Chicken (21 d)	Wheat-Oats	*C. perfringens*	Zn-bacitracin	↑BW	↓Intestinal lesion scores ↓*C. perfringens.*	(Ao *et al.*, 2012)
Chicken (30 d)	Corn	*C. perfringens*	Enramycin, 0.1 g/kg	↓FCR ↑Dressed yield	↑ Villus height (duodenum and jejunum; d 30)	(Abudabus, 2012)
Chicken (43 d)	Corn	*Eimeria spp.* + *C. perfringens*	Enramycin (Starter: 125 ppm; Grower: 100 ppm; Finisher: 62 ppm)	↑BW ↓FCR	-	(Pereira *et al.*, 2015)
Chicken (28 d)	Corn	*E. coli*	Colistin sulfate, 20 ppm	↑BW and ↑ADG	↑Digestive enzyme activities	(Zhang *et al.*, 2016)
Chicken (10 d)	Wheat	-	Zinc bacitracin, 50 ppm Avilamycin, 15 ppm	↓FCR -	↑ Microbiota diversity (cecum) -	(Crisol-Martínez *et al.*, 2017)
Chicken (28 d)	Corn	*C. perfringens*	Enramycin, 10 ppm	-	↓Histology score lesion (ileum) ↓Lamina propia thickness ↓ Inflammatory cell infiltration.	(Belote *et al.*, 2018)
Weaned piglet (28 d)	Corn	-	Chlortetracycline, 55 ppm + Virginiamycin, 27.5 ppm	↑ ADG	-	(Stahly *et al.*, 1980)
Weaned piglet (-)	Corn	*E. coli F18*	Colicin E1, 16.5 ppm	↑ADG ↓Faecal score	↓ *Coliforms* and *E. coli* (ileum and faeces) ↓ TNFβ- mRNA ileal tissue	(Cutler *et al.*, 2007)
Weaned piglet	Corn	-	Chlortetracycline, 0.1%	↑AFI ↑ADG ↓FCR	↓ *Clostridium* spp. (faeces)	(Choi *et al.*, 2011)

(28 d)						
Weaned piglet (28 d)	Corn	-	Lincomycin, 110 ppm	↑AFI ↑ADG	↓ *Coliforms* (faeces)	(Namkung *et al.*, 2011)
Weaned piglet (28 d)	Corn	-	Chlortetracycline, 100 ppm	↑BW ↑ADG ↑AFI ↓FCR ↓ Diarrhoea rate	↓ *E. coli* (duodenum. jejunum, cecum)	(Wang *et al.*, 2012)
Weaned piglet (14 d)	Corn	-	Chlortetracycline, 75 ppm	↑ADG ↓FCR ↓Faecal score	↓*Clostridium* and *E. coli* (ileum, colon) ↓ Intestinal permeability ↓ IL-6 and TNF-α (ileal mucosa)	(Song *et al.*, 2013)
Weaned piglet (12 d)	Corn	*E. coli K88*	Colistin sulfate, 50 ppm + Zinc bacitracin, 100 ppm + Olaquindox, 100 ppm	↑ ADG ↑ADFI ↓Diarrhoea rate	↑ IgA mucosal (ileum) ↓ Intestinal permeability ↑ Villus height: Crypt depth ratio (duodenum, ileum).	(Pan *et al.*, 2017)
Weaned piglet (28 d)	Corn	-	Chlortetracycline, 300 ppm + Colistin sulphate, 60 ppm	↑ ADG	↑ Villus height (duodenum,) ↑TGF-β ↓IFN-γ ↑ Microbial richness and diversity (ileum). ↑ Spirochaetes, Tenericutes, Euryarchaeota, Verrucomicrobia ↓Chlamydiae (ileum)	(Yu *et al.*, 2017; Zhu *et al.*, 2017)

[1] Body weight = BW; Feed conversion ratio = FCR; Average daily gain = ADG; Average daily feed intake = ADFI [2] Tumor necrosis factor β = TNFβ; Interleukin 6 = IL-6; Tumor necrosis factor α = TNFα; Transforming growth factor β = TGF-β; Interferon gamma = IFN-γ.

The main theories attempting to explain their mechanisms suggest that antimicrobials may reduce the energetic cost of the digestive tract by reducing the activity of gastrointestinal microbiota, and limiting nutrient losses due to inflammatory response (Collier *et al.*, 2003). They may also modify intestinal microbiota populations towards a bacterial community that is prone to stimulating animal growth (Dibner and Richards, 2005). Antimicrobials show higher responses and functionality in animal groups with poor performance and in diets with compromised nutrient values (Cowieson and Kluenter, 2019).

Unravelling the mechanisms of how antibiotics might influence growth performance through changes in gut microbiota would help to identify alternative high-productivity strategies in poultry and swine diets. With this objective in mind, Crisol-Martinez *et al.* (2017) showed that two antibiotics (avilamycin and zinc bacitracin) produced different results in growth performance and caecal microbiota composition of chickens. In contrast to avilamycin, zinc bacitracin improved feed efficiency, as also shown by Ao and Choct (2013). Avilamycin reduced the abundance of two taxa, *Catabacteriaceae* and a phylo-type closest to *Clostridium spiroforme* (93%) in the caecal microbiota. The former has been found to be in higher abundance in chickens showing poor growth performance (Stanley *et al.*, 2016). The latter taxon is a pathogenic bacterium that tends to inflame the gastrointestinal tract (Stiles *et al.*, 2014), inducing diarrhoea and colitis in some animals (Borriello and Carman, 1983). Zinc bacitracin showed a much greater impact on gut microbiota composition and diversity than avilamycin (Crisol-Martínez *et al.*, 2017). Zinc bacitracin promoted a lower abundance of the highly dominant genus *Lactobacillus,* which was linked with improved growth performance (Torok *et al.*, 2011). It has been suggested that *Lactobacillus* is a genus associated with the production of the bile salt hydrolase enzyme (Begley *et al.*, 2006; Ridlon *et al.*, 2006). Reduced numbers of lactobacilli in antibiotic-treated chickens can reduce bile salt hydrolase enzyme activity, increasing the abundance of conjugated bile salts and ultimately leading to increased energy digestibility and growth performance (Lin *et al.*, 2013). In this respect, there is currently discussion as to whether lactobacilli are truly beneficial or not. In addition, other genera, such as *Bacteroides* spp., were also associated with improved growth in chickens and considered effective degraders of carbohydrates (Collier *et al.*, 2003; Stanley *et al.*, 2013).

20.3 The way towards a drug free program

In 1996, the European Union (EU) established restrictions on the use of antibiotics as growth promoters (Directive 96/23/EC; European Commission, 1996). Additionally, the Scientific Steering Committee stated in its opinion of 28 May 1999 that: '*the use of antibiotic agents from classes which are or may be used in human or veterinary medicine (i.e. where there is a risk of selecting for cross-resistance to drugs used to treat bacterial infections) should be phased out as soon as possible and ultimately abolished'*. Seven years later, the use of antibiotics as growth promoters was completely banned (Regulation Nº1831/2003).

In this context, the EU authorities called for the development of alternative substances and methods of farm management, feeding, hygiene, etc., to replace the need for those antibiotics. The latest report on the sales of veterinary antibiotics for 2016 (European Surveillance of Veterinary Antimicrobial Consumption, ESVAC), published in October 2018, shows that sales of antibiotics for use in animals in Europe fell was by 20% between 2011 and 2016. Furthermore, institutions like Health Canada and the United States Food and Drug Administration have also called for voluntary withdrawal of antibiotic growth promoters (AGPs) in livestock animals (Kuehn, 2014). However, antimicrobials are still widely used in food producing animals when veterinarians prescribe them for therapeutic uses. According to

the (EMA ESVAC, 2018), across the 30 included European countries, oral formulations accounted for 90.1% of all antimicrobial use in food-producing animals (mg/PCU). Most classes are available as a premix (40.8%, mostly used for systemic prevention), oral powder, and oral solutions for drinking water to treat acute disease outbreaks. Antimicrobial premixes and preventive or metaphylactic uses in large groups of pigs and poultry were the main forms of use in those countries where antimicrobial consumption remained high. However, oral formulations lead to high exposure of the gastrointestinal microbiome to antimicrobials, which is of particular concern in terms of a potential source of resistant bacteria.

Some EU Member States have implemented reduction strategies successfully and with favourable results, especially in northern Europe. These strategies include national reduction targets, benchmarking of antimicrobial consumption, restrictions on the use of specific critically important antimicrobials, together with improvements to animal management procedures and husbandry to reduce inherent disease risk. Other actions include increasing the responsibility placed on veterinarians when prescribing antimicrobials, controlling group treatments, especially premixes for preventive and metaphylactic antimicrobial use, and a requirement for antimicrobial susceptibility testing prior to use of high priority critically important antimicrobials.

There is also increasing worldwide interest in animal production schemes based on low or antibiotic-free exposure. This scenario represents a greater challenge in terms of subsequent putative problems for the swine and poultry industries. Hence, there is a growing need to find effective alternatives to control infectious diseases in the animal industry, including enhancing the ability of animals to cope with pathogens. Possible alternatives include probiotics and prebiotics, competitive exclusion, bacteriophages, immunomodulators and organic acids, but also feed enzymes.

In some countries, a reduction in the use of antimicrobials is happening alongside an increase in the use of zinc oxide, especially for post-weaning piglets, which has also prompted concerns about the environmental impact as well as the potential for co-selection of resistant bacteria. In poultry, a prospective study involving 1.55 million birds was conducted on eight commercial broiler farms in Quebec (Canada) to evaluate the impact of the removal of antibiotic growth promoters and anticoccidial drugs by a drug-free program including improved brooding conditions, anticoccidial vaccination, essential oil-based feed additives, and water acidification (Gaucher *et al.*, 2015). Their results showed that it is possible to raise broiler chickens using a drug-free program in the Canadian production system. However, the new alternative program was associated with reduced performance, as well as with a higher risk of experiencing gut health problems such as clinical necrotic enteritis (NE) and subclinical enteritis for certain farms.

The joint European Food Safety Authority/European Medicines Authority also reviewed published information available on specific measures applied by the European Member States (MSs), including circumstances and diseases demanding the use of antimicrobials, such as:

- Broilers, for coccidiosis, necrotic enteritis, dysbacteriosis
- Laying hens, for enteritis caused by *Escherichia coli*, avian intestinal spirochaetosis
- Weaner piglets, for diarrhoea, postweaning colibacillosis
- Growing pigs, for proliferative enteropathy by *Lawsonia intracellularis*, swine dysentery, ileitis, *Salmonella* spp.

20.4 Digestive diseases in poultry: a likely preventive role for feed enzymes

The digestive tract is a major site for potential exposure to pathogens. The lumen normally contains feed and its constituents, resident and transient microbial populations, endogenous nutrients, and secretions from the gastrointestinal (GI) tract. The GI tract acts as a selective barrier between the host tissues and its luminal environment. Some compounds may contribute to intestinal integrity (e.g. butyrate). However, a wide range of factors associated with diet, the presence of pathogens, the environment, and management can negatively affect the delicate equilibrium among the components of the digestive function, and subsequently impair growth rate and feed efficiency (Celi *et al.*, 2017).

Feed enzymes contribute to digestion and may modulate microbiota populations, either through changes in the physico-chemical properties of digesta or by modulating the availability of substrates for intestinal fermentation. More detailed information on these effects and their mechanisms have been largely described in previous Chapters of this meeting. The present review briefly explores the likely role of feed enzymes in helping the digestive function and with the prevention of diseases, and their likely contribution to effective removal of prophylactics and therapeutics from feed antibiotics on swine and poultry farms. Therefore, it will only focus on those pathogens or disorders that mostly require the use of antimicrobials to treat or prevent GI disorders.

Dysbacteriosis is a non-specific enteritis following a disturbance in the equilibrium of the gut microbiota, similar to small intestinal bacterial overgrowth in human medicine (Abu-Shanab and Quigley, 2010). In broilers, dysbacteriosis and necrotic enteritis are still a problem and major indications for group antimicrobial treatment (Persoons *et al.*, 2012). Other factors linked to antimicrobial uses are wet litter, the use of a live vaccine against infectious bursal disease (involving immunosuppression), and addition of finely ground wheat to feed, which may predispose to necrotic enteritis outbreaks (Annett *et al.*, 2002). Incorporating a high level of dried distillers grains (DDGS) in the diet of broiler chickens may also increase susceptibility to NE (Barekatain *et al.*, 2013). On the other hand, whole or coarsely ground wheat or maize can be protective (M'Sadeq *et al.*, 2015).

Rosen (2003) suggest that exogenous enzymes are the best-characterised prospects to replace AGPs, after a review that compared between the AGPs and the most important groups of additives including enzymes, prebiotics, probiotics, acids and phytobiotics. Although, there are no published studies comparing enzymes to antimicrobials in challenging conditions, the primary aim of the present review was to select studies on the efficacy of the enzyme on performance and health parameters (e.g. reduced morbidity or mortality). Table 20.2 summarises the results of peer-reviewed published articles on the action of *in-feed* carbohydrases in challenging conditions. Most reports describe experimental trials with diets containing wheat and/or barley as challenging conditions. Arabinoxylans (AX) are a major antinutritional factor of wheat for monogastric animals, especially broilers. It cannot be digested in the small intestine and generates a viscous chyme (Choct, 1999), which leads to gut health problems, greater incidence of NE than corn-based diets (Jia *et al.*, 2009) and intestinal inflammation, decreased growth performance, and detrimental litter conditions caused by sticky droppings.

Dietary supplementation of xylanase for wheat based feeds has become a routine approach in the poultry industry. In theory, xylanases could also be a good candidate for antibiotic replacement. Xylanase increases growth performance (Zhang *et al.*, 2014), inhibits pathogens

in the gut (Józefiak *et al.*, 2007; Liu and Kim, 2016), and alleviates the impairment of intestinal mucosa barrier induced by a *Clostridium perfringens* challenge (Liu *et al.*, 2012). Studies have demonstrated that xylanase reduces the viscosity of feedstuffs and increases fat digestibility, especially in young animals fed on barley or wheat, and saturated fat sources (Ouhida *et al.*, 2000).

AX consist of water-extractable and water-unextractable polymers, which can form covalent and noncovalent interactions with other dietary components. Therefore, complete enzymatic degradation of AX, particularly of the water-unextractable chains, requires the synergistic effects of endoxylanase in combination with debranching enzymes, such as arabinofuranosidase and ferulic acid esterase, which enables xylanase to access the AX backbone (Lei *et al.*, 2016). Therefore, the simultaneous supplementation of side-chain cleaving enzymes and xylanase reduces viscosity more efficiently than xylanase alone (Lei *et al.*, 2016).

Bedford and Cowieson (2012) also showed that xylanase in wheat-based diets may result in an increased concentration of short-chain xylo-oligosacharides (XOS) in the cecum of broilers. Results from an *in vitro* fermentation trial showed that AX hydrolysate exerted proliferative effects of *Bacillus subtilis* and *Lactobacillus brevis*, with significant increases in the amount of unbranched short-chain fatty acids (SCFAs) and reductions in the digesta pH. It was shown that these bacteria become metabolically more active, showing numerous nutritional benefits for the host, including an increased level of extracellular enzymes, such as proteases, lipases, celluloses, xylanases and phytases (Sen *et al.*, 2012). An increase in the levels of colonic or caeca *Bifidobacterium* spp. has been also reported in several studies using XOS in rats or in mice (Campbell *et al.*, 1997; Hsu *et al.*, 2004; Santos *et al.*, 2006). This increase was accompanied by significant decreases in counts of *Enterobacteriaceae* and/or sulphite-reducing bacteria. In contrast to AX hydrolysates, the fermentation of fructooligosaccharides generated more branched SCFAs (Lei *et al.*, 2016), which were likely to have been released from protein fermentation. Therefore, xylanase appears to show positive results in viscous as well as in non-viscous diets. In the latter, xylanase activity may provide small products (i.e.- XOS) that remain largely unabsorbed in the small intestine and show a prebiotic and growth promoting effect in poultry by selectively stimulating the growth and/or activity of a limited number of bacteria in the hindgut (Munyaka *et al.*, 2016; Ribeiro *et al.*, 2018).

Different studies describe the prebiotic concept in poultry for different challenging conditions. Thus, the mannan and xylooligosaccharides might modulate caecal microbiota in *Salmonella enteritidis* challenged chickens (Eeckhaut *et al.*, 2008; Pourabedin *et al.*, 2017). XOS stimulated the growth of *Lactobacilli* and butyrate-producing *Clostridium* cluster XIV (De Maesschalck *et al.*, 2015; Yacoubi *et al.*, 2018). Additionally, xylanase inclusion in broiler diets have showed improved the butyrate production (González-Ortiz *et al.*, 2016; Lee *et al.*, 2017; Masey-O'neill *et al.*, 2014). Butyrate is known to provide energy to the epithelial cells, and exerts anti-inflammatory properties by inhibiting nuclear factor kappa B transcriptional activity (Segain *et al.*, 2000) and T-lymphocyte infiltration in the intestinal mucosa (Yacoubi *et al.*, 2018). In addition, butyric acid increases mucosal barrier function by increasing the production of mucin and antimicrobial peptides (Sunkara *et al.*, 2011), and prevents necrotic enteritis from pathogenic infection (Timbermont *et al.*, 2010). Supplementation of the diet of laying hens (Ding *et al.*, 2018) with XOS enhanced intestinal health (increased villus height and VH:CD ratio), and immune function (increased contents of IgA, TNF-α, IgM and IL-2). Yan *et al.* (2017) has shown that a rye-wheat based diet containing feather meal in addition to a mild *Eimeria* challenge induced subclinical enteritis in broilers. However, a carbohydrase was able to counteract these effects by reducing digesta viscosity and ileal *C. perfringens* counts.

Table 20.2. Effects of enzymes in performance and gut health of chickens challenged.

Diet (study length, d)	Challenge	Type of Enzymes	Response for enzymes vs. positive control[1]	Combination products	Response for combination vs. positive control[2]	Reference
Wheat (33 d)	*Campylobacter jejuni* (nalidixic acid resistance) (5d)	Xylanase, 0.1%	↓Jejunal viscosity ↓*C. jejuni* caecal count ↑Neutral and sulphated mucins in goblet cells	-	-	(Fernandez *et al.*, 2000)
Wheat (42 d)	*S. typhimurium*	Xylanase, 16,000 BXU	↑ BW ↓FCR ↑ADG ↓*Salmonella* contamination	*L. plantarum* (10^6cfu/g)	↑BW ↓FCR ↑ADG ↓*Salmonella* contamination	(Vandeplas *et al.*, 2009)
Wheat (39 d)	*C. perfringens* (13d)	Multicarbohydrase enzyme: (Cellulose, 60 U; Pectinase, 1,400 U; Xylanase, 1,200 U; Glucanase, 800 U; Mannanase, 500 U; Galactanase, 30 U).	↓FCR (13 d)	-	-	(Jia *et al.*, 2009)
Corn (39 d)			↑ BW ↑ FI and ↓FCR (13 d) ↑ADG	-	-	
Wheat (21 d)	*C. perfringens*	Xylanase, 5,500 U/kg	↓Intestinal lesion score ↑Villus height/crypt depth ratio in jejunum ↓Plasma endotoxin levels ↑Occludin mRNA expression	-	-	(Liu *et al.*, 2012)

Wheat-Maize (42 d)	*S. heidelberg*	Xylanase, 2,000 U/kg	↑WG ↓FCR ↓ Horizontal transmission	Cinnamaldehyde and thymol (100g/t)	↑WG ↓FCR ↓Horizontal transmission	(Amerah *et al.*, 2012)
Wheat (21 d)	*C. perfringens*	Xylanase, 5,500 BXU	↑AME and CP digestibility ↑Digestive enzyme activities ↑mRNA nutrient transporters	-	-	(Guo *et al.*, 2014)
Wheat (35 d)	*C. perfringens*	Multi-enzyme complex, 500 ppm: (Xylanase, 2´424,000 IU; Glucanase, 506,600 IU; Manase, 322,800 IU)	↑FI ↓FCR ↓SIgA ↑Villus:crypt ratio ↓Endotoxin plasma	Thymol (25%) Carvacrol (37%) (60 mg/kg)	Non interaction	(Sun *et al.*, 2015)
Wheat (21 d)	*C. perfringens*	Xylanase, 200 ppm	↓ FCR ↑AME and CP digestibility ↑mRNA nutrient transporters	Citric acid 30 mg/kg	Non interaction	(Hosseini *et al.*, 2016)
Wheat (21 d)	*Eimeria* oocysts (9d) + *C. perfringens* (14d)	-	↑CP digestibility -	Arabinoxylans (2%) Xylanase (16000 BXU)	↓FCR ↑SCFAs	(Keerqin *et al.*, 2017)

[1-2] Body weight = BW; Feed conversion ratio = FCR; Average daily gain = ADG; Feed intake = FI; WG= weight gain; Apparent metabolizable energy = AME; Crude protein = CP; Secretory immunoglobulin A = SIgA; Short-chain fatty acids = SCFAs

20.5 Digestive diseases in swine; the likely role of feed enzymes

Performance of piglets in nurseries may be variable. Average mortality is approximately 4.8% (but may range widely from 2.8% to 6.9% or higher) with average feed-to-gain ratios from 1.53 to 1.74 (SIP-consultors 2018, personal communication). There are many factors affecting performance in nurseries, including the sanitary and welfare status of the farm (single vs multiple animal origin, high vs low quality water supply, and low vs high density). Among the several factors triggering diarrhoea in pigs, enterotoxigenic strains of *E. coli* is a major one (Williams *et al.*, 2001).

High protein diets increase luminal concentration and epithelial exposure to putatively toxic metabolites (such as ammonia, hydrogen sulphide and biogenic amines) and increase the risk of post-weaning diarrhoea. Thus, the use of diets with low levels of dietary protein provided with high-quality protein sources may help to reduce the risk of intestinal disease in young pigs (Pieper *et al.*, 2016). Dietary protein reduction decreases the indigestible nitrogen fraction while amino acid imbalance and excess is avoided.

With feedstuffs of lower quality, more undigested dietary proteins will enter the hindgut, promoting putrefactive fermentation bacteria. In plant ingredients, the structural properties of proteins may play a major role in resistance to denaturation and gastrointestinal digestion. For example, the β-sheet structures of raw legume proteins and the intermolecular β-sheet aggregates, arising upon heating, were highly negatively correlated with feed digestibility values ($r = -0.980$) (Carbonaro *et al.*, 2012). Lower protein digestibility with β-conformations relates to the highly hydrophobic character of these structures. Moreover, complex interactions between cell wall carbohydrates and proteins pose resistance to the intestinal digestion of vegetable ingredients. Thus, carbohydrase enzyme supplementation may provide a strategy to degrade the covalent unions between water insoluble carbohydrates and protein and facilitate protein digestibility. The main effects of enzymes on the performance and gut health of pigs are summarized in Table 20.3.

As described for poultry, changes in the intestinal environment, including viscosity and substrate fermentation, may also modulate the progression or expression of infectious intestinal disease (Hopwood *et al.*, 2004a; Pluske *et al.*, 2003). However, the relevance of viscosity on the intestinal contents of pigs appears minor in relation to poultry nutrition and gut function, and the effects of dietary carbohydrase are less consistent (Torres-Pitarch *et al.*, 2017). Hopwood *et al.* (2004) reported that pearl barley altered the intestinal microenvironment (higher viscosity) and predisposed to post-weaning colibacillosis in piglets. However, enzyme addition (enzyme mix contained β-glucanase (250 units/g), xylanase (400 units/g) and a-amylase (1000 units/g)) failed to decrease intestinal viscosity, and tended to maintain or increase the proliferation of enterotoxigenic *E. coli*.

Recently, Zhang *et al.* (2014) assessed the effects of dietary supplementation with an exogenous multi-enzyme preparation in 35- to 65-d-old piglets on apparent total tract digestibility (ATTD), growth performance, digestive enzyme activities, and selected microbial populations in faeces. Inclusion of the multi-enzyme preparation in the diet increased the counts of *Lactobacillus* spp. and *Bacillus subtilis*, and reduced the numbers of *Salmonella* spp. and *E. coli* in faeces. The results coincided with enhanced activity of amylase, lipase and protease in the small intestine, and with a tendency for higher average daily feed intake (ADFI) and average daily gain (ADG).

Table 20.3. Effects of enzymes in performance and gut health of pigs.

Phase (study length, d)	Diet	Type of Enzymes	Performance[1]	Gut health[2]	Reference
Growing (27 d)	Wheat-barley-rye	NSP-degrading enzymes, 0.01% (Xylanase, 22,000 U/g; β-Glucanase, 2,000 U/g)	↑BW ↑ADG	↑Villus: crypt ratio	(Willamil *et al.*, 2012)
Post-weaning (28 d)	Corn	Multi-enzyme complex, 150 ppm: (Amylase, 2,000 U/g; Protease, 40,000 U/g ; Xylanase, 20,000 U/g)	↑ADG ↓ FCR	↑Acetic, propionic and butyric acid (cecum and colon), ↑ *Lactobacilli* (cecum), ↓ *E. coli* (colon), ↑ Digestibility GE	(Yi *et al.*, 2013)
Post-weaning (10 d)	Corn	Xylanase, 700 LXU/kg	-	↓Digesta viscosity (jejunum) ↑ AID of DM, OM	(Passos *et al.*, 2015)
Post-weaning (14 d)	Corn	Protease, 200 and 300 ppm	↑BW ↑ADG ↑ADFI↓ FCR	↑Villus height ↑Villus: crypt ratio (duodenum, jejunum, ileum) ↑CP digestibility, ↑ Pancreatic, amylase, trypsin pepsin activity (stomach), ↓Diarrhoea index	(Zuo *et al.*, 2015)
Post-weaning to Finishing (145 d)	Corn	Xylanase, 9,000 U/kg	↓Mortality ↓ FCR	-	(Zier-Rush *et al.*, 2016)
Post-weaning (42 d)	Corn	Xylanase, 0.010%	↑ADG ↓ FCR	↑*Lactobacilli* counts (faecal) ↓ Faecal score, ↑ ATTD of DM, GE, N, ↓Blood urea nitrogen ↓NH_3 and H_2S emissions	(Lan *et al.*, 2017)
Post-weaning (28 d)	Wheat-corn	Xylanase, 100 - 2,000 U/kg	↑ADG	↑ ATTD DM, CP, NDF, ADF, Ca, P. ↓ *Lachnospiraceae* (50%) ↑*Prevotellaceae* (175%)	(Dong *et al.*, 2018)

[1] Body weight = BW; Feed conversion ratio = FCR; Average daily gain = ADG; Average daily feed intake = ADFI

[2] Gross energy = GE; Apparent ileal digestibility = AID; Dry matter = DM; Organic matter = OM; Crude protein = CP; Apparent total tract digestibility = ATTD; NDF = Neutral detergent fiber; ADF= Acid detergent fiber

Xylanase supplementation of a wheat-corn-soybean diet (including 10% wheat bran) for weanling pigs also increased growth performance and nutrient digestibility, when the enzyme was supplemented at 2,000 U/kg (Dong *et al.*, 2018). Productive changes with xylanase were associated with diminished growth of harmful pathogenic bacteria, such as *Escherichia* spp. and *Shigella* spp. in the colon, and a significant increase in phylum Bacteroidetes. Similarly, Jiang *et al.*, (2015) showed that an enzyme combination (xylanase and β-glucanase) increased the villus-to-crypt ratio and reduced the number of mucosal macrophages in the ileum compared with the control group. Dietary enzymes also reduced faecal *Lactobacillus* spp. and *E. coli* counts but increased the Lactobacillus-to-coliforms ratio.

Due to the growing interest in the swine industry in feeding fibrous coproducts, the use of exogenous carbohydrases has received increasing interest. Li *et al.* (2018) evaluated the impact of dietary xylanase and/or a carbohydrase enzyme blend in weaned pigs fed high fibre diets (including wheat middlings and DDGS from 5 to 10%). The authors reported that a combination of multiple enzymes (containing cellulase, β-glucanase, and xylanase) was effective for improving the growth performance of nursery pigs. However, enzymes did not affect the ATTD. The authors then suggested the likely role of other mechanisms, such as improving intestinal barrier function. The multiple carbohydrase combination also improved small intestinal barrier integrity, and decreased immune activation (lower IgA, and ileal IL-22 mRNA abundance).

20.6 Conclusions

It has been suggested that through a better understanding of dysbacteriosis and its management, antimicrobial consumption for gastrointestinal diseases in young animals could be reduced, e.g. by improving nutrient digestibility and modulating the intestinal microbiota. However, a limited number of reports on carbohydrase as an alternative to antimicrobials are available in the current literature, the number of reports being higher in poultry than in pigs.

The experience with antibiotic use in animal production clearly shows an improvement in weight gain, meat quality and feed efficiency by 1-10%, with the highest effects in challenging conditions and poor performance groups. The main mechanism behind antibiotics is dysbacteriosis control, and the modulation of microbiota with subsequent effects on intestinal integrity. In poultry, viscosity plays a major predisposing role on intestinal dysbiosis, like NE. When poultry diets contain viscous cereals like barley, wheat and rye, the risk of enteritis is even higher. Dietary NSP-enzymes work by reducing the viscosity of the digesta in the small intestine, so that digesta passage and the nutrient digestion rate increase, providing less substrate and less time for the putrefactive fermentation organisms to proliferate. This may restore the normal and efficient endogenous enzymatic digestion of nutrients in the small intestine. Therefore, the inclusion of enzymes that reduce viscosity represents a method for controlling the intestinal environment by limiting the growth of anaerobic bacteria such as *C. perfringens* or *E. coli*.

Moreover, arabinoxylo-oligosaccharides (AXOS) have the potential to be an efficacious prebiotic in broiler and pig diets. Degradation of dietary AX through xylanase inclusion may release XOS with such benefits as: changed relative abundance of specific microbial populations (i.e. *Bifidobacterium*), increased production of SCFAs, and induction of different cytokine expressions that are correlated with pathogen reduction colonization (i.e. *Salmonella* enteritidis). This prebiotic concept opens opportunities to find complementary strategies, which are worth exploring.

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ARTICLE II

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Maize nutrient composition and the influence of xylanase addition

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ABSTRACT

This study assessed differences in nutrient composition, physical characteristics, and xylo-oligosaccharide content with or without xylanase treatment by maize genotype and the grain position on the cob. Ten cobs each from sixteen maize varieties sowed in the same field were collected and classified considering the grain's position on the cob (basal vs apical). The majority of physicochemical characteristics were influenced by an interaction between genetic background and grain position ($P < 0.05$); however, moisture, crude protein, starch, ash and soluble arabinose:xylose ratio differed between maize varieties and grain on cob position, without interaction. Xylanase addition increased the concentration of soluble compounds and xylotriose content in the aqueous phase following incubation *in vitro* ($P < 0.05$) and in the case of xylotriose the amounts released varied with grain position and variety. In conclusion, maize genotype and grain position on the cob significantly influenced chemical composition and oligosaccharide content when treated with xylanase, which may contribute to nutrient variability between maize samples.

1. Introduction

Maize is the most common energy source used in commercial monogastric diets, especially in the American, Southern Europe and most Asian countries where maize grain is the primary cereal for poultry feeds (Dei, 2017). However, the nutritional value of maize for livestock feeding can vary (Cowieson, 2005). Genotype, agronomic conditions and pre- and post-harvest processing can affect the chemical characteristics of cereal grains (Gehring et al., 2013). Indeed genetics has been demonstrated as an important source of biochemical and nutrient variability (Rodehutscord et al., 2016; Uribelarrea et al., 2004). The nutrient composition of maize can also be affected by the position of the grain on the cob and grain size (Nadeem et al., 2014). Consequently, the smaller, extreme apical grains can produce nutritional variability, which could affect the final composition of animal diets. Little is known about how grain position on the cob and their interaction with genetic factors contribute to variations in the chemical composition and physical characteristics of maize for poultry feeds. Characterization of the variation in nutritional value of maize grains that result from these factors may help define appropriate breeding objectives and improve the accurate use of additives that depend on nutrient composition, such as enzymes, in order to improve the feeding value of cereal grains for livestock nutrition. Xylanase is commonly added, particularly when viscous cereals such as wheat is used in poultry diets, in order to reduce intestinal viscosity of birds by degradation of soluble arabinoxylans (AX) (Choct et al., 2004). However, the benefits obtained by degradation of endosperm cell walls, which includes the release of encapsulated nutrients ("cage effect") and/or through a prebiotic effect as a result of generation of short-chain oligosaccharides from polysaccharide AX (Bedford and Apajalahti, 2000; Khadem et al., 2016). However, these effects also contribute to the responses noted but these effects can be heterogeneous and result in variable animal responses, depending on the quantity and nature of the cereal fiber polysaccharides present.

It was hypothesized that maize genetic background and grain position on the cob might influence chemical composition, physical characteristics, and response to xylanase supplementation. The present study aimed to investigate the differences in nutrient composition, physical characteristics and short-chain xylo-oligosaccharide (XOS) content of basal or apical grains obtained from different maize hybrids harvested from the same field and under similar environmental growing and fertilizing conditions.

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Abbreviations

NIRS	near infrared spectroscopy
SolDM	solubility of dry matter
WRC	water retention capacity
NDF	neutral detergent fiber
ADF	acid detergent fiber
PSI	protein solubility index
NSP	total non-starch polysaccharides
AX	total arabinoxylans
AME	apparent metabolizable energy
XOS	xylo-oligosaccharides
HPLC	high performance liquid chromatography
ELSD	evaporative light scattering detector
A:X	arabinose:xylose
P	phosphorus
AA	amino acids
DAD	diode array detector
HILIC	hydrophilic interaction liquid chromatography
SLM	supported liquid membrane
PROC GLM	general linear model procedure

Table 1
Maize samples.

Varieties	Trial Code	Maize Yield (kg/ha)
KWS-7661	Variety 1	19,621
KWS-16772	Variety 2	15,890
KWS-kxb9391	Variety 3	<8000
Kontigos	Variety 4	15,746
KWS-7651	Variety 5	18,415
Kelindos	Variety 6	15,856
KWS-6553	Variety 7	16,260
KWS-4565yg	Variety 8	15,481
Kefieros	Variety 9	18,276
KWS-9393	Variety 10	<8000
KWS-7569	Variety 11	16,568
KWS-7562	Variety 12	18,656
Kefrancos	Variety 13	14,897
KWS-7554	Variety 14	17,221
KWS-kbx9392	Variety 15	<8000
KWS-2679yg	Variety 16	18,332

2. Materials and methods

2.1. Maize samples

Sixteen maize varieties were sown in the same field and environmental growing conditions (Gimenells, Catalunya, Spain), including fertilizer and harvesting. The rainfall of the area was 207 mm in the cultivation period and the average minimum/maximum temperatures varied between 15.2 and 29.3 °C (AEMET, 2020). The planting density was 92,000 seeds/ha. Irrigation was with fixed cane sprinkling and, from sowing until an average plant height of 50 cm, at a rate of 1 h of watering per day, applied at night. From 50 cm until the plants had 12-14 leaves, they were watered according to the need for soil moisture, based on rainfall and visual assessment of the plants. From 12 to 14 leaves until the flowering spike is lost, water was applied for 30 min daily, while from flowering to harvest, plants were watered for 1 h daily at a rate of 50 min at night and 10 min at the time of maximum heat during the day. No pesticide was applied during cultivation, the fertilizer scheme was 170 kg of N/ha before sowing, 50 kg of liquid N/ha at 8 leaves, and 50 kg of liquid N/ha plus 5 L of organic fertilizer, through the irrigation system, at 12 leaves. Maize hybrids were sown in April 2018 and harvested in October 2018. The size of the land area and the harvest weights of each variety were used for the calculation of maize yield per hectare (Table 1). The plot for each variety consisted of eight rows with 17 cm of separation between plants within the row, and 70 cm of separation between rows of the same variety. The cobs used in the present study were collected at random from the experimental field; briefly, for each variety a total of 50 cobs were obtained from groups of five cobs in ten sampling points that followed a diagonal pattern along the four central rows from each variety plot. Ten cobs were then randomly selected from these 50 for each hybrid. For each cob, all grains were collected according to their position. Briefly, grain cob position was defined as apical or basal, with the line of differentiation between categories established subjectively considering the shape and size of the grains for each individual cob. The differences in the shape and size of the grains are shown in Fig. 1. Total grains from each position were oven dried at 60 °C for 6 h, weighed (portion weight, PW), and stored in a cold room at 4 °C. The proximate and physiochemical analyses were obtained for each hybrid and cob position using near infrared spectroscopy (NIRS), with results expressed on a fresh weight basis.

Eight maize samples (variety 1, 2, 7, 11, 12, 13, 14 and 16) were selected, based mainly on starch, protein and non-starch polysaccharide (NSP) content and were subsequently analyzed for solubility of dry matter (solDM), water retention capacity (WRC) and xylo-oligosaccharides (XOS, including X_2: xylobiose; X_3: xylotriose and X_4: xylotetraose) release when incubated with xylanase *in vitro*.

2.2. Maize proximate and physio-chemical analysis

The fresh weight proximate and physiochemical characteristics of maize samples (16 varieties & 2 grain positions) viz: apparent metabolizable energy (AME), crude protein, starch, crude fat, crude fiber, neutral detergent fiber (NDF), acid detergent fiber (ADF), protein solubility index (PSI), vitreousness, phytic acid phosphorus, total non-starch polysaccharides (NSP), total arabinoxylans (AX), soluble AX, total arabinose:xylose ratio (A:X) and soluble A:X were predicted by NIRS (Foss DS2500, Hilleroed, Denmark) using calibrations provided by the Feed Quality Service of AB Vista (Marlborough, England, United Kingdom).

2.3. Dry matter solubility and water retention capacity

Dry matter solubility and WRC (Anguita et al., 2006) for five cobs from the eight selected varieties (variety 1, 2, 7, 11, 12, 13, 14 and 16), with two portions (apical and basal) and two levels of xylanase inclusion (with and without) were determined following an *in vitro* procedure which simulates gastric pH (n = 160). In short, the samples were milled and 0.5 g of each sample was weighed into a 10 mL screw cap tube and incubated with 5 mL of 0.1 M sodium phosphate buffer and 2 mL of 0.2 M hydrochloric acid (pH = 2.5). Additionally, 0.5 mL of a solution of liquid xylanase (Econase XT, 16,000 BXU/mL; one BXU is defined as the amount of enzyme that produces one nmol reducing sugars from birchwood xylan in 1 min at 50 °C and pH 5.3.) was added to half of the samples at a dose of 16 BXU/mL. Tubes were kept at 41 °C for 2 h in a horizontal shaking water bath. The amount of sample submitted to analysis was recorded (W0) as well as the weight of the screw cap tube plus the sample (W1). After incubation, the tubes were centrifuged for 20 min at 2000×*g*. The supernatant was carefully removed and tubes were kept upside down for 10 min to ensure that the non-retained water

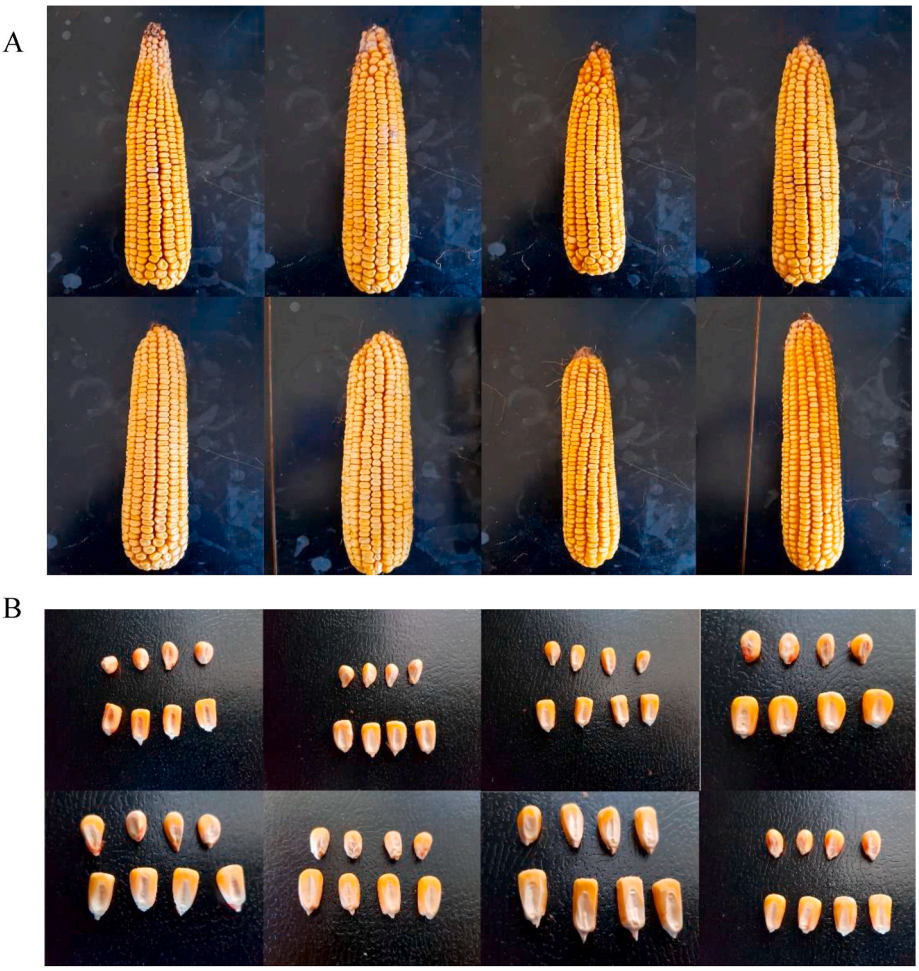

Fig. 1. From the top left to the bottom right, in both images, samples of cobs (A) and the corresponding grain category (upper: apical and lower: basal) (B) of maize 1, 2, 7, 11, 12, 13, 14 and 16 used in the *in vitro* assay are presented.

was drained. The supernatant was used for XOS determination. Tubes with sample were then weighed (W2), dried in the oven at 103 °C for 16 h to ensure the complete drying of the insoluble residue, and then weighed again (W3). The solubility of the DM was calculated as follows:

$$SolDM = \{\frac{W1 - W3}{W0}\}$$

Water retention capacity determined after centrifugation is expressed as grams of water retained by the total amount of sample incubated:

$$WRC_{DM} = \{\frac{W2 - W3}{W0}\}$$

2.4. Xylo-oligosaccharides determination

Determination of XOS was performed by high-performance liquid chromatography with an evaporative light scattering detector analysis (HPLC-ELSD), adapting the method described by Pu et al. (2017). Briefly, supernatant collected after centrifugation (20 min at 2000×*g*) in the SolDM and WRC analysis were used in these determinations. Xylose (X1; Merck Life Science S.L., Madrid, Spain), xylobiose (X2; Prod Code. O-XBI), xylotriose (X3; Prod Code. O-XTR) and xylotetraose (X4; Prod Code. O-XTE) were obtained from Megazyme (Wicklow, Ireland). Standards were used in a 250 ppm solution concentration with milli Q water to optimize the instrument parameters, and 0, 1, 2, 3, 4, and 10 mg/L solutions were prepared with milli Q water to obtain the calibration line. The samples and standards were filtered through 22 μm syringe filters. Analysis of the 162 standards and samples was carried out on an Agilent 1100 HPLC equipped with a DAD Agilent detector and an Agilent 1260 ELSD infinity detector. The HPLC column used was a Luna® 3u HILIC 200 A (150 × 2 mm), Phenomenex. The ELSD temperature was set at 80 °C and air was used as nebulizer gas with a flow rate of 1 SLM. The injection volume was 1 μL. Column temperature was set at 35 °C and flow rate was 1.0 mL/min. Despite high sensitivity, good precision, simple operation and rapid analysis by the HPLC-ELSD method, and being validated with purified samples of XOS in other studies (Li et al., 2016; Pu et al., 2017), the values obtained from this method are considered semi-quantitative, since the non-purified samples could contain compounds that could interfere with the results.

2.5. Statistical analysis

The cob was considered the experimental unit for all variables. The

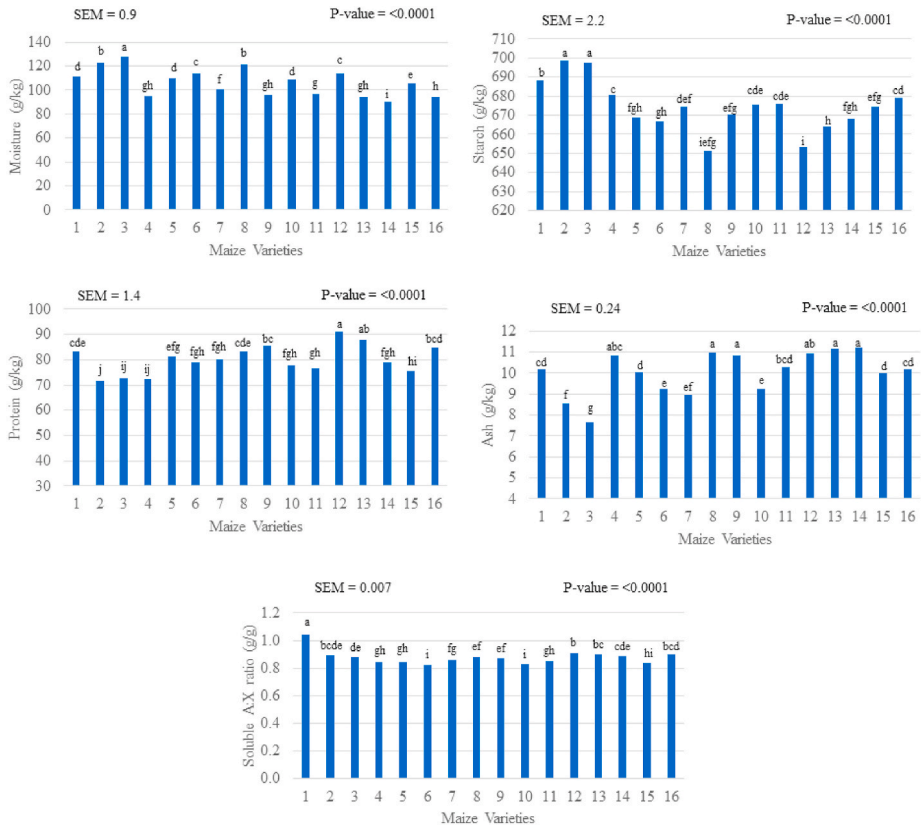

Fig. 2. Effects of maize genotype on physicochemical components. Only parameters without significant interaction are shown. Moisture, protein, ash, and soluble A:X ratio (Soluble arabinose:xylose ratio). The physicochemical components are expressed in as is basis. Each maize variety is a mean of 20 samples obtained from 10 cobs with two portions (n = 320).

physicochemical characteristics were analyzed by two-way ANOVA to identify genetic and grain cob position effects and the interaction between them. The solDM, WRC and XOS were analyzed by three-way ANOVA to identify genetic, grain cob position and xylanase effects and the interaction between them. The PROC GLM procedure was performed for both statistical analyses using SAS software (SAS, 2014). Significantly different means were separated using Tukey's HSD test. Significance was declared at a probability $P \leq 0.05$ and tendencies were considered when *P*-values were between >0.05 and < 0.10. Spearman's non-parametric correlation analysis was used to explore the associations between NIRS nutrient predictions, solDM, WRC, and XOS semi-quantitative estimation, using corrplot package of R 3.6.1.

3. Results

3.1. Maize physicochemical analyses

The effects on physicochemical composition of the maize genotype and the grain cob position are showed in Figs. 2 and 3, as well as their interaction are shown in Tables 2 and 3. Predicted poultry AME, crude fat, crude fiber, NDF, ADF, total NSP, soluble NSP, total AX, soluble AX, phytic phosphorus, PSI, vitreousness, total A:X ratio, and portion weight were influenced by an interaction between genetics and grain cob position ($P < 0.05$). The poultry AME, phytic P, A:X ratio, PSI, and portion weight were higher in grains from the basal position compared to those from the apical position; however, these differences changed according to each variety. The contents of crude fiber, total NSP and AX were higher in the apical cob, while vitreousness and crude fat were higher in basal grains in most of the hybrids. In spite of the interaction, total NSP (range: 55.6–81.3 g/kg), soluble NSP (range: 1.0–8.5 g/kg), total AX (range: 38.8–50.0 g/kg), and soluble AX (range: 2.2–5.3 g/kg) showed the highest variability due to maize genotype.

Moisture, protein, starch, ash, and soluble A:X ratio differed ($P < 0.005$) by genetic and grain cob position as individual factors. Except for ash, these factors were higher in basal grains than in those from the apical portion (moisture: 104 vs 108 g/kg; crude protein: 78 vs 81 g/kg; starch: 672 vs 676 g/kg; ash: 10.2 vs 9.7, g/kg and soluble A:X: 0.86 vs 0.88 g/g). The average and standard deviation of these components considering the genetics were; moisture: 106 ± 11.6 g/kg; crude protein: 80 ± 5.6 g/kg; starch: 674 ± 13.3 g/kg; ash: 10 ± 1.0 g/kg and soluble A:X 0.87 ± 0.05.

3.2. In vitro analyses

Results from the SolDM, WRC and semi-quantitative determination of XOS in maize samples after the *in vitro* incubation with xylanase are shown in Table 4. An interaction between variety and grain cob position was observed for solDM, WRC, xylobiose, xylotriose and xylotetraose. The solDM and WRC were highest in the apical grains in six and five of the eight varieties, respectively. The contents of xylobiose and xylotetraose were highest in the apical grains in four and five of the eight varieties, respectively, while xylotriose content was higher in apical

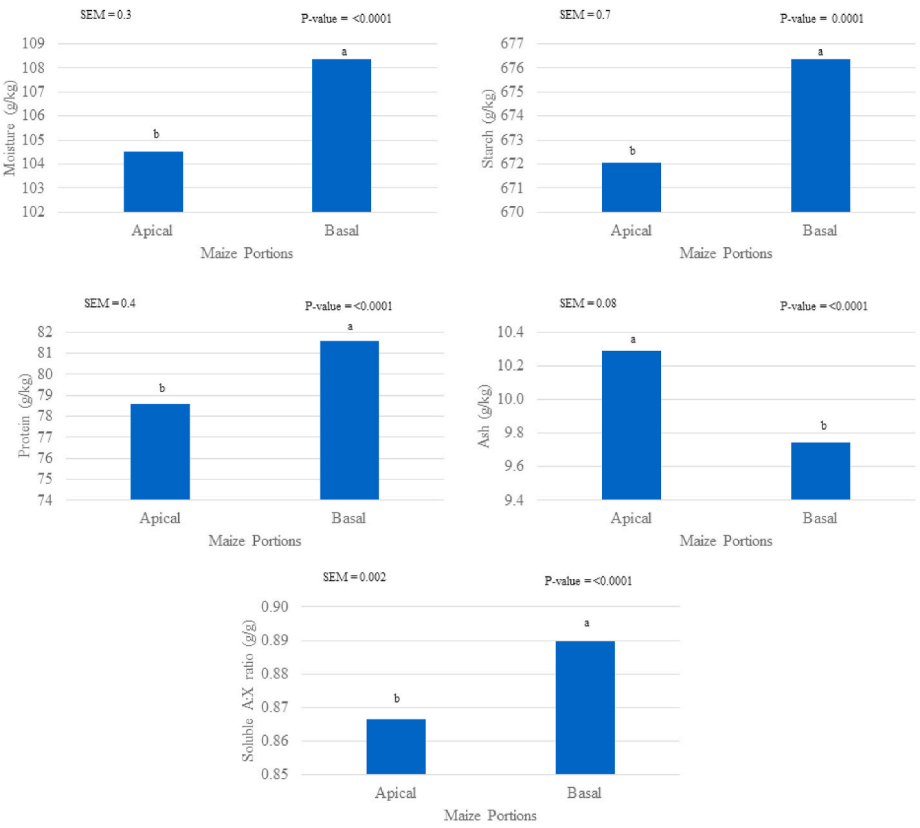

Fig. 3. Effect of grain cob position on physicochemical components. Only parameters without significant interaction are shown. Moisture, protein, ash, and soluble A:X ratio (Soluble arabinose:xylose ratio). The physicochemical components are expressed on an as is basis. Each portion is a mean of 160 samples obtained from 10 cobs of the 16 maize varieties (n = 320).

grains in seven of the eight hybrids (Fig. 4). Furthermore, two interactions were detected for xylotriose, the first between the genetic variety and xylanase, and the second between the grain portion and xylanase. The xylotriose content was increased in all hybrids when xylanase was added (Figs. 5 and 6). Similarly, higher xylotriose content was observed when xylanase was added in both grain cob positions; however, the response was greater in the grains of the apical portion compared those from the basal portion.

3.3. Correlations

Fig. 7 shows the correlation matrix and significance for the relationships between all parameters. Significant correlations ($P < 0.05$) were observed among physicochemical maize values. For example, predicted poultry AME was positively correlated with PSI (r = 0.83), fat (r = 0.71), soluble A:X ratio (r = 0.68), and negative correlated with crude fiber (r = −0.50), total NSP (r = −0.51), soluble NSP (r = −0.70), and soluble AX (r = −0.67). Starch was negatively correlated with crude fiber (r = −0.72), total NSP (r = −0.48), soluble NSP (r = −0.47), total AX (r = −0.40), and ash (r = −0.63). Crude protein was positively correlated with crude fiber (r = 0.47), total A:X ratio (r = 0.49), phytic P (r = 0.51), and vitreousness (r = 0.82), and negatively correlated with starch (r = −0.52). Soluble AX was negative correlated with PSI (r = −0.74), crude fat (r = −0.76), and AME (r = −0.60). Vitreousness was positive correlated with soluble A:X ratio (r = 0.61) and PSI (r = 0.62). Maize yield was positive correlated with crude protein (r = 0.33), soluble A:X ratio (r = 0.37), PSI (r = 0.32), and vitreousness (r = 0.49). Additionally, significant correlations ($P < 0.05$) between physicochemical maize values and *in vitro* analysis were observed. The solDM in samples was negative correlated with phytic P (r = −0.60). Xylobiose was negatively correlated with PSI and crude fat (r = −0.42 & r = −0.60, respectively). Xylotriose was positively correlated with WRC (r = 0.60). The xylotetraose was positively correlated with soluble NSP and soluble AX (r = 0.54 & 0.63, respectively), and negatively correlated with poultry AME (r = −0.61), crude fat (r = −0.56), soluble A:X ratio (r = −0.40), and PSI (r = −0.54).

4. Discussion

In the current study, the proximate analysis by NIRS of 16 maize varieties showed an effect of genotype on all parameters; however, most of these parameters also show an interaction with the grain position on the cob. The physicochemical characteristics which were not subject to a genotype:position interaction were moisture, crude protein, starch, ash, and soluble A:X ratio, but, as described, these were influenced by genotype. Rodehutscord et al. (2016) showed that genotype is an important factor influencing nutrient composition in several cereals used for animal feeding. The results obtained for moisture (97 ± 5.9 g/kg), crude protein (93.5 ± 9.18 g/kg DM), starch (740 ± 28.2 g/kg DM) and ash (13.3 ± 1.51 g/kg DM) of the 27 maize genotypes analyzed by Rodehutscord et al. (2016) are similar to those reported in the present study. Similarly, physicochemical composition mean values were also in

Table 2
Effects of maize variety and grain cob position on non-fiber physicochemical components [a,b,c].

Varieties/ Portions	Poultry AME	Moisture	Crude Protein	Starch	Crude Fat	Phytic P	Ash	PSI	Vitreousness	PW
	kJ/kg		g/kg					%		g/cob
	Apical/Basal	Apical/ Basal	Apical/ Basal	Apical/ Basal	Apical/ Basal	Apical/ Basal	Apical/ Basal	Apical/ Basal	Apical/Basal	Apical/ Basal
Variety 1	15,181^{b}/ 15,455^{a}	109/112	81/86	689/688	47^{b}/50^{a}	2.2^{cdef}/2.4^{a}	10.3/10.0	43^{b}/47^{a}	61^{cd}/62^{bc}	56^{jkl}/ 193^{efgh}
Variety 2	14,866^{cd}/ 15,057^{b}	120/125	69/74	699/698	42^{cd}/43^{c}	1.9^{klm}/ 2.1^{ghijk}	8.8/8.3	36de/40^{c}	55^{q}/57^{mno}	69^{ij}/209^{cde}
Variety 3	14,788^{cd}/ 14,916^{c}	124/132	73/73	696/698	40^{ed}/42^{cd}	1.9^{ijkl}/ 2.1^{defgh}	7.9/7.3	35^{def}/37 d	55^{pq}/56^{opq}	52^{jkl}/186^{gh}
Variety 4	14,129^{ijkl}/ 14,255^{hij}	92/97	73/72	680/682	35^{hijk}/ 36^{ghijk}	2.0^{hijkl}/ 2.1^{fghijk}	11.2/10.5	29^{ijklm}/ 31^{hi}	57^{klmno}/ 58^{jklmn}	68^{ijkl}/182^{gh}
Variety 5	14,096^{klm}/ 14,263^{hi}	107/112	82/81	665/672	35^{ijkl}/ 35^{hijk}	1.9^{jkl}/ 2.1^{efgh}	10.6/9.5	31^{hi}/33^{fgh}	59^{ghi}/58^{hij}	74^{i}/209^{cde}
Variety 6	13,852°/ 13,998^{lmn}	111/116	80/78	665/668	31°/33^{mno}	1.8^{n}/1.9^{ijkl}	9.3/9.1	28^{jklm}/3^{1hi}	58^{ijkl}/58^{ijkl}	59^{ijkl}/181^{h}
Variety 7	13,928^{no}/ 14,185^{jkl}	100/101	78/83	673/676	31°/32^{mno}	2.1^{efghij}/ 2.2^{cd}	9.4/8.5	27^{lm}/29^{ijkl}	58no/57^{lmno}	60^{ijkl}/ 200^{defg}
Variety 8	13,944^{no}/ 14,113^{jkl}	118/124	82/85	652/650	35^{hijk}/ 37^{fgh}	1.9^{lm}/2.0^{ijkl}	10.9/11.0	32^{gh}/37^{d}	58^{ijkl}/60^{fgh}	60^{ijkl}/237^{ab}
Variety 9	14,125^{ijlk}/ 14,557^{e}	95/96	84/87	663/678	36^{ghijk}/ 37^{fghi}	2.2^{defg}/2.3^{bc}	11.2/10.5	31^{hij}/ 35^{defg}	58^{ijk}/59^{efg}	62^{ijkl}/219^{bc}
Variety 10	14,013^{lmn}/ 14,115^{jkl}	106/110	77/79	673/678	32^{mno}/ 32no	1.9^{lm}/2.0^{klm}	9.6/8.9	29^{ijkl}/30^{ijk}	58^{jklmn}/ 58^{ijklm}	50^{l}/183^{gh}
Variety 11	13,963^{mno}/ 14,389^{fgh}	95/98	73/80	676/677	33^{lmn}/ 36^{ghij}	2.0^{klm}/ 2.1^{efghi}	10.2/10.4	26^{m}/34^{efg}	57no/59^{ghi}	62^{ijkl}/ 198^{defgh}
Variety 12	14,169^{jkl}/ 14,464^{efg}	110/116	88/93	646/660	39^{ef}/37^{ghij}	1.9^{klm}/ 2.0^{ijkl}	11.9/9.9	37^{d}/41^{c}	61^{cd}/63^{ab}	68^{ijk}/212^{cd}
Variety 13	14,207^{jkl}/ 14,520^{ef}	93/94	85/90	660/668	36^{ghijk}/ 37^{fgh}	2.2defg/ 2.4^{ab}	11.5/10.8	34^{efg}/37^{d}	59^{fgh}/61de	74^{i}/190^{fgh}
Variety 14	13,970^{mno}/ 14,359^{gh}	88/91	77/81	664/673	34^{klm}/ 37^{ghij}	2.2^{defg}/ 2.4^{ab}	11.6/10.8	30^{ijk}/34^{efg}	57^{klmno}/59^{hij}	56^{ijkl}/ 208^{cdef}
Variety 15	13,882^{no}/ 14,212^{ijk}	103/107	74/78	672/677	32no/35^{jkl}	1.8^{mn}/ 2.2^{defg}	10.3/9.7	27^{klm}/31^{hi}	57^{nop}/58^{ijkl}	51^{kl}/ 198^{defgh}
Variety 16	14,370^{gh}/ 14,754^{d}	92/95	83/86	679/679	33^{lmn}/38^{fg}	2.0^{hijkl}/ 2.2^{defg}	9.8/10.5	35^{def}/42^{bc}	61^{def}/63^{a}	66^{ijkl}/250^{a}
SEM[d]	50.2	1.3	1.9	3.1	0.8	0.04	0.33	0.9	0.4	6.4
P-value										
Variety	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Position	<0.0001	<0.0001	<0.0001	0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Variety*Position	0.001	0.204	0.605	0.184	0.004	0.031	0.091	<0.0001	0.002	<0.0001

[e] Values in the same column not sharing a common letter are significantly different ($P < 0.05$).
[a] Apparent metabolizable energy (AME), phosphorus (P), protein solubility index (PSI) and portion weight (PW).
[b] The physicochemical components are expressed on an as is basis.
[c] Data are a mean of 10 samples obtained from 10 cobs with two portions each (n = 320).
[d] Standard error of the mean.

agreement with FEDNA (2019) values reported for Spanish maize; however, the standard deviation for many, including crude protein and starch (5 and 13 g/kg, respectively), could result in large differences between random samples and thus would affect the final nutritional value of diets based on such grains and consequently animal performance. The NIRS technology has become an important analytical tool in the field of animal nutrition, due to its practicality, ease of use, low cost, and speed in obtaining results. However, the accuracy is reliant on a detailed database of samples and the calibrations developed from this database.

Several studies have shown that the variability contributed by genotype for wheat, triticale, maize, and rye for nutrient digestibility was low with respect to ruminants (Krieg et al., 2017) but higher in poultry (Zuber and Rodehutscord, 2017). The lesser effect in ruminants could be explained by the fact that their digestive process is based largely on bacterial fermentation.

Vitreousness, the ratio of hard to soft endosperm, has been showed to be an indicator of nutrient digestibility in maize used in animal diets. In this regard, a negative effect on digestion of starch and NDF has been associated with high vitreous maize endosperm in dairy cows (Lopes et al., 2009). Similarly, in broiler chickens fed maize-based diets, the presence of a significant content of hard (high vitreousness) endosperm reduced the nutritional value (Kaczmarek et al., 2013). The present study identified variation in vitreousness with genotype and grain cob position, which suggests the proportion of hard endosperm varies with these factors.

The WRC was also influenced by genetic background, which is likely linked to differences in fiber components between varieties (Robertson and Eastwood, 1981). The high variation contributed by genotype for total and soluble NSP, and AX observed in the current study suggests that it could be important to consider these components in plant breeding and subsequently to take these into account in feed formulation. The antinutritive effect of soluble NSP is linked with increased digesta viscosity, with negative changes in digesta transit time and modification of intestinal mucosa and microbiota of the gut. Although the quantity of soluble AX is lower in maize compare than other cereals,

Table 3
Effects of maize variety and grain cob position on fiber parameters[a,b,c].

Varieties/ Portions	Crude Fiber	NDF	ADF	Total NSP	Soluble NSP	Total AX	Soluble AX	Total A:X ratio	Soluble A:X ratio
	g/kg								
	Apical/ Basal	Apical/ Basal	Apical/Basal	Apical/Basal	Apical/Basal	Apical/Basal	Apical/Basal	Apical/Basal	Apical/Basal
Variety 1	19^{jk}/21^{ghij}	99^{bc}/103^{ab}	41^{b}/46^{a}	59^{nop}/57^{op}	1.0^{m}/1.0^{m}	$40^{hijklmno}$/43^{efg}	2.2^{p}/2.1^{p}	$0.72^{ijklmno}$/ 0.70^{opqr}	1.02/1.06
Variety 2	14^{l}/15^{l}	93^{cdef}/95^{cd}	$35^{fghijkl}$/38^{defg}	61^{mno}/55^{pqr}	1.0^{m}/1.0^{m}	40^{jklmno}/39^{op}	3.1^{mn}/2.7^{o}	0.70^{qr}/0.69^{r}	0.86/0.91
Variety 3	13^{l}/14^{l}	89^{efghi}/95^{cd}	33^{klmnop}/ 38^{bcde}	57^{opq}/54^{qr}	1.0^{m}/1.0^{m}	38^{op}/39^{mno}	3.4^{klm}/2.9^{no}	0.69^{pqr}/0.68^{s}	0.86/0.90
Variety 4	20^{ghijk}/ 20^{hijk}	84^{hijkl}/82^{ijkl}	29^{pq}/30^{opq}	76^{bc}/72^{cdef}	8.3^{abc}/ 8.2^{abcde}	45^{cde}/43^{def}	4.9^{cdefgh}/ 4.7^{gh}	0.71^{lmno}/0.70^{nopq}	0.85/0.85
Variety 5	20^{ghijk}/ 21^{ghijk}	80^{kl}/86^{ghijk}	31^{nopq}/ 34^{hijklm}	67^{ghijk}/ 64^{jklmn}	6.2^{ghi}/5.4^{hij}	$41^{fghijklmn}$/ $42^{fghijkl}$	4.6^{h}/4.6^{h}	0.73^{bcdefg}/ $0.72^{fghijkl}$	0.83/0.85
Variety 6	20^{hijk}/21^{ghi}	82^{ijkl}/ 86^{ghijk}	34^{hijklm}/ 36^{efghij}	62^{klmno}/ 63^{jklmn}	6.9^{cdefg}/ 6.7^{efgh}	39^{nop}/ $41^{ghijklmno}$	5.0^{cdefg}/ 5.0^{bcdefg}	0.74^{bcde}/ $0.72^{efghijkl}$	0.81/0.83
Variety 7	22^{fgh}/22^{efgh}	91^{defgh}/ 88^{efghi}	35^{ghijkl}/ 36^{efghij}	71^{defg}/67^{ghijk}	8.6ab/ 6.5^{fghi}	43^{efg}/43^{efgh}	5.4^{a}/5.2^{abcd}	$0.74^{defghijk}$/ $0.72^{fghijkl}$	0.85/0.86
Variety 8	23^{def}/22^{efg}	93^{cdef}/94^{cde}	$37^{defghij}$/38^{defg}	69^{defghi}/ 66^{hijklm}	6.9^{efg}/ 7.1^{cdefg}	43^{efghij}/ $42^{fghijklm}$	3.9^{ij}/3.6^{jk}	$0.73^{efghijkl}$/ 0.72^{mnop}	0.86/0.88
Variety 9	23^{cdef}/ 20^{hijk}	95^{cd}/79^{kl}	33^{jklmno}/30^{q}	77^{bc}/68^{efghij}	6.9^{defg}/ 6.0^{ghi}	47c/44^{def}	5.1^{bcdefg}/ 4.7^{gh}	0.73^{bcdefg}/ $0.71^{cdefghi}$	0.87/0.87
Variety 10	19^{ijk}/19^{k}	79^{kl}/78^{l}	29q/ 32lmnopq	67^{ghijk}/ 61^{lmno}	7.3^{bcdefg}/ 5.1^{ij}	$41^{ijklmno}$/ 40^{klmno}	4.9^{efgh}/4.7^{gh}	0.74^{bcdef}/ 0.73^{cdefgh}	0.82/0.82
Variety 11	22^{efg}/22^{fgh}	91^{defg}/81^{jkl}	32^{lmnopq}/ 31^{mnopq}	73^{bcde}/68^{fghij}	9.1^{a}/ 7.8^{abcdef}	43^{ef}/$42^{fghijklmn}$	5.2^{efgh}/4.6^{gh}	0.72^{jklmno}/ 0.72^{klmno}	0.83/0.86
Variety 12	25^{a}/21^{fgh}	94^{cdef}/82^{ijkl}	41^{bc}/40^{bcd}	66^{ghijkl}/51^{r}	5.4^{hij}/2.3^{lm}	42^{ghijk}/37^{p}	3.4^{abcde}/3.2^{h}	$0.73^{defghijk}$/ 0.75^{ab}	0.90/0.91
Variety 13	25^{ab}/25^{abcd}	95^{cd}/ 88^{defghi}	37^{defgh}/38^{cdef}	76^{bc}/70^{defgh}	6.4^{ghi}/6.2^{ghi}	48^{bc}/46^{cd}	4.9^{defgh}/ 4.8^{fgh}	0.74^{bcd}/0.73^{cdefgh}	0.88/0.91
Variety 14	25^{ab}/25^{abc}	108^{a}/99^{bc}	40^{bcd}/38^{cdef}	85^{a}/78^{b}	8.5^{ab}/6.5^{fgh}	50^{a}/49^{ab}	5.4^{ab}/ 5.1^{abcdef}	$0.73^{defghij}$/ $0.72^{hijklmn}$	0.87/0.90
Variety 15	21^{ghijk}/ 21^{fgh}	87^{fghij}/ 87^{fghij}	33^{mnlopq}/ $35^{fghijkl}$	74^{bcd}/68^{fghij}	8.3^{abcd}/ 6.3^{ghi}	43^{efghi}/44^{def}	5.3^{abc}/4.8^{gh}	0.73^{cdefg}/ $0.72^{ghijklm}$	0.81/0.85
Variety 16	23^{bcde}/ 21^{fgh}	88^{efghij}/76^{l}	41^{b}/33^{ijklmn}	64^{ijklmn}/60^{no}	3.4^{kl}/4.5^{jk}	42^{fghij}/40^{lmno}	4.8^{gh}/4.0^{i}	0.76^{a}/0.74^{abc}	0.87/0.90
SEM[d]	0.7	2.5	1.2	1.9	0.5	0.9	0.11	0.005	0.009
P-value									
Variety	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Position	0.117	0.001	0.081	<0.0001	<0.0001	0.003	<0.0001	<0.0001	<0.0001
Variety*Position	0.002	<0.0001	<0.0001	0.028	0.001	0.002	0.027	0.021	0.091

[e] Values in the same column not sharing a common letter are significantly different ($P < 0.05$).
[a] Neutral detergent fiber (NDF), acid detergent fiber (ADF), non-starch polysaccharides (NSP), arabinoxylans (AX), ratio arabinose: xylose (A:X).
[b] The physicochemical components are expressed on an as is basis.
[c] Data are a mean of 10 samples obtained from 10 cobs with two portions each (n = 320).
[d] Standard error of the mean.

such as wheat, barley, rye, and triticale, the present study showed that genetics can play an important role in soluble AX variability in maize.

The increment of soluble dry matter content when xylanase was included in the incubation of maize samples in the *in vitro* simulation suggests a release of nutrients and/or soluble NSP and oligosaccharide production. Xylanase supplementation clearly can result in significant release of oligosaccharides when used with different cereals but maize seems to be much more intransigent to such effects compared with wheat of other small grain cereals (Dale, 2020; Morgan et al., 2017). However, there is no information concerning the variability in XOS production from maize samples when treated with xylanase; indeed Dale (2020) reported no production of XOS from maize samples when incubated with and without xylanase. However, the results of the present study have shown that XOS contents increased, albeit marginally, with xylanase addition in all maize samples, with different effects on xylobiose, xylotriose and xylotetraose release depending upon the maize sample itself. The current results show a marginal increment in xylotriose and/or reduction in xylotetraose contents with xylanase supplementation. This study demonstrates that there are clear differences in XOS contents between maize samples whether they are treated or not with xylanase. The amount of XOS likely is dependent upon the simulation conditions used, as it has been shown that xylanase production of XOS can be greater under more acidic conditions (Morgan et al., 2017). However, despite the benefits described about the HPLC-ELSD method, and the fact that the ELSD is not limited by the optical properties or functional groups of the analytes and has been widely used in the detection of chromophore-free compounds, such as carbohydrates (Ma et al., 2014), the results from this study should be confirmed by other methods, since maize extracts are complex and could contain compounds that could interfere with the analysis.

The grain cob position influenced moisture, crude protein, starch, ash and soluble A:X ratio. The apical and basal portions in this study represent 23 ± 2% and 77 ± 2% of the total grain cob weight, respectively. Apical grains contained less moisture, crude protein, starch and soluble A:X ratio, and higher total NSP, soluble NSP and other fiber compounds, suggesting the differences in nutrient availability of a maize sample can be due to grain position. In this regard, our results also showed a negative correlation between crude fiber, total NSP and total AX with AME, SolDM and PSI, suggesting that these fiber compounds influence nutrient availability. Previous studies showed that the size of the cereal grain could affect the physicochemical characteristics (Nadeem et al., 2014), possibly related to the amount of NSP, which has

Table 4
Effects of maize variety, grain cob position and xylanase supplementation on dry matter solubility (SolDM), water retention capacity (WRC) and xylo-oligosaccharides (XOS) quantification[a].

Main factors	g/100 g				
	WRC	SolDM	Xylobiose	Xylotriose	Xylotetraose
Maize varieties					
Variety 1	131^{bc}	11.0	0.26	0.12	0.66
Variety 2	127^{cd}	11.8	0.27	0.09	0.75
Variety 7	128^{cd}	10.7	0.32	0.07	0.94
Variety 11	139^{a}	10.9	0.29	0.12	0.86
Variety 12	127^{cd}	12.7	0.25	0.08	0.81
Variety 13	129^{bc}	10.7	0.24	0.07	0.83
Variety 14	134^{ab}	10.8	0.27	0.07	0.79
Variety 16	123^{d}	10.5	0.23	0.06	0.77
SEM[b]	2.0	0.08	0.007	0.005	0.015
Position					
Apical	130	11.3	0.28	0.09	0.81
Basal	129	11.0	0.26	0.08	0.79
Xylanase					
With	131	11.6^{a}	0.26	0.10	0.78^{b}
Without	128	10.7^{b}	0.27	0.07	0.82^{a}
SEM	1.0	0.04	0.004	0.003	0.008
Probabilities					
Variety	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Position	0.793	<0.0001	<0.0001	0.001	0.093
Xylanase	0.076	<0.0001	0.285	<0.0001	0.006
Variety*Position	0.323	<0.0001	<0.0001	0.028	0.033
Variety*Xylanase	0.201	0.403	0.802	0.004	0.990
Position*Xylanase	0.082	0.178	0.648	0.014	0.739
Variety*Position*Xylanase	0.508	0.716	0.998	0.391	0.999

[abc] Values in the same column not sharing a common letter are significantly different ($P < 0.05$).
[a] Data for variety effect are a mean of 20 samples obtained from 5 cobs with two portions and two levels of xylanase inclusion (n = 160).
[b] Standard error of the mean.

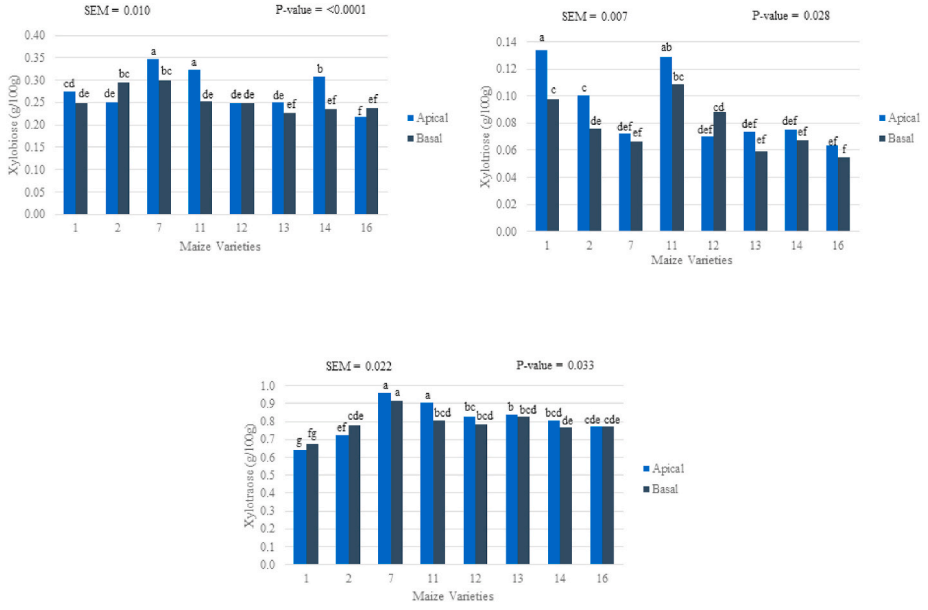

Fig. 4. Interaction effect of maize genotype and grain cob position on xylobiose, xylotriose and xylotetraose production. Data are a mean of 10 replicates obtained from 5 cobs with two portions and two levels of xylanase inclusion each (n = 160).

been shown to affect the nutritional value of the maize (Kaczmarek et al., 2013). Indeed, one of the strategies to minimize such variation in nutrient availability is the use of xylanase to reduce variability in animal performance resulting from dietary AX (González-Ortiz et al., 2016). In the current work, xylanase application interacted with grain position, showing a greater production of xylotriose from the apical grains compared to those from the basal, which is likely related to the higher NSP values in the apical grains and/or lower soluble A:X ratio.

The interaction between genotype and grain position showed differences for many of the physicochemical characteristics of maize, including predicted poultry AME, crude fat, crude fiber, NDF, ADF, NSP, soluble NSP, AX, soluble AX, total A:X ratio, phytic acid phosphorus, PSI, vitreousness, and PW. As suggested previously, the interaction between AX and other major nutrients could have a negative effect on nutrient availability. Arabinoxylan physico-chemical characteristics, such as gelling capability, depends on many characteristics, including molecular weight, side-chain ferulic acid concentration and A:X ratio (Izydorczyk and Biliaderis, 1995), and AX-protein associations can also play an

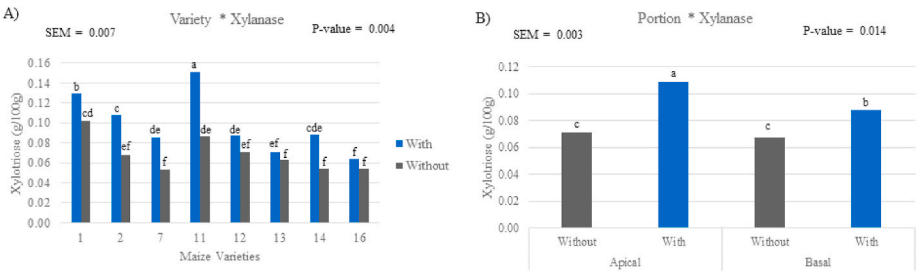

Fig. 5. Interaction effect of xylanase inclusion with the maize variety (A) and grain cob position (B) on xylotriose production. Data are a mean of 10 replicates for Variety*Xylanase and 40 samples for Portion*Xylanase, obtained from 5 cobs with two portions and two levels of xylanase inclusion each (n = 160).

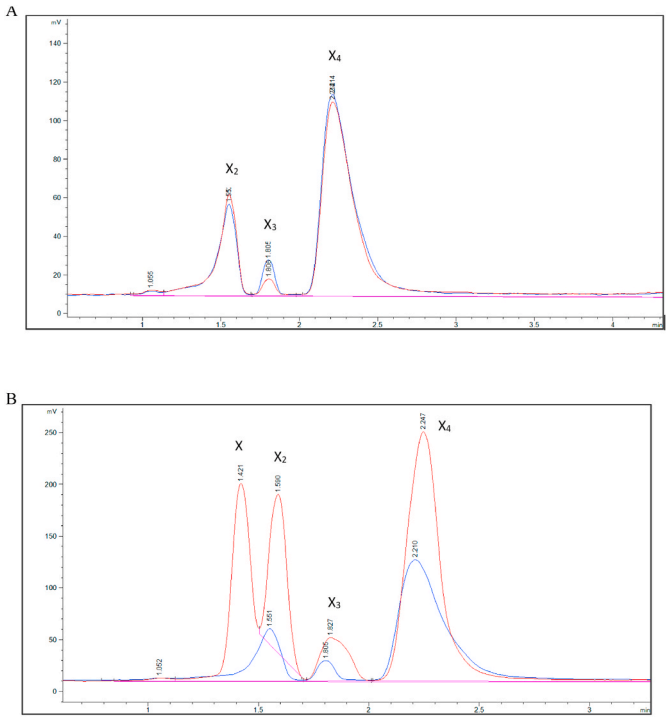

Fig. 6. Chromatogram of xylose (X), xylobiose (X2), xylotriose (X3) and xylotetraose (X4) semi-quantification in a maize sample. (A) Maize sample incubated without (red) and with (blue) xylanase. (B) Maize sample without xylanase (blue) and enriched with standards (red). The numbers in the graphs express time retention of the peak. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

important role, for example on the digestibility of the protein fraction (Méndez-Encinas et al., 2019). These factors add to the problem of the deficient levels of lysine and tryptophan in maize (Larkins et al., 2017), so variance in their digestibility could exacerbate this and influence animal performance significantly. The amount and packing of protein within the grain is partly reflected in the vitreousness (Paulsen et al., 2003), and a potential reduction of access to digestive enzymes, with consequent lower digestibility. Non-starch polysaccharide anti-nutritional effects are considered minimal in maize, because it is believed to have a relatively low soluble AX content with a minor impact on animal digesta viscosity. However, in this study it was demonstrated that soluble AX ranged from 2.2 to 5.3 g/kg, depending on genetics. These values highlight that some maize could behave similar to wheats in promoting viscosity issues. An improvement has been observed in poultry performance and nutrient digestibility when carbohydrases are included in maize-basal diets, suggesting that the availability of the nutrients is compromised by these fiber compounds (Cordero et al., 2019). However, modulation of the microbiota by the supply of XOS produced from AX breakdown by xylanase represents an alternative method to increase the relative abundance of bacteria producing butyrate in the animal gut, as has been demonstrated by direct feeding of XOS (Cordero et al., 2019; Onrust et al., 2015). In this context, xylanase supplementation of maize-based diets could represent a valuable strategy to control the negative effects of AX. The improvement shown in the SolDM fractions when xylanase was added may indicate an increased nutrient release and/or oligosaccharide production.

The present results suggest that genetic background and the grain position on the cob, to a greater and lesser extent, contributed to differences in nutrient composition, while the xylanase supplementation could be used to improve the nutritional value of maize through the increment of soluble compounds (nutrient releasing) and microbiota modulation by XOS production. The eight maize samples selected for detailed analysis in the present study were assessed in a broiler *in vivo* trial that showed differences on performance and nutrient digestibility associated to the nutritional variability produced by the maize genotype, showing the impact of this variability under practical conditions.

5. Conclusion

In conclusion, maize genotype and grain position on the cob have an important influence on the physicochemical composition and oligosaccharide contents and may contribute to nutrient variability. The NSP compounds were close related maize predicted AME for poultry. Apical grains have lower nutritional value and higher fiber compounds, which could compromise the final composition of animal diets. Xylanase supplementation increased dry matter solubility and xylotriose content *in vitro*, suggesting an improvement in the nutrient availability if oligosaccharide production also occurred within the gastrointestinal tract of broilers.

CRediT authorship contribution statement

Diego Melo-Durán: Conceptualization, methodology, investigation, data curation and software, formal analysis, writing-original draft preparation. **José Francisco Pérez:** Conceptualization, methodology, investigation, writing-original draft preparation and supervision. **Gemma González-Ortiz:** Conceptualization, methodology, writing-review and editing and supervision. **Sandra Villagómez-Estrada:** investigation and formal analysis. **Michael R. Bedford:** Writing-review and editing. **Hadden Graham:** Writing-review and editing. **David Sola-Oriol:** Conceptualization, methodology, investigation, writing-original draft preparation and supervision.

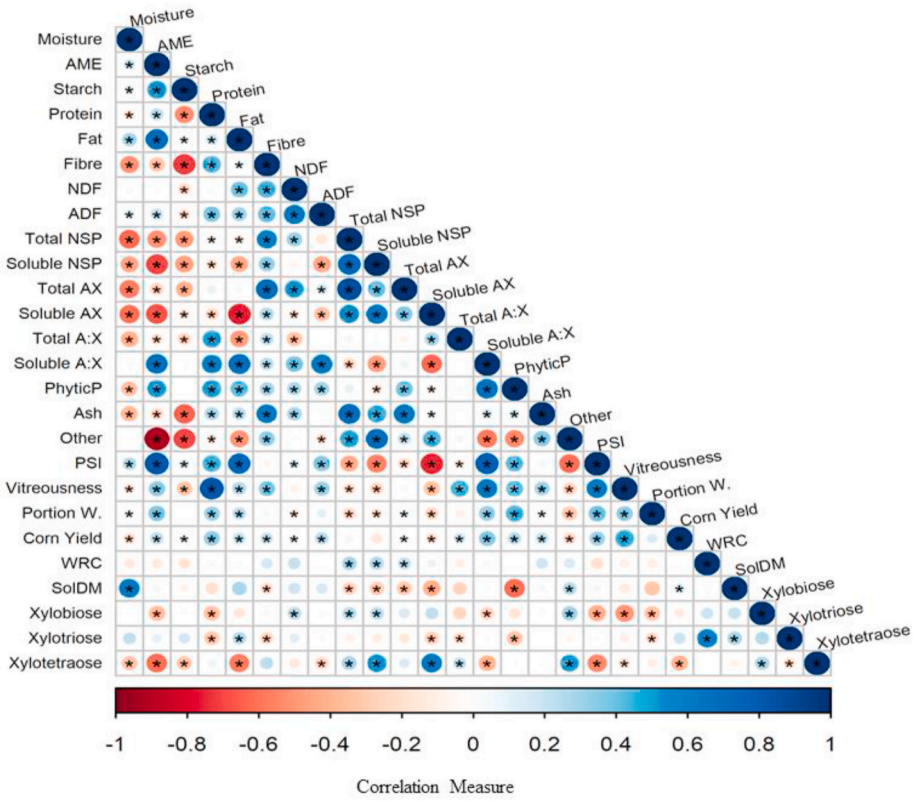

Fig. 7. Correlations between nutrient content of maize varieties and *in vitro* analysis. *In vitro* values only include the eight varieties analyzed. The * symbol indicates a statistically significant correlation ($P < 0.05$). The scale colors (Spcobman's ρ from −1 to +1) indicate whether the correlation is positive (blue colored circles) or negative (red colored circles). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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ARTICLE III

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Growth performance and total tract digestibility in broiler chickens fed different corn hybrids

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ABSTRACT The aim of the present study was to investigate the variability in nutrient digestibility associated with corn genetic background and its influence on the feeding value for broiler chickens. A total of 960 1-day-old male broiler chicks (Ross 308) were distributed in eight treatments, with 12 pens per treatment and 10 birds per pen in a 42-day study. Eight corn samples (Variety 1 to Variety 8) were selected based on their nutrient composition. A fixed amount of each corn (577 g/kg in the starter diets and 662 g/kg in the finisher diets) was used to formulate feeds. Diets were offered *ad libitum* in pellet form. Performance parameters were determined at d 21 and d 42, and excreta samples collected at d 21 to determine energy, organic matter and dry matter (**DM**) whole-tract digestibility. The results revealed a decrease ($P < 0.05$) in body weight (**BW**) and feed intake in birds fed variety 8 compared to other varieties at d 21. The lowest whole tract DM and energy apparent digestibility were also observed for the variety 8 diet ($P < 0.05$), together with varieties 3 and 5. Energy digestibility was higher in varieties 2, 4 and 7 ($P < 0.05$). Multivariate analysis revealed that corn protein concentration was positively correlated with vitreousness (r = 0.60, $P = 0.054$) and the arabinose:xylose ratio (r = 0.67, $P < 0.05$) and negatively correlated with starch (r = -0.62, $P < 0.05$). Soluble non-starch polysaccharide content was negatively correlated with the protein solubility index (r = -0.88, $P < 0.05$). In addition, corn protein concentration was negatively correlated ($P < 0.05$) with 21-d BW (r = -0.71) and weight gain (r = -0.62). In conclusion, the corn genetic background influenced the nutrient digestibility and growth performance of broiler chickens. The content and nature of the non-starch polysaccharides were found to be two of the main factors affecting the solubility and availability of nutrients in corn, and could be the reason for the negative effects on the performance of broiler chickens as shown in the present study.

Keywords: corn, near-infrared spectroscopy, total tract digestibility, chickens

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INTRODUCTION

Corn is the most common energy source used in commercial animal diets, especially in American, Southern European and Asian countries, where corn grain is the primary cereal for all poultry feed (Larbier et al., 1994; Dei, 2017). Due to its high dietary inclusion rate, corn can contribute up to 65% of metabolizable energy and 20% of protein in poultry diets (Gehring et al., 2013; Naderinejad et al., 2016). Although corn has a high and consistent nutritional value for livestock, its feeding value can be very variable (Summers, 2001; Cowieson, 2005). Genetics, agronomic conditions, proximate composition, and pre- and post-harvest processing are considered major factors affecting the nutrient variability. Of these, genetics has been demonstrated to be an important source of biochemical and nutrient variability (Uribelarrea et al., 2004; Reynolds et al., 2005). Phenotypic characteristics such as the grain-filling duration, related to physiological maturity, and composition, growth rate and moisture of the kernel are specific for each genotype and could affect the nutrient value (Seebauer et al., 2010; Prado et al., 2014). The main differences in corn composition include protein solubility, zein content, amylose to amylopectin ratio, and vitreousness (Gehring et al., 2013). Moreover, the apparent metabolizable energy (**AME**) value of corn can fluctuate by more than 470 kcal/kg from batch to batch (Cowieson, 2005).

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Near-infrared spectroscopy (**NIRS**) is an efficient tool for assessing the nutritive value of raw ingredients before manufacturing the feed, and thus it enhances precision feed formulation (Pujol et al., 2007). In addition to predicting the conventional composition, NIRS can also be used to predict the non-conventional composition, including anti-nutritional factors (Rahman et al., 2015). The anti-nutritional factors of corn include non-starch polysaccharides (**NSP**), phytate, lectins, and resistant starches (Englyst, 1989; Eeckhout and De Paepe, 1994; Cowieson, 2005). In this context, it was hypothesized that the corn genetic background could influence energy and nutrient digestibility, and thus broiler performance. Therefore, the aim of the present study was to research the differences in growth performance and energy and nutrient digestibility in broilers fed diets based on eight different corn hybrids harvested in the same field/area and under similar environmental growing and fertilizing conditions.

MATERIALS AND METHODS

Birds and Housing

A total of 960 Ross 308-day-old male broilers were purchased from a local hatchery. Upon arrival, birds were individually weighed and assigned to 96 floored pens in an environmentally controlled room, with ten birds per pen. Each pen (150 cm x 75 cm) was equipped with a bell feeder and drinker. Test diets and water were provided *ad libitum* throughout the trial. During the first 2 d, the temperature was set at 32°C and was gradually reduced to 20°C until the end of the trial (d 42). For the first 10 d, 23 h of light were provided and this was reduced to 18 h from d 11 onwards.

Corn Samples

Sixteen corn varieties were sowed in the same field with the same environmental growing conditions (Gimenells, Catalunya, Spain), including fertilizer and harvesting. The rainfall of the area was 207 mm in the cultivation period and the average minimum/maximum temperatures varied between 15.2 and 29.3°C (AEMET, 2020). The planting density was 92,000 seeds/ha. Fixed cane sprinkling was used for irrigation from sowing until an average plant height of 50 cm, at a rate of 1 h of watering per day, applied at night. From 50 cm until the plants had 12 to 14 leaves, they were watered according to the need for soil moisture, based on rainfall and visual assessment of the plants. From 12 to 14 leaves until the flowering spike was lost, water was applied for 30 min daily; and from flowering to harvest, plants were watered for 1 h daily at a rate of 50 min at night and 10 min at the time of maximum heat during the day. No pesticide was applied during cultivation, the fertilizer scheme was 170 kg of N/ha before sowing, 50 kg of liquid N/ha at 8 leaves, and 50 kg of liquid N/ha plus 5 L of organic fertilizer, through the irrigation system, at 12 leaves. The maize hybrids were sown in April 2018 and harvested in October 2018. The size of the land area and the harvest weights of each variety were used to calculate maize yield per hectare. The plot for each variety consisted of 8 rows with 17 cm of separation between plants within the row, and 70 cm of separation between rows of the same variety. The cobs used in the present study were collected at random from the experimental field. Briefly, for each variety a total of 50 cobs were obtained from groups of five cobs in 10 sampling points that followed a diagonal pattern along the 4 central rows from the plot of each variety. Eight corn samples (variety 1 to variety 8) were selected considering their nutrient composition, in order to cover a wide range of nutrient variation, mainly focused on protein, starch and NSP content. The corn proximate and physiochemical analyses are the averages from 10 cobs of each hybrid selected randomly from the experimental field, analyzed by NIRS and expressed on a fresh weight basis.

Experimental Diets

The experimental diets based on corn and soybean-meal were formulated to meet all nutrient requirements recommended by FEDNA (2018) (Table 1). Broilers were fed in 2 different phases: starter, from 0 to 21 d of age; and finisher, from 22 to 42 d of age. A fixed amount of each corn (577 g/kg in the starter diets and 662 g/kg in the finisher diets) was used in the formula regardless of their chemical composition. The nutrient contents of each corn hybrid, determined by NIRS, are shown in Table 2. Diets were formulated to have the same content of protein (starter: 200 g/kg and finisher: 180 g/kg), ether extract (starter: 7.7 g/kg and finisher: 5.5 g/kg) and corrected nitrogen AME (AMEn) (starter: 2,900 −2,940 kcal/kg and finisher: 3,045−3,087 kcal/kg). In order to include the same quantity of corn and be isonitrogenous, an ingestible ingredient (silicon dioxide; IBERSIL) was added to the diets as required. Diets were pelleted at 70°C using a pellet press (Pellet Mill 3020-4) with a capacity of 4,500−5,500 kg/h and using a 2.2 mm die for starter diets and 3.3 mm die for finisher diets. Diets contained 5 g/kg of titanium dioxide as an indigestible marker for estimating nutrient digestibility.

Experimental Procedures

The experimental procedures were approved by the Animal Experiment Committee of the Universitat Autònoma de Barcelona, and were in compliance with the European Union guidelines for the care and use of animals in research (European Parliament, 2010).

Birds were weighed individually at d 0, 21 and 42, and feed consumption was determined by pen per diet. Body weight (**BW**) uniformity was evaluated as the BW coefficient of variation. Mortality was monitored twice daily, and the weights of dead birds were used to adjust the feed conversion ratio. Excreta samples were collected at d 21 to determine energy, organic matter (**OM**) and dry matter (**DM**) digestibility. The excretas were collected in plastic containers and immediately frozen at -20°C. Excretas

Table 1. Ingredients and calculated composition of the experimental diets[1].

Diets	d 0–21		d 22–42	
Ingredients, (g/kg)	Mean	Range	Mean	Range
Corn	577	-	662	-
Soybean meal 47	317	311–331	263	256–269
Soy oil	53	50–55	33	30–36
DL-Methionine	3.3	3.2–3.4	2.8	2.7–2.9
Lysine HCl	3.6	3.3–3.8	2.8	2.6–3.1
Threonine	1.3	1.2–1.4	1	0.9–2.0
L-Valine	1.2	1.1–1.3	0.3	0.2–0.5
Salt	3	-	3	-
Limestone	11	-	11	-
Monodicalcium phosphate	10	-	9	-
Vitamin premix[2]	4	-	4	-
Sepiolite	14	0–24	8	0–17
Phytase[3]	0.1		0.1	
Calculated composition (g/kg)				
AMEn kcal/kg	2,920	2,900–2,950	3,064	3,032–3,087
Crude protein	200	-	180	-
Calcium	9.3	-	9.0	-
Available P	4.5	-	4.3	-
Fat	77	-	55	-
D Met+Cys	8.6	-	7.6	-
D Lys	11.8	-	10.0	-
Analyzed composition, (g/kg)				
Gross energy, Kcal/kg	4,150	4,075–4,224	3,938	3,914–3,958
Dry matter	910	906–922	870	864–874
Crude protein	196	190–199	170	168–179
Ether extract	74	71–76	50	48–52
Ash	110	104–138	90	61–103
Crude fiber	20	14–24	20	15–22
NFD	60	50–63	60	55–65

[1]Eight diets with different corn hybrid varieties were obtained; using NIRS predictions, all diets were formulated in order to contain the same calculated nutrient composition.

[2]Provided per kg of feed: vitamin A (retinol acetate) 10,000 UI; vitamin D (vitamin D3) (cholecalciferol) 4,800 UI; vitamin E/tocopherol) 45 mg; vitamin K3 (MNB, menadione nicotinamide bisulfate) 3 mg; vitamin B1 (tiamin mononitrate) 3 mg; vitamin B2 (riboflavin) 9 mg; vitamin B6 (piridoxin chlorohydrate) 4.5 mg; vitamin B12 (cyanocobalamine) 0.04 mg; nicotinamide 51 mg; pantothenic acid (calcium D-pantothenate) 16.5 mg; biotin (D-(+)-biotin) 0.15 mg; folic acid 1.8 mg; choline chloride 350 mg; iron (iron sulfate monohydrate) 54 mg; zinc (Zn, zinc oxide) 66 mg; manganese (Mn, manganese oxide) 90 mg; iodine (I, calcium iodine anhydrate) 1.2 mg; selenium (Se, sodium selenate) 0.18 mg; copper (Cu, copper sulfate pentahydrate) 12 mg; ethoxiquin 4 mg; D,L-malic acid 60 mg; fumaric acid 75 mg; sepiolite 907 mg; vermiculite 2001 mg; colloidal silica 45 mg.

[3]Quantum Blue 5G, AB Vista, Marlborough, UK; 5,000 FTU/g.

were then oven-dried and ground to pass through a 0.5 mm screen in a grinder before the analyses.

Sample Analyses

Corn Proximate and Physiochemical Analyses The fresh weight proximate and physiochemical characteristics of corn samples (10 per variety) included AMEn, crude protein, starch, crude fat, crude fiber, neutral detergent fiber, acid detergent fiber, protein solubility index (**PSI**), vitreousness, total NSP, total arabinoxylan (**AX**), and soluble AX. These were determined with NIRS (Foss DS2500, Hilleroed, Denmark) using calibrations provided by AB Vista (Feed Quality Service). To validate the NIRS predicted data, 16 corn samples were also analyzed for moisture, protein, starch and fat bywet chemistry, and NSP components using high performance liquid chromatography, following the method of Englyst et al. (1994).

Diet and Excreta Nutrient Analyses The contents of DM, ash, and gross energy (**GE**) were analyzed in feed and excreta, as well as crude protein (CP) in feed. Proximate analyses were performed according to AOAC (2005) Official Methods: Method 968.06 (CP), Method 934.01 (DM), Method 942.05 (ash). GE was determined using an isoperibolic calorimeter (Parr Instrument Company, Moline, Illinois, USA).

Whole-tract apparent digestibility of crude protein, energy, DM, and OM were calculated with the index method using the following equation:

$$\textit{Whole Tract Apparent Digestibility} = 1 - (([Ti_D]/[Ti_E]) \times ([N_E]/[N_D]))$$

Where $[Ti_D]$ is the concentration of Ti in the diet, $[Ti_E]$ is the concentration of Ti in excreta, $[N_E]$ is the nutrient content in the excreta, and [N] is the nutrient content in the diet.

DM Solubility and Water Retention Capacity The hydrolysis capacity of the nutrients and the ability to retain water by the fiber components (NSP) were determined using the solubility of the DM (solDM) and water retention capacity (**WRC**), respectively. All corns and diets (n = 5) were analyzed using a modification of the protocol described by Anguita et al. (2006). Samples were treated following an *in vitro* procedure that simulates gastric pH. In short, 0.5 g of each sample was weighed into a 10 ml screw cap tube and incubated with 5 ml of 0.1M sodium phosphate buffer and 2 ml of 0.2M hydrochloric acid (pH = 2.5). Tubes were kept at 41°C for 2 h in a horizontal shaking water bath. The amount of sample submitted to analysis was recorded (W0) as well as the weight of the screw cap tube plus the sample (W1). After incubation, the tubes were centrifuged for 20 min at 2000 × g. The supernatant was carefully removed and tubes were kept upside down for 10 min to ensure that the non-retained water was drained. Tubes with sample were weighed (W2) then dried in the oven at 100°C for 16 h to ensure the complete drying of the insoluble residue, and then weighed again (W3). The solubility of the DM was calculated as follows:

$$SolDM = \left\{\frac{W1 - W3}{W0}\right\}$$

WRC determined after centrifugation is expressed as gram of water retained by the total amount of sample incubated:

$$WRC_{DM} = \left\{\frac{W2 - W3}{W0}\right\}$$

Statistical Analysis

Corn sample and pen were considered the experimental unit for all variables. The nutrient composition of all

Table 2. Proximate and physiochemical analysis (g/kg fresh weight) of corn hybrid samples measured by near-infrared spectroscopy (NIRS)[1].

Varieties	Trial Code	AMEn[2] (kcal/kg)	Protein (g/kg)	Starch (g/kg)	Fat (g/kg)	Crude Fiber (g/kg)	NDF[3] (g/kg)	ADF[4] (g/kg)	PSI[5] (%)	Vitreou.[6] (%)	Total-NSP[7] (g/kg)	Total-AX[8] (g/kg)	Soluble-AX[8] (g/kg)
Kws-16772	Variety 1	3,599^{b}	73.7^{d}	698.0^{a}	43.1^{b}	14.8^{c}	94.9^{ab}	37.5^{bc}	40.2^{bc}	57.1^{d}	54.8^{d}	38.7^{cd}	2.7^{e}
Kws-7569	Variety 2	3,439^{d}	80.4^{cd}	676.5^{bc}	36.3^{c}	21.5^{ab}	81.1^{c}	31.0^{d}	33.9^{d}	58.9^{c}	67.5^{b}	41.3^{bcd}	4.6^{b}
Kws-7554	Variety 3	3,432^{de}	80.9^{cd}	672.5^{cd}	36.7^{c}	24.7^{a}	98.5^{ab}	37.9^{bc}	33.9^{d}	58.6^{cd}	77.8^{a}	49.6^{a}	5.1^{ab}
Kws-2679yg	Variety 4	3,526^{c}	82.6^{cd}	679.4^{bc}	37.8^{c}	21.6^{ab}	76.4^{c}	33.7^{cd}	42.7^{b}	63.2^{a}	60.1^{bcd}	39.4^{cd}	4.0^{c}
Kerubino	Variety 5	3,390^{e}	82.7^{bc}	676.1^{bc}	32.1^{d}	21.7^{ab}	87.4^{bc}	35.9^{bcd}	29.4^{e}	57.2^{d}	66.5^{bc}	42.9^{bc}	5.2^{a}
Kws-7661	Variety 6	3,694^{a}	85.6^{abc}	687.7^{ab}	49.7^{a}	20.6^{b}	102.9^{a}	46.1^{a}	47.4^{a}	62.1^{ab}	57.6^{cd}	42.9^{bc}	2.1^{f}
Kefrancos	Variety 7	3,471^{d}	90.3^{ab}	668.0^{cd}	37.2^{c}	24.6^{a}	88.2^{bc}	38.1^{bc}	37.3^{cd}	60.7^{b}	69.7^{ab}	45.8^{ab}	4.8^{ab}
Kws-7562	Variety 8	3,457^{d}	93.2^{a}	660.3^{d}	36.9^{c}	21.4^{ab}	81.6^{c}	39.6^{b}	40.5^{bc}	63.1^{a}	50.7^{d}	36.6^{d}	3.2^{d}
	SEM[8]	10	1.94	3.21	0.83	0.78	2.89	1.31	0.78	0.35	2.23	1.09	0.11
P value													
Variety		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[1]Data are means of 10 replicates from each corn hybrid.
[2]Apparent metabolizable energy for poultry.
[3]Neutral detergent fiber.
[4]Acid detergent fiber.
[5]Protein solubility index.
[6]Vitreousness.[7]Non-starch polysaccharides.
[8]Arabinoxylans.
[9a-f]Standar error mean Values in the same column not sharing a common letter are significantly different ($P < 0.05$).

corns, broiler performance, solDM, WRC and coefficients of digestibility were analyzed by one-way ANOVA to identify treatment effects, using PROC GLM (SAS 9.4). Significantly different means were separated using Tukey adjust. Significance was declared at a probability $P \leq 0.05$ and tendencies were considered when P values were between >0.05 and <0.10. Spearman's non-parametric correlation analysis was used to explore the associations between NIRS nutrient predictions, solDM, WRC and performance. A regression analysis between NIRS and wet chemistry of NSP and AX values, and correlation plot between NIRS nutrient content predictions and performance, nutrient digestibility and *in vitro* corn and feed results were created using a linear model function and the corrplot package of R 3.6.1., respectively.

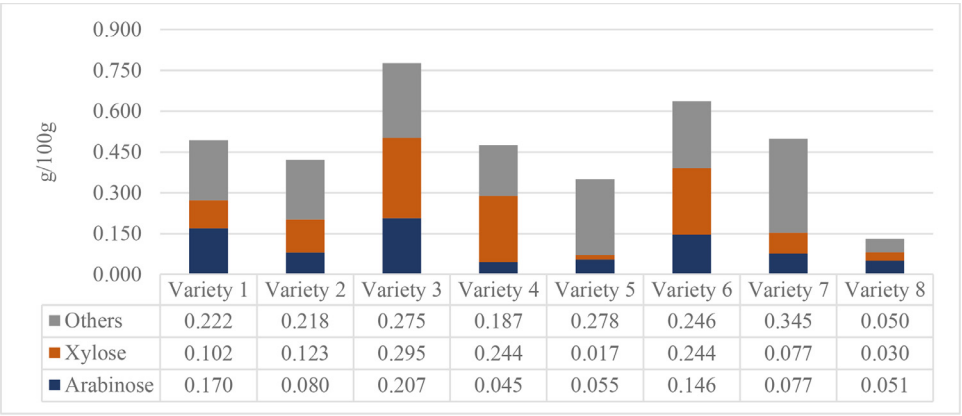
RESULTS

Corn and Diets Analyses

The corn hybrid samples differed in nutritional composition (Table 2). The average contents of the main components were: AMEn 3,501 kcal/kg; starch 677 g/kg; crude protein 83.7 g/kg; crude fiber 21.4 g/kg; crude fat 38.7 g/kg; total NSP 63.1 g/kg; total AX 42.2 g/kg; soluble AX 4.0 g/kg; ratio A:X 0.73; PSI 38.2%; and vitreousness 60.2%. The non-starch polysaccharide content in the corn samples is shown in Figure 1. Overall, the sum of components (crude protein + starch + crude fat + total NSP) came to approximately 863 g/kg of fresh weight, with the remaining presumably being mainly moisture and sugars (mono-, di- and oligosaccharides + fructans). The regression analyses between NIRS and wet chemistry values for moisture, protein, starch, fat, NSP and AX are shown in Figure 2. The proximate analyses of starter diets were in accordance with the expected values for protein and ether extract. Minor differences in GE were observed, in a range from 4,075 to 4,224 kcal/kg. In the finisher diets, the CP showed higher variability (range 168−179 g/kg), as the analyzed contents in all diets were lower than the calculated value. Diet GE showed less variability, with a range from 3,925 to 3,958 kcal/kg.

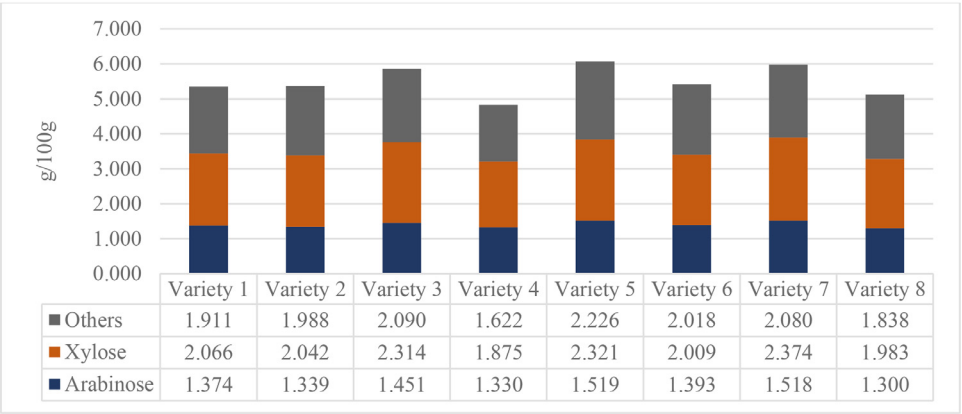
DM Solubility and WRC

Results from the SolDM and WRC of corn and feed after *in vitro* incubation are shown in Table 3. The SolDM and WRC were influenced by corn variety ($P < 0.05$ and $P < 0.001$, respectively). Variety 2 had a higher WRC compared to varieties 1 and 5. The other varieties were intermediate. Variety 8 had the highest solDM followed by variety 1, with no statistically significant differences between other varieties. In the starter diets, no differences were observed in WRC ($P = 0.11$). Differences in solDM of the starter diets were observed ($P = 0.0001$), as those formulated with varieties 1 and 8 had higher levels of solDM than the other diets. In the finisher diets, significant differences were observed for WRC ($P < 0.0001$) and solDM ($P < 0.0001$). Diets



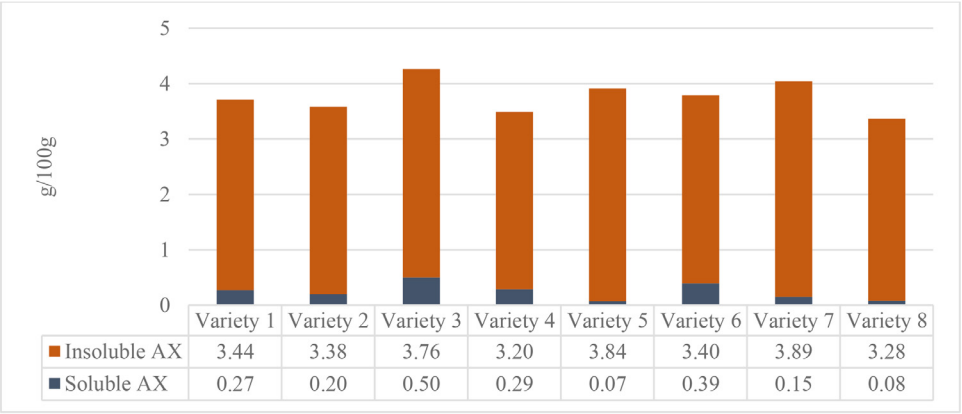
	Variety 1	Variety 2	Variety 3	Variety 4	Variety 5	Variety 6	Variety 7	Variety 8
Others	0.222	0.218	0.275	0.187	0.278	0.246	0.345	0.050
Xylose	0.102	0.123	0.295	0.244	0.017	0.244	0.077	0.030
Arabinose	0.170	0.080	0.207	0.045	0.055	0.146	0.077	0.051

A



	Variety 1	Variety 2	Variety 3	Variety 4	Variety 5	Variety 6	Variety 7	Variety 8
Others	1.911	1.988	2.090	1.622	2.226	2.018	2.080	1.838
Xylose	2.066	2.042	2.314	1.875	2.321	2.009	2.374	1.983
Arabinose	1.374	1.339	1.451	1.330	1.519	1.393	1.518	1.300

B



	Variety 1	Variety 2	Variety 3	Variety 4	Variety 5	Variety 6	Variety 7	Variety 8
Insoluble AX	3.44	3.38	3.76	3.20	3.84	3.40	3.89	3.28
Soluble AX	0.27	0.20	0.50	0.29	0.07	0.39	0.15	0.08

C

Figure 1. Soluble (A) and insoluble (B) non-starch polysaccharide and arabinoxylan (C) content in the corn samples.

containing varieties 3, 4, 5, and 6 had the highest WRC, varieties 1 and 2 had intermediate results, and varieties 7 and 8 had the lowest values. The solubility of the DM in variety 8 finisher feed was higher compared to the others except for the variety 1 diet.

Growth Performance

The effect of diets based on the individual corn hybrids on broiler chicken BW and growth performance is described in Table 4. Overall broiler mortality was 8.1% (data not shown), and no differences were observed between the experimental treatments ($P > 0.05$). Although no significant effects of feeding broiler chickens different corn varieties were observed for the whole period or the finisher phase ($P > 0.10$), a significant trend was observed in weight gain ($P = 0.096$). Birds fed varieties 2 and 6 had the highest BW gain, and those fed with varieties 1, 3, and 8 had the lowest BW gain. Overall feed intake was not influenced by corn variety ($P > 0.10$); however, it is interesting to highlight that birds

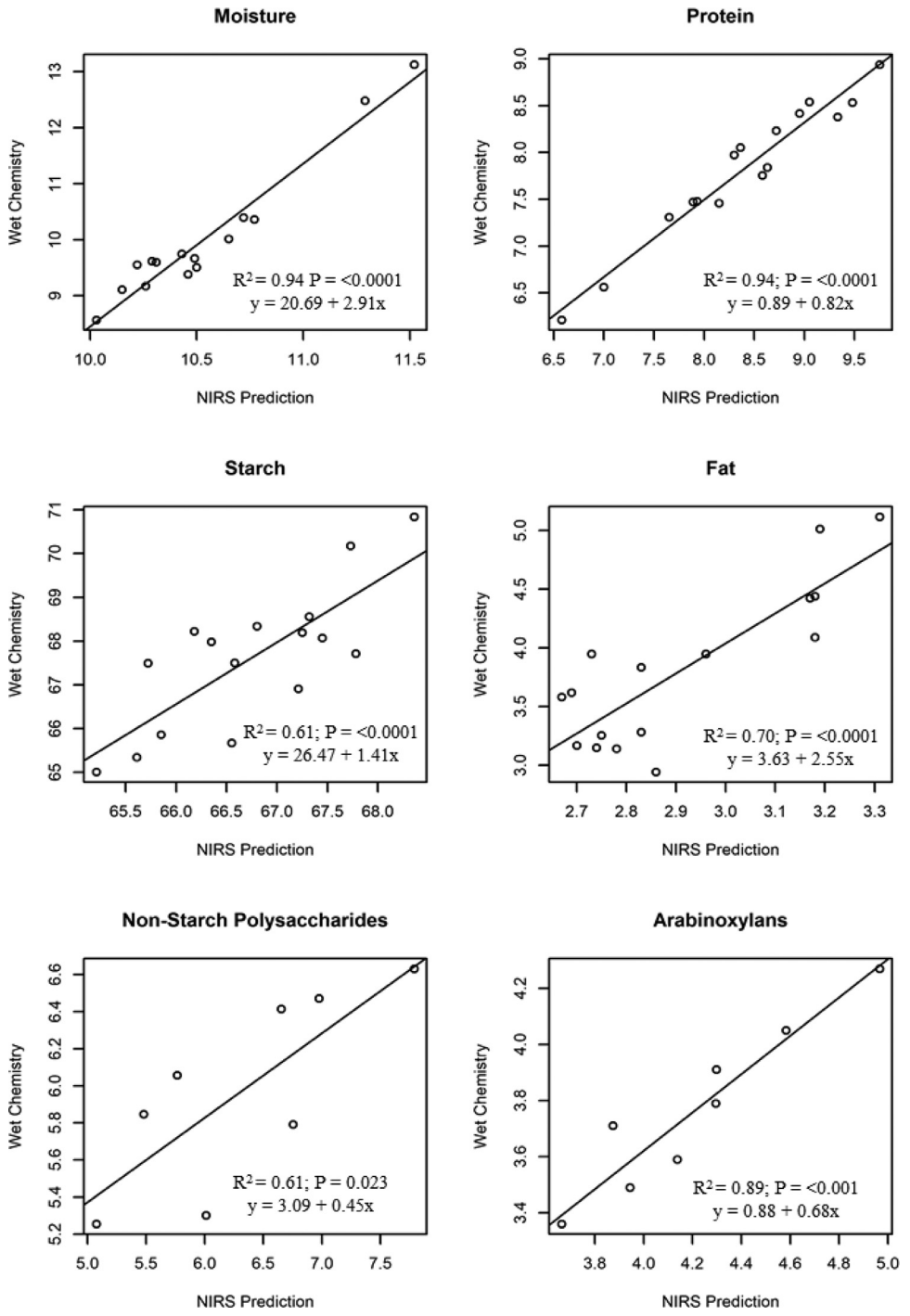


Figure 2. Regression analysis between wet chemistry and NIRS predicted values for moisture, protein, starch, fat, NSP and AX using the 8 corn varieties.

Table 3. Effect of corn hybrid samples and feeds on water retention capacity (WRC, g:g) and dry matter solubility (SolDM, g:g) at pH 2.5[1].

	Variety 1	Variety 2	Variety 3	Variety 4	Variety 5	Variety 6	Variety 7	Variety 8	SEM[2]	*P* value
Corn										
WRC	1.23^{b}	1.39^{a}	1.32^{ab}	1.26^{ab}	1.25^{b}	1.27^{ab}	1.32^{ab}	1.30^{ab}	0.031	0.020
SolDM	0.116^{b}	0.099^{c}	0.100^{c}	0.101^{c}	0.103^{c}	0.102^{c}	0.101^{c}	0.123^{a}	0.0012	<0.0001
Feed d 0−21										
WRC	2.17	2.16	2.18	2.23	2.24	2.19	2.20	2.11	0.029	0.110
SolDM	0.139^{a}	0.130^{b}	0.123^{b}	0.129^{b}	0.129^{b}	0.126^{b}	0.127^{b}	0.139^{a}	0.0021	0.0001
Feed d 21−42										
WRC	1.88^{ab}	1.90^{ab}	1.92^{a}	1.94^{a}	2.00^{a}	1.96^{a}	1.77^{b}	1.77^{b}	0.032	<0.0001
SolDM	0.170^{ab}	0.159^{c}	0.164^{bc}	0.165^{bc}	0.156^{c}	0.166^{bc}	0.163^{bc}	0.175^{a}	0.0023	<0.0001

[1]Data are means of 5 replicates per sample.
[2]Standard error of the mean.[3abc]Values in the same row not sharing a common letter are significantly different ($P < 0.05$).

Table 4. Effect of corn hybrid sample on body weight (BW), weight gain, feed intake and feed conversion ratio (FCR).[1]

	Variety 1	Variety 2	Variety 3	Variety 4	Variety 5	Variety 6	Variety 7	Variety 8	SEM[2]	*P* value[3]
d 0–21										
BW (g)	1,036^{ab}	1,039^{ab}	998^{ab}	1,043^{a}	1,018^{ab}	1,011^{ab}	993^{ab}	949^{b}	21	0.033
Weight gain (g/d)	994	997	956	1,001	976	966	960	907	21	0.056
Feed Intake (g/d)	1,253^{ab}	1,233^{ab}	1,216^{ab}	1,286^{a}	1,210^{ab}	1,203^{ab}	1,256^{ab}	1,168^{b}	23	0.021
FCR (g/g)	1.263	1.238	1.273	1.285	1.241	1.246	1.309	1.291	0.0183	0.065
d 21–42										
BW (g)	3,028	3,140	3,016	3,070	3,042	3,095	3,068	2,975	38	0.100
Weight gain (g/d)	1,984	2,084	2,018	2,027	2,024	2,113	2,067	2,019	33	0.130
Feed Intake (g/d)	3,695	3,778	3,677	3,663	3,603	3,814	3,755	3,668	53	0.113
FCR (g/g)	1.866	1.816	1.825	1.807	1.782	1.807	1.818	1.819	0.0253	0.544
d 0–42										
Weight gain (g/d)	2,978	3,081	2,974	3,028	3,001	3,080	3,028	2,926	40	0.096
Feed Intake (g/d)	4,949	5,011	4,892	4,949	4,813	5,016	5,011	4,836	64	0.147
FCR (g/g)	1.662	1.626	1.644	1.634	1.606	1.630	1.655	1.654	0.015	0.205

[1]Data are means of 8 pens each with 10 birds in the starter and finisher periods.
[2]Standard error of the mean.
[3ab]Values in the same row not sharing a common letter are significantly different ($P < 0.05$)

Table 5. Effects of corn variety on total tract digestibility and intake of energy, dry matter (DM) and organic matter (OM) at d 21.[1]

	Total tract apparent digestibility			Intake of digestible nutrients		
	DM	OM	Energy	DM(g)	OM(g)	Energy (kcal)
Variety 1	0.69^{ab}	0.72^{abc}	0.73^{ab}	868^{a}	901^{a}	3,824^{a}
Variety 2	0.68^{ab}	0.72^{abc}	0.74^{a}	844^{ab}	899^{a}	3,747^{ab}
Variety 3	0.67^{abc}	0.70^{cd}	0.71^{bc}	820^{ab}	858^{ab}	3,678^{ab}
Variety 4	0.69^{ab}	0.73^{ab}	0.74^{a}	896^{a}	940^{a}	3,979^{a}
Variety 5	0.67^{bc}	0.70^{bcd}	0.72^{ab}	817^{ab}	860^{ab}	3,693^{ab}
Variety 6	0.68^{ab}	0.72^{abc}	0.73^{ab}	821^{ab}	870^{ab}	3,679^{ab}
Variety 7	0.70^{a}	0.73^{a}	0.74^{a}	862^{a}	908^{a}	3,777^{ab}
Variety 8	0.65^{c}	0.69^{d}	0.70^{c}	764^{b}	812^{b}	3,404^{b}
SEM[2]	0.087	0.081	0.091	20.9	21.4	91.9
P value[3]						
Variety	0.008	0.048	0.003	0.0004	0.001	0.001

[1]Data were obtained by pooling 10 birds per pen, with 8 pens per treatment.
[2]Standard error of the mean.
[3abcd]Values in the same column not sharing a common letter are significantly different ($P < 0.05$).

fed varieties 5 and 8 tended to have low feed consumption for the entire period compared to the other diets. BW and FI were influenced by treatments in the starter period ($P < 0.05$). Birds fed the variety 8 diet had a lower BW and FI than those fed with the variety 4 diet, while the other corns gave intermediate performances.

Whole-Tract Apparent Digestibility and Intake of Digestible Nutrients

The effects of corn source on energy, DM and OM whole-tract apparent digestibility and intake of digestible nutrients at d 21 are shown in Table 5. The variety 8 diet had lower apparent digestibility of DM, OM and energy compared to diets with varieties 1, 2, 4, 6 and 7 ($P < 0.05$). Intakes of digestible DM, OM and energy were lower with variety 8 compared to varieties 1 and 4 (764 g vs 868 and 896 g; 812g vs 901 and 940g; 3404 kcal vs 3824 and 3979 kcal, $P < 0.05$). Variety 7 gave a higher intake of digestible DM and OM, and variety 2 gave a higher intake of digestible OM than variety 8.

Correlations

Figure 3 shows the correlation matrix and significance for the relationships between all parameters. Significant correlations ($P < 0.05$) were observed among physiochemical corn values. For example, AMEn was positively correlated with PSI (r = 0.88) and fat (r = 0.98), and negatively correlated with soluble NSP (r = -0.83) and soluble AX (r = -0.88). Protein was positively correlated with the total arabinose:xylose ratio (A:X) (r = 0.67) and vitreousness (r = 0.60, $P = 0.054$) and negatively correlated with starch (r = -0.62). Soluble AX was negatively correlated with PSI (r = -0.88), fat (r = -0.83) and AMEn (r = -0.88).

In addition, significant correlations ($P < 0.05$) between physiochemical corn values and *in vitro* analysis, 21-d performance and digestibility were observed. DM solubilities of corn and starter feeds were negatively correlated with total NSP (r = -0.79 & -0.76, respectively). DM solubility of the starter feed was negatively correlated with crude fiber content (r = -0.69), total AX (r = -0.88), phytic phosphorus (r = -0.90) and corn yield (r = -0.81). DM solubility of finisher feeds was negatively correlated with soluble NSP (r = -0.80). BW and weight gain at d 21 were negatively correlated with corn protein content (r = -0.71 & -0.62, respectively), while BW at d 21 was positively correlated with corn starch content (r = 0.74). DM solubility of corn hybrids was negatively correlated with BW at d 42 (r = -0.57) and overall weight gain (r = -0.57). Corn harvest yield was positively correlated with phytic phosphorus (r = 0.83),

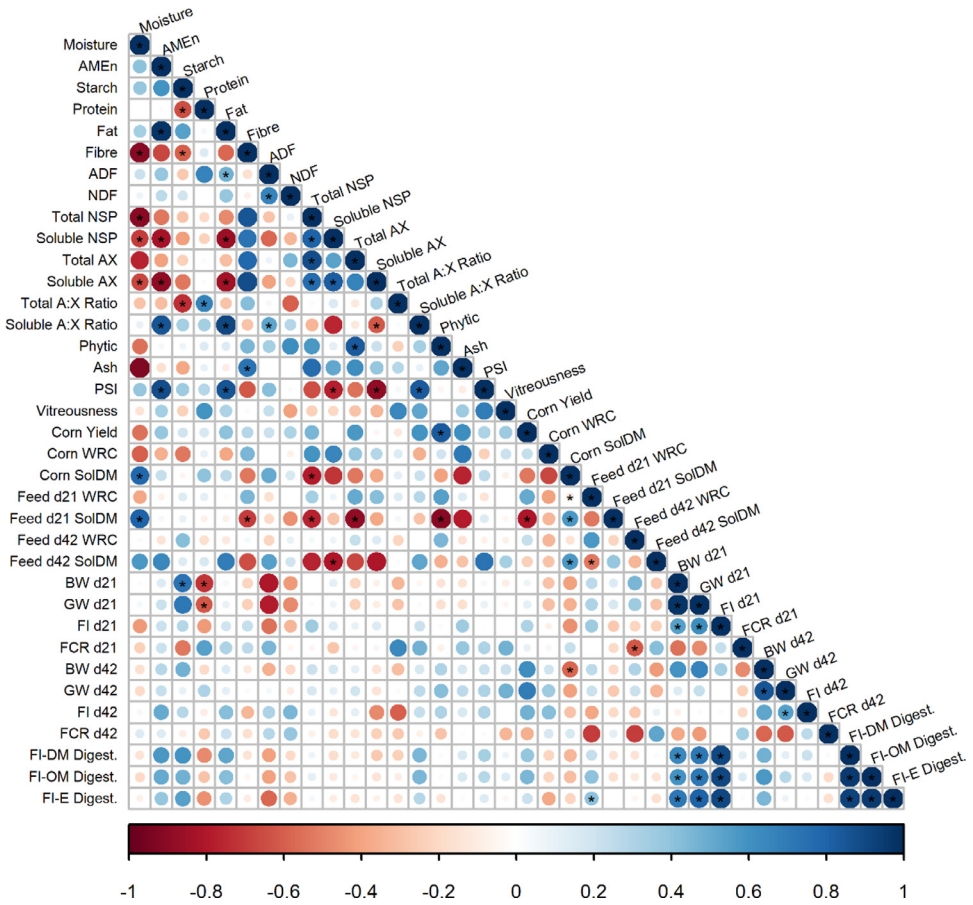


Figure 3. Correlations between NIRS predicted nutrient content and broiler performance, nutrient digestibility and *in vitro* corn and feed results (WRC and solDM). The * ($P < 0.05$) symbol indicate a statistically significant correlation. The scale colors (Spearman's ρ from -1 to +1) indicate whether the correlation is positive (blue) or negative (red) between factors.

and DM (r = 0.79), OM (r = 0.79), and energy (r = 0.74) digestibility.

DISCUSSION

Corn nutrient composition can be influenced by many factors, such as genetics and environmental conditions. Genetic selection can influence several kernel characteristics and thus the high heritable parameters, including kernel weight, volume, endosperm type, degree of damage, density and kernel breakage (Johnson and Russell, 1982; LeFord and Russell, 1985; Chen et al., 2016). In addition, the genetic background can also produce variation in the nutritional and anti-nutritional components in corn (Reynolds et al., 2005). Potentially anti-nutritional factors of corn, such as NSP, could interact with major nutrients, reducing their availability and consequently impairing bird digestibility and performance. Based on these considerations, the aim of the present study was to test the effects of anti-nutritional factors and nutrient availability of corn hybrids on broiler performance and nutrient digestibility.

In the current study, the proximate analysis by NIRS of 8 corn varieties showed a range in nutrient content of CP (73−93 g/kg), starch (660−698 g/kg) and crude fiber (14.8−24.7 g/kg). Masey O'Neill et al. (2012) reported nutritional variation within one hybrid harvested in different regions of China with similar ranges of CP, starch and crude fiber (76−85 g/kg, 742 −757 g/kg and 25.9−26.5 g/kg, respectively). Although the range in nutrients reported here was low, it revealed that the genetic background is a consistent source of nutrient variation, which is probably as important as the geographical factors. The results of the present study also showed differences in total NSP (50.7−77.8 g/kg), PSI (29.4−47.4%) and total AX (36.6−49.6 g/kg). Some expected correlations were observed, such as a negative correlation between protein and starch, as previously described in other studies (Uribelarrea et al., 2004; Masey O'Neill et al., 2012), while the positive correlation between crude protein and A:X ratio might suggest a relationship between AX structure and protein. A higher A:X ratio indicates more arabinose single-sugar side chains on the xylan backbone, and has been related to the solubility of AX (Rosicka-Kaczmarek et al., 2016).

AX physicochemical characteristics, such as gelling capability, depends on many characteristics, including molecular weight, side-chain ferulic acid concentration and A:X ratio (Izydorczyk and Biliaderis, 1995). AX-protein associations can also play an important role, especially the nature and quantity of the protein fraction (Méndez-Encinas et al., 2019). Furthermore, the negative correlation between PSI and soluble NSP and AX

suggests that protein solubility is affected by these soluble fiber components. Thus, the availability for digestion of corn protein could depend on the interaction with other corn kernel components, including fiber AX. In addition, the results from this study showed a positive correlation between protein content and vitreousness, with varieties 8 and 1 having the highest and lowest vitreousness and protein contents, respectively. Genotype, maturity at harvest and growing environment are factors that affect corn vitreousness (Corona et al., 2006; Masoero et al., 2011). Therefore, cereal endosperm vitreousness or hardness can influence the particle size distribution of subsequent diets (Lentle et al., 2006; Amerah et al., 2008) and thus the nutritional value of corn for chickens (Kaczmarek et al., 2013). In addition, corn diet particle size distributions have been related to the development of the gastrointestinal tract, performance and digestibility in broiler chickens (Jacobs et al., 2010). However, a negative correlation between corn solDM and d 42 BW and weight gain was observed in this study. This suggests that soluble corn compounds such as soluble NSP could decrease nutrient availability and consequently performance. This would explain why birds fed the variety 8 diet, which has the highest solDM, had a lower BW and FI at d 21 and a decrease in whole tract DM, OM and energy digestibility compared to those fed the other corns. Corn variety 8 also showed the highest CP content. In this regard, zeins, or corn prolamins, and starch have been related to endosperm texture (Lee et al., 2006). Thus, the amount and packing of this protein within the kernel could affect physical properties such as vitreousness (Paulsen et al., 2019) and access to digestive enzymes (Liu et al., 2016; Pan et al., 2017). Overall, these factors could explain part of the effects observed in the current study on broiler performance and digestibility when the animals were fed different corn hybrids.

Corn solubility could be affected by the nature of the zeins, which are divided into several subclasses with different molecular weights and solubilities (Esen, 1986; Lee et al., 2006). It is thought that the matrix in the endosperm is associated with the nature of the protein/starch interactions, and this may influence the nutritional value of the cereal (Kaczmarek et al., 2013), and thus affect the protein and amino acid digestibility (Kaczmarek et al., 2011). It is plausible that the negative correlation observed between protein content and d 21 BW and weight gain could be related to the profile of corn zeins, given that the high-protein variety 8 had a negative effect on nutrient digestibility and bird performance. It is clear that an interaction between nutrients and other compounds influences the physicochemical characteristics and nutrient value of corn and, consequently, their behavior in feed processing and digestion processes.

Despite the small number of samples in this study, regression results between NSP and AX, predicted by NIRS, and the wet chemistry values showed that NIRS is an effective nutrient prediction tool. This is supported by there being no differences in the 42-d bird performances when they were fed eight different diets formulated with NIRS data to be iso-nitrogenous and iso-energetic. NIRS could also be used to make decisions about adding carbohydrase, such as xylanase, in order to prevent the negative effects of AX, the major component of NSP.

In conclusion, the results from the present study show that corn genetic background influenced nutrient digestibility and growth performance of broiler chickens fed diets based on these corns. The content and nature of the NSP are shown as two of the main factors affecting the solubility and availability of nutrients in corn, with possible negative effects on the performance of broiler chickens. The NIRS prediction for NSP and AX could be useful for improving feed formulation or the efficiency of any fiber utilization enhancer in corn-based diets.

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DISCLOSURES

On behalf of the authors, we wish to confirm that all authors have read and approved and that there are no other persons who satisfied the criteria for authorship but are not listed. Moreover, we confirm that all the authors listed in the manuscript have been approved one to each other.

We also wish to confirm that there are known conflicts of interest associated with this publication and that there has not been financial support for this work that could have influenced its outcome.

We also assume that although the corresponding author is the sole contact with the Editor in chief, he is responsible for communicating with the other co-author about the submission and the progress of the manuscript revision and final approval of proofs.

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ARTICLE IV

Melo-Durán, D., Pérez, J.F., González-Ortiz, G., Sala, R., Villagómez-Estrada, S., Bedford, M.R., Graham, H., Solà-Oriol, D., 2020. Influence of particle size and xylanase in corn-soybean pelleted diets on performance, nutrient utilization, microbiota and short-chain fatty acid production in young broilers. Animals. 10(10), 1904. doi: 10.3390/ani10101904

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Influence of Particle Size and Xylanase in Corn-Soybean Pelleted Diets on Performance, Nutrient Utilization, Microbiota and Short-Chain Fatty Acid Production in Young Broilers

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Simple Summary: The use of enzymes, such as xylanase, in poultry production has the objective of improving the productive value of diets by releasing nutrient content and boosting animal performance, with the consequent reduction in feed costs and environmental impact. Additionally, xylanase could have benefits in the intestinal microbiota through the prebiotic effect of oligosaccharides produced from the arabinoxylans present in the diet. In broilers, little is known of how feed processing of the diet, such as pelletizing, particle size, and grinding method, could affect enzyme efficacy in corn-based diets. The present study aimed to understand the effects of corn particle size and xylanase supplementation in pelleted diets on the performance, nutrient utilization, short-chain fatty acid concentration, gut microbiota, and intestinal development of young broilers. Our results have shown evidence of the importance of coarse particles on the gut development and digestibility of nutrients, and along with the xylanase, has shown benefits on microbiota modulation and in reducing the variation in body weight.

Abstract: The objective of this study was to investigate the effects of particle size and xylanase supplementation in corn-based pellet diets on the performance and digestive traits in young broilers. A total of 512 male Ross 308 broilers were used in a 21-day study. The treatments were designed in a 4 × 2 factorial arrangement with four levels of geometric mean diameter (D_{gw}) of corn (540, 660, 1390, and 1700 µm), and two levels of xylanase (0 or 16,000 BXU/kg diet). Feeding coarse corn diets (1390 and 1700 µm D_{gw}) and xylanase supplementation showed an inferior coefficient of variation of body weight. Higher gizzard weight, microbiome alpha-diversity, and clustered separately beta-diversity ($p < 0.05$) were observed in birds fed coarse diets. The addition of xylanase promoted changes in relative bacteria abundance, increasing Lachnospiraceae, Defluviitaleaceae, Bacteroidaceae, Bacillaceae, Eggerthellaceae, and Streptococcaceae families in the 1700 µm group, and Christensenellaceae and Lachnospiraceae families in the 540 µm D_{gw} group. In conclusion, xylanase supplementation and particle size of corn interact in the intestinal environment, showing changes in microbial composition. Coarse diets and xylanase supplementation showed improved body weight homogeneity, which might be related to a better gut development and microbiota modulation.

Keywords: microbiota; nutrient digestibility; particle size; short-chain fatty acids; xylanase

1. Introduction

The main objective of grinding poultry feed ingredients is to crush the grain cell wall and increase the surface for the digestive enzymes; however, it is important to obtain an optimal particle size distribution (PSD). The PSD needs to be adapted to the physiological capabilities of the birds in order to improve nutrient digestibility and animal performance, and to facilitate further feed processing, such as mixing, pelleting, or extrusion/expansion [1,2]. In poultry diets, coarse particles play a physical role in gut development and function, increasing the size of digestive organs such as the gizzard [3], and the retention time in the proximal portion of the digestive tract. In this way, positive effects, such as improved nutrient digestibility and reduced litter moisture content, have been described with coarse grain particle diets, either with corn or barley [4–7]. In Europe, grinding ingredients for poultry diets are mostly undertaken with hammer mills, usually fitted with a screen between 3 and 4.5 mm in size [8], and commonly presented in a pellet form, since the benefits are greater than in mash presentation. However, pelletizing applies an additional grinding of particles (secondary grinding), which is mainly caused by rollers of the pellet press [9]. This increases the extra micro-particles and reduces the percentages of coarse particles [10]. In addition to their effects on gut development of chickens, particle size and form might also influence the activity of digestive enzymes based on their different surface area. Xylanase is commonly added when viscous cereals, such as wheat, are used in poultry diets in order to reduce digesta viscosity of birds by degradation of soluble arabinoxylans [11]. Moreover, xylanase also shows benefits when used in corn diets [12,13], due to the disruption of the cell wall, which releases encapsulated nutrients ("cage effect"), and/or through the prebiotic effect of xylo-oligosaccharides (XOS) or arabinoxylo-oligosaccharides (AXOS) produced from corn arabinoxylans [14–16]. In broiler diets, the efficacy of supplemental xylanase was influenced by the wheat particle size on pellet diets [17], being enhanced in the presence of whole wheat [18]. However, with regard to the degree of grain grinding in corn base diets, there are no previous studies examining the interaction between xylanase supplementation and corn particle size in poultry diets. Thus, we hypothesize that the coarse corn particles could improve the response of xylanase supplementation in corn-based diets. Therefore, the aim of the present study was to investigate the effects of particle size and xylanase supplementation on the performance, nutrient utilization, short-chain fatty acid concentration, gut microbiota, and intestinal morphology of young broilers fed with corn-based pelleted diets.

2. Materials and Methods

2.1. Ethics Statement

The experimental procedures were approved by the Animal Experiment Committee (CEEAH) of the Universitat Autònoma de Barcelona (number code: CEEAH 10167), and were in compliance with the European Union guidelines for the care and use of animals in research [19].

2.2. Birds and Housing

A total of 512 Ross 308 day-old male broilers were purchased from a local hatchery. Upon arrival, birds were individually weighed and assigned to 64 wire-floored battery cages with eight birds per cage. Each cage (62 cm × 48 cm × 37 cm) was equipped with a line feeder and a nipple waterer. Test diets and water were provided ad libitum throughout the trial. The room was environmentally controlled and pre-heated to 34 °C two days prior to the start of the study. During the first two days, the temperature was set at 32 °C, and was gradually reduced to 20 °C at the end of the trial (d 21). The birds were given 24 h of light for the first 2 days, which was reduced to 23 h of light and 1 h of dark from d 3 to d 10, and 18 h of light and 6 h of dark from d 11 until the end of the experimental period.

2.3. Experimental Diets

The experimental design was a 4 × 2 factorial arrangement, consisting of four levels of geometric mean particle size diameter (D_{gw}) for corn (540, 660, 1390 and 1700 μm), and two levels of xylanase supplementation, 0 or 100 g/ton of xylanase (Econase XT 25P, AB Vista, Marlborough, UK; 160,000 BXU/g). Corn geometric mean diameter (D_{gw}) was measured in the feed-mill, prior to the pelleting process, using the procedure of three-sieve analysis [20]. Experimental diets (Table 1) were offered in pellet form, and met the nutrient recommendations for broilers from d 0 to d 21 [21]. Corn was obtained from a commercial batch at a local feed-mill. The four particle sizes of corn (540, 660, 1390, and 1700 μm) were obtained using a horizontal hammer mill with different configurations, corresponding to 1500, 1200, 600, and 450 rpm mill speed, and 5, 5, 8, and 8 mm mill screen size, respectively. The soybean meal for all diets was milled at the same configuration of 540 μm D_{gw} (speed 1500 rpm; screen size 5 mm). The diets were cold pelleted (70 °C) using a pellet press (Pellet Mill 3020-4) with a capacity of 4500–5500 kg/h, and using a 2.2 mm die. The particle size spectrum was characterized by dry sieving using a method described by Baker and Herrman [22] for corn, soybean meal and mash diets, and wet sieving using a method described by Miladinovic [23] for pelleted diets. The D_{gw} and geometric standard deviation (S_{gw}) were then determined. Diets contained 0.5% titanium dioxide as an indigestible marker for the estimation of nutrient digestibility.

Table 1. Ingredient and calculated composition of the d 0 to 21 experimental diets [1].

Items	Geometric Mean of Corn (μm)			
	540	660	1390	1700
Ingredients, %				
Corn		53.4		
Soybean meal 47		38.4		
Palm oil		2.50		
Soy oil		2.69		
Salt		0.31		
DL-Methionine		0.29		
Lysine HCl		0.14		
Threonine		0.03		
Limestone		0.97		
Mono dicalcium phosphate		0.94		
Vitamin premix [2]		0.40		
Calculated composition (%, as feed)				
Apparent Metabolizable Energy, kcal/kg		2900		
Crude protein		22.3		
Calcium		0.90		
Available P		0.45		
Crude fat		7.68		
D Me + Cys		0.98		
D Lys		1.18		
Analyzed composition [3], %				
Gross energy, kcal/kg		4264		
Dry matter		90.3		
Crude protein		22.3		
Starch		27.7		
Ash		5.6		

[1] Hydrochloric acid (HCl), phosphorus (P), methionine (Me), cysteine (Cys), lysine (Lys). [2] Provided per kg of feed: Vitamin A (retinil acetate) 10,000 UI; Vitamin D (Vitamin D_3) (Colecalciferol) 4800 UI; Vitamin E/acetate de tot-rac-3- tocopheril) 45 mg; Vitamin K_3 (MNB Menadiona nicotinamida bisulfit) 3 mg; Vitamin B_1 (Tiamin mononitrat) 3 mg; Vitamin B2 (Riboflavin) 9 mg; Vitamin B6 (Piridoxin Chlorhidrate) 4.5 mg; Vitamin B12 (cyanocobalamine) 0.04 mg; Nicotinamida 51 mg; Pantotenic Acid (Calcium D-pantotenate) 16.5 mg; Biotin (D-(+)-biotin) 0.15 mg; Folic Acid 1.8 mg; Choline chloride 350 mg; Iron (Iron sulphate monohydrate) 54 mg; Zinc (Zn, zinc oxide) 66 mg; Manganes (Mn,

Manganes oxide) 90 mg; Iodine (I, Calcium Iondine Anhydrate) 1.2 mg; Selenium (Se, Sodium Selenate) 0.18 mg; Copper (Cu, copper Sulphate Penthahydrate) 12 mg; Etoxiquin 4 mg; D,L-Malic acid 60 mg; Fumaric acid 75 mg; Sepiolite 907 mg; Vermiculite 2001 mg; Colloidal silica 45 mg. [3] The range of analyzed nutrients was; gross energy from 4259 to 4269 kcal/kg, dry matter from 90.1 to 90.6%, crude protein from 21.3 to 22.5, ash from 5.4 to 5.7% and starch from 25.7 to 28.6 % (as is).

2.4. Experimental Procedures

Birds were weighed individually on d 0 and 21. Body weight (BW) uniformity was expressed as the coefficient of variation (CV) of BW. Feed consumption was determined by cage, and mortality was monitored twice daily. The weights of dead birds were used to adjust the feed conversion ratio (FCR). From d 19, excreta samples from all cages were collected over a 48-h period using aluminum trays. Feather down and feather scales were removed. At the end of the collection period, the excreta were collected in plastic containers, immediately frozen at −20 °C, and then freeze dried. On d 21, three birds from each of the 64 cages were randomly selected and euthanized by cervical dislocation. The complete gastrointestinal tract (GIT) was removed immediately from the abdominal cavity and dissected. The empty weights of the total GIT and each of its sections were recorded. Digesta contents from the ileum and cecum were collected separately on a cage basis by gently squeezing the contents out of the relevant sections from the three animals per cage, and pooled. The ileal digesta and excreta were ground to pass through a 0.5 mm screen in a grinder, before the analysis for apparent nutrient digestibility measurements. Cecal contents were analyzed for short chain fatty acid (SCFA) concentration. An additional aliquot of cecal contents from the 540 and 1700 μm treatments (with and without xylanase inclusion) were frozen at −80 °C for microbiota 16S rRNA gene sequence analysis.

2.5. Samples Analyses

The contents of dry matter (DM), ash, crude protein (CP) and gross energy (GE) were analyzed in feed, ileal digesta and excreta. Additionally, starch analyses were performed according to the 996.11 of Association of Official Analytical Chemists (AOAC) International (2000) method for both feed and ileal contents. Diet proximate analyses were performed according to the AOAC (2005) [24] Official Methods: Method 968.06 (CP), Method 934.01 (DM), Method 942.05 (ash), and GE was determined by using an isoperibolic calorimeter (Parr, Parr Instrument Company, Moline, Illinois, USA). Activity of xylanase was determined using the method of analysis recommended by the supplier. Xylanase activity was determined at pH 5.3 and 50 °C, using birchwood xylan as a substrate.

Apparent ileal and total tract digestibility of CP, GE, DM and organic matter (OM) were calculated by the index method using the following equation:

$$Ileal\ and\ total\ tract\ apparent\ digestibility\ = ((Ti_D/Ti_M)/(N_M/N_D)) \quad (1)$$

where Ti_D is the concentration of the Titanium (Ti) in the diet, Ti_M is the concentration of Ti in ileal digesta or excreta, N_M is the nutrient content in ileal digesta or excreta and N_D is the nutrient content in the diet.

The estimate of total intake of digestible nutrients was obtained using the coefficients of digestibility, diet nutrient composition, and total feed intake.

2.6. Pellet Quality

Pellet quality was determined as pellet durability index (PDI), which measures the portion of fines generated during standardized mechanical handling, using Holmen pellet tester (NHP 100, Norfolk, UK). Duration of treatment was 30 s, and fines were removed before and after the treatment using the sieve with 2 mm openings diameter.

2.7. Dry Matter Solubility and Water Retention Capacity

Solubility of the DM (SolDM) and water retention capacity (WRC) for all diets were evaluated ($n = 7$). Samples were treated following an in vitro procedure that simulates gastric and small intestine

pH [25], separately. In short, 0.5 g per duplicate of each sample was weighed into 10 mL screw cap tubes and incubated with 5 mL of 0.1 M sodium phosphate buffer (Na_2HPO_4) and 2 mL of 0.2 M hydrochloric acid (HCl) to simulate gastric pH (pH = 2.5), and 7 mL of 0.1 M sodium phosphate buffer to simulate small intestine pH (pH = 5). Tubes were kept at 41 °C for 120 min in a horizontal shaking water bath. The final volume of liquid was 7 mL both in the gastric and in the small intestine incubation. The amount of sample submitted for analysis was recorded (W0), as well as the weight of the screw cap tube plus sample (W1). After incubation, WRC was measured by centrifugation for 20 min at 2000× *g*. The supernatant was carefully removed, and the tubes were kept upside down for 10 min to ensure that the non-retained water was drained. Tubes with the sample were then weighed (W2), and dried in the oven at 100 °C for 16 h to ensure the complete drying of the insoluble residue, and then weighed again (W3). The solubility of the dry matter was calculated as follows:

$$SolDM = \frac{W1 - W3}{W0} \quad (2)\ (3)$$

Water retention capacity determined after centrifugation is expressed as the gram of water retained by the total amount of sample incubated:

$$WRC = \frac{W2 - W3}{W0} \quad (4)\ (5)$$

2.8. Short-Chain Fatty Acids

The SCFA in the ceca was analyzed as free acids by gas chromatography using pivalic acid as an internal standard [26]. Briefly, one mL of H_2O was mixed with 1 g of cecal content, and then one mL of 20 mM pivalic acid solution was added as an internal standard. After mixing, one mL of perchloric acid was added and the SCFA were extracted by shaking the mixture for 5 min. After centrifugation, perchloric acid in the supernatant was precipitated by adding 50 μL of 4 M KOH in 500 μL of supernatant. After 5 min, saturated oxalic acid was added and the mixture incubated at 4 °C for 60 min and then centrifuged. Samples were analyzed by gas chromatography using a glass column packed with 80/120 Carbopack B-DA/4% Carbowax 20M stationary phase (Supelco, Bellefonte, PA, USA), using helium as the carrier gas and a flame ionization detector. The acids measured were acetic, propionic, butyric, valeric, iso-butyric, 2-methyl-butyric, iso-valeric, and lactic acid.

2.9. Microbial Diversity Analysis

The DNA extracted from cecal samples was subjected to 16S ribosomal RNA gene sequence-based analysis to examine the profile of the bacterial communities. The V3-V4 region of the bacteria 16S ribosomal RNA gene were amplified by PCR (95 °C for 3 min, followed by 25 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s and 72 °C for 5 min) using primers F5′-barcode-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCW GCA G-3′ and R5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′. A negative control of the DNA extraction was included, as well as a positive Mock Community control, to ensure quality control. After 25 cycles of amplifications, 550 pb amplicons were obtained. The Illumina Miseq sequencing 300 × 2 approach was used. Raw sequencing reads were quality clipped, assembled, and compared with available genomic sequences in the databases using proprietary Software, and were validated and subsequently completed with the Kraken Metagenomics [27] and QIIME [28] software. Taxonomic assignment of phylotypes was performed using a Bayesian Classifier trained with Silva database version 132 (99% OTUs full-length sequences; [29].

2.10. Statistical Analysis

Individual weights were used to calculate the CV of BW within each cage. Normal distribution and homoscedasticity of variances was checked prior to the analysis by using the Shapiro-Wilk test and Levene's test for UNIVARIATE and General Linear Model (GLM) procedures, respectively. Cage

was the experimental unit for all other variables. Performance, SCFA, solDM, WRC, relative organ weights and coefficients of digestibility were analyzed as a factorial arrangement, using a 2-way ANOVA to identify any interaction between particle size and xylanase inclusion, with PROC GLM (SAS 9.4, SAS Institute Inc., Cary, NC, USA). In addition, orthogonal polynomial contrasts were used to test the linear and quadratic effects of increasing levels of particle size of the diets in PDI. Biostatistical analysis for microbiota was performed in open source software R-Studio v.3.6.1. (Boston, MA, USA). Diversity was analyzed at operational taxonomic units (OTU) level using a vegan package [30]. Richness and alpha diversity were calculated with raw counts based on Simpson, Shannon and Inverse-Simpson estimators. Beta diversity was evaluated by multivariate ANOVA based on dissimilarities, using the adonis function. Finally, differential abundance analysis was performed with taxa relative abundances under a zero-inflated log normal mixture model, *p*-values were corrected by false-discovery rate (FDR) using the metagenome Seq package [31]. Due to factorial arrangement, the main effects are discussed for responses in which the interaction was not significant. Significantly different means were separated using Tukey's Honestly Significant Difference (HSD) test. Significance was declared at a probability $p \leq 0.05$ and tendencies were considered when *p*-value was between >0.05 and <0.1.

3. Results

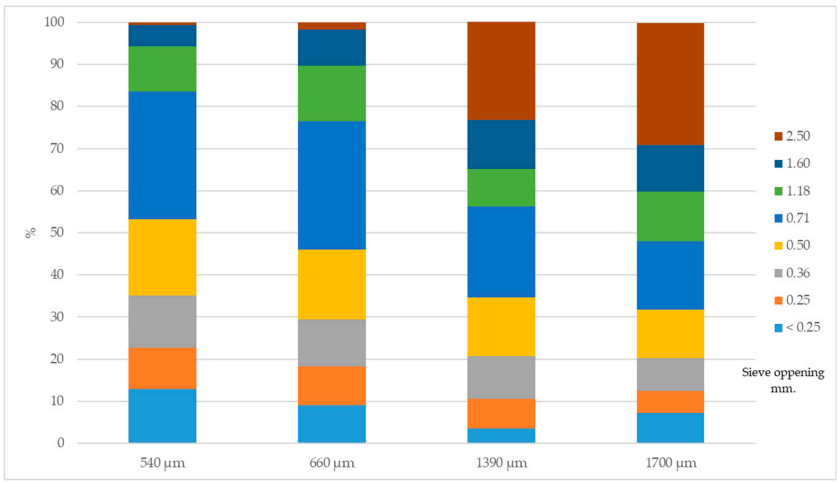
3.1. Feed Analyses and Diet Particle Size Analysis

The measurements of the nutrient composition (Table 1) and enzyme activities (Table 2) of all diets were all close to expected. Graphic comparisons of the particle size distributions of mash and pelleted diets (Figure 1) obtained by wet sieving, showed that pelleting reduced the relative proportion of coarse particles (>1000 μm) and increased the proportion of fine particles (<500 μm) in all diets. Geometric mean diameter (D_{gw}) of pelleted diets manufactured using a ground corn with D_{gw} 540, 660, 1390, and 1700 μm were determined to be 488, 549, 583, and 637 μm, respectively, with corresponding standard deviation (S_{gw}) values of 2.03, 2.13, 2.11, and 2.32 μm (Table 3).

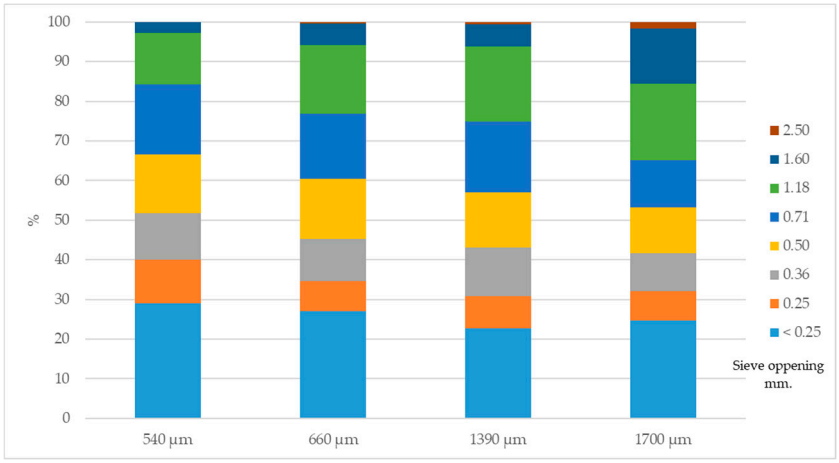
Table 2. Analyzed enzyme activities of xylanase in diets (d 0 to 21).

Diets [1]	Xylanase [2] (BXU/kg)
540	<2000
660	<2000
1390	<2000
1700	<2000
540 + Xylanase	12,400
660 + Xylanase	13,900
1390 + Xylanase	12,700
1700 + Xylanase	14,200

[1] Diets consisted of a four geometric mean diameter (D_{gw}) for corn (540, 660, 1390, and 1700, μm), and two levels of xylanase supplementation (0 or 16,000 BXU/kg diet). [2] One BXU is defined as the amount of enzyme that produces one nmol reducing sugars from birchwood xylan in ones at 50 °C and pH 5.3.



(**a**)



(**b**)

Figure 1. Particle size distribution of the three particle sizes of the mash (**a**) and pelleted diets (**b**).

Table 3. Geometric mean diameter (D_{gw}) and standard deviation (S_{gw}) of corn, mash, and pellet feed [1].

D_{gw} (µm)			S_{gw} (µm)		
Corn	**Mash Diet**	**Pellet Diet**	**Corn**	**Mash Diet**	**Pellet Diet**
540	617	488	2.33	2.79	2.03
660	701	549	2.37	2.85	2.13
1390	1032	583	2.54	3.01	2.11
1700	1170	637	2.48	3.04	2.32

[1] The four D_{gw} particle sizes (540, 660, 1390, and 1700, µm) of corn corresponding to 1500, 1200, 600, and 450 rpm mill speed, and 5, 5, 8, and 8 mm mill screen size, respectively. The soybean meal for all diets was milled at 1500 rpm and 5 mm screen size for all diets.

3.2. Pellet Quality, Solubilization of Dry Matter and Water Retention Capacity

Pellet quality, determined by PDI, differed (linear, $p = 0.014$; quadratic, $p = 0.006$; Figure 2) due to mash particle size, with 540 µm D_{gw} having an inferior PDI (87.1%) than 1390 µm (91.3%); 660 and 1700 µm D_{gw} corn diets had intermediate values (89.5% and 89.2%, respectively).

The SolDM and WRC results after the in vitro incubation at pH 2 or 5 are shown in Table 4. At pH 2, 540 µm D_{gw} diet increased SolDM compared with the two largest particle sizes, as did xylanase use compared to the controls (0.144 g vs. 0.126 g, $p = <0.0001$). An interaction between particle size and xylanase inclusion was observed at pH 5 for SolDM ($p = 0.002$), whereby an increment in SolDM was observed with the 660, 1390, and 1700 µm D_{gw} corn diets when xylanase was added but not in the 540 µm D_{gw} diet. Water retention capacity was greater with the 1390 and 1700 µm diets compared

with the 660 and 540 μm D_{gw} diets at pH 2, but no effects were noted at pH5 or with xylanase supplementation.

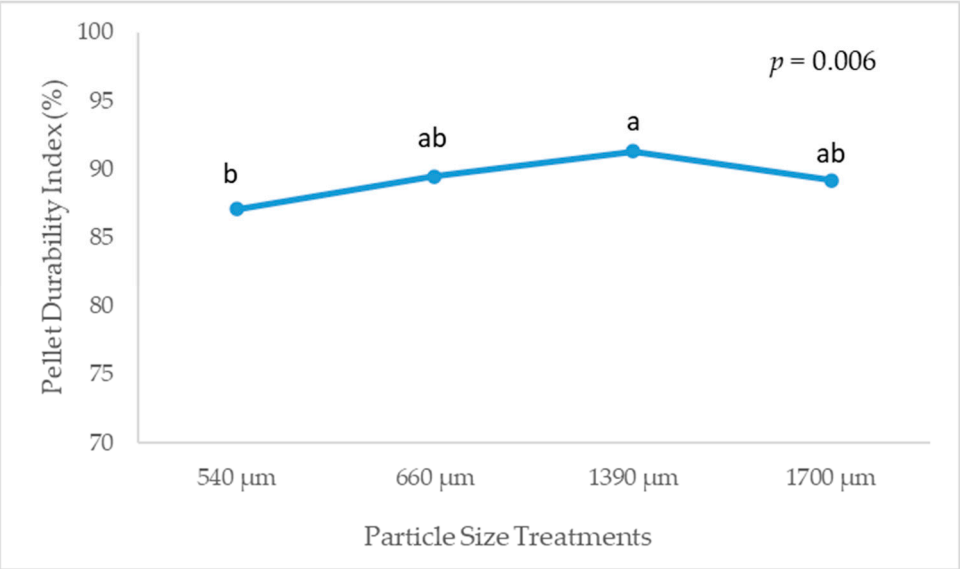


Figure 2. Effect of corn particle size on the pellet durability index ($n = 4$).
[a,b] Treatments with different letters are statistically different ($p < 0.05$).

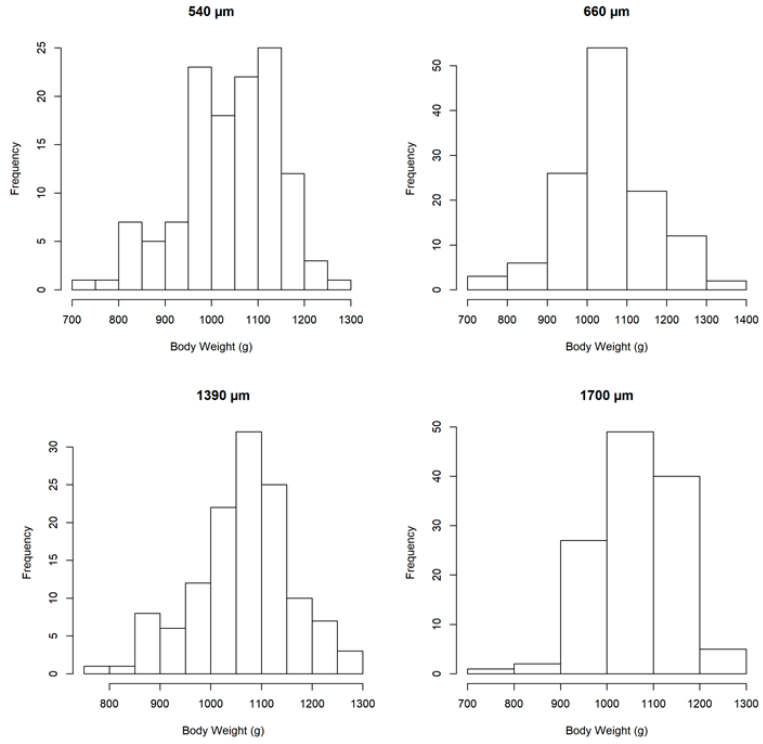
Table 4. Effects of particle size and xylanase inclusion on the in-vitro analysis [1,2].

		pH 2		pH 5	
Particle Size	**Xylanase**	**SolDM**	**WRC**	**SolDM**	**WRC**
540	-	0.136	2.16	0.149 [a]	2.25
	+	0.146	2.21	0.157 [a]	2.27
660	-	0.126	2.22	0.135 [b]	2.28
	+	0.143	2.11	0.153 [a]	2.23
1390	-	0.122	2.23	0.133 [b]	2.37
	+	0.141	2.27	0.156 [a]	2.31
1700	-	0.120	2.26	0.122 [b]	2.35
	+	0.147	2.28	0.154 [a]	2.24
SEM [3]		0.0033	0.044	0.0031	0.058
Main effect					
Particle size	540	0.144 [a]	2.18 [b]	0.153	2.27
	660	0.135 [ab]	2.17 [b]	0.144	2.26
	1390	0.132 [b]	2.25 [a]	0.144	2.34
	1700	0.133 [b]	2.27 [a]	0.138	2.30
SEM		0.0021	0.031	0.0002	0.043
Xylanase	-	0.126 [b]	2.22	0.135	2.32
	+	0.144 [a]	2.22	0.155	2.28
SEM		0.0013	0.018	0.0014	0.030
p-value [4]					
Particle Size		0.013	0.015	<0.0001	0.443
Xylanase		<0.0001	0.993	<0.0001	0.164
Particle size x Xylanase		0.051	0.105	0.002	0.880

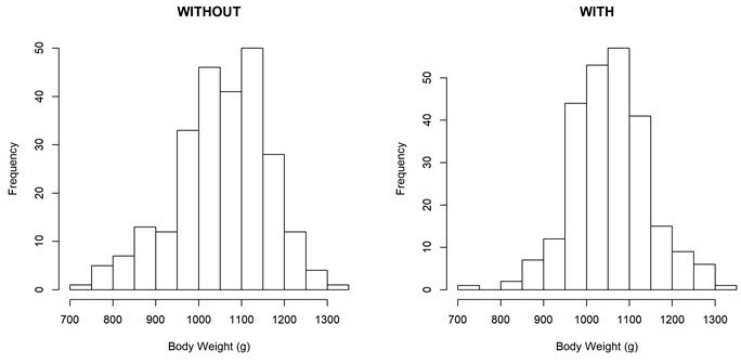
[1] Data are mean of 5 replicates for each treatment. [2] Dry matter solubility (SolDM) and water retention capacity (WRC) at pH 2 and pH 5 [3] Standard error of the mean. [4ab] Values in the same column with different letters are statistically different ($p < 0.05$).

3.3. Growth Performance

The effect of particle size and xylanase inclusion on BW and growth performance is described in Figure 3 and summarized in Table 5. Overall broilers mortality was 2.15% (data not shown), and no differences were observed between the experimental treatments ($p = 0.576$). No interactions ($p > 0.05$) were observed between particle size and xylanase supplementation on broiler performance. A statistical trend ($p = 0.083$) was observed for average daily feed intake (ADFI), showing a decrease in the 540 µm D_{gw} corn diet compared to the 1390 µm diet (55.7 vs. 57.3, g/bird/d). The BW CV was significantly affected by both particle size and xylanase. Birds fed the 1390 µm and 1700 µm D_{gw} corn diets had inferior CV compared to those on the 540 and 660 µm D_{gw} corn diets (8.3% & 7.4% vs. 9.9% & 9.2%; $p = 0.044$). Xylanase decreased BW CV (7.7%) compared to the birds fed control diets (9.7%; $p = 0.003$).



(**a**)



(**b**)

Figure 3. Effect of particle size (**a**) and xylanase inclusion (**b**) on the distribution of body weight (BW).

Table 5. Effects on performance [1] of particle size and xylanase inclusion [2].

Particle Size	Xylanase	BW (g)	ADG (g/d/bird)	ADFI (g/d/bird)	FCR (g/g)	CV (%)
540	-	1032	47.5	55.4	1.17	12.1
	+	1048	48.0	56.1	1.18	7.6
660	-	1050	48.0	56.6	1.18	10.4
	+	1059	48.8	56.9	1.17	8.1
1390	-	1076	49.5	58.2	1.18	8.1
	+	1053	48.2	57.5	1.19	8.5
1700	-	1060	48.6	56.9	1.17	8.1
	+	1066	48.6	57.8	1.18	6.6
SEM [3]		14.0	0.69	0.84	0.014	0.89
Main effect						
Particle size	540	1040	47.4	55.7	1.18	9.9 [b]
	660	1055	48.4	56.8	1.17	9.2 [b]
	1390	1064	48.8	57.9	1.18	8.3 [a]
	1700	1063	48.6	57.3	1.17	7.4 [a]
SEM		9.9	0.48	0.59	0.010	0.63
Xylanase	-	1055	48.4	56.8	1.17	9.7 [b]
	+	1057	48.3	57.1	1.18	7.7 [a]
SEM		7.0	0.34	0.42	0.007	0.44
p-value [4]						
Particle Size		0.280	0.189	0.083	0.886	0.044
Xylanase		0.836	0.848	0.637	0.486	0.003
Particle size x Xylanase		0.520	0.537	0.762	0.835	0.074

[1] Body weight (BW), average daily gain (ADG), average daily feed intake (ADFI), 0–21 day feed conversion ratio (FCR) and BW coefficient of variation (CV). [2] Data are means of 8 pens with 8 birds (d 0–21) by treatment. [3] Standard error of the mean. [4ab] Values in the same column with different letters are statistically different ($p < 0.05$).

3.4. Apparent Ileal and Total Tract Digestibility

No interactions in ileal and total tract (Tables 6 and 7) digestibility were observed between particle size and xylanase. Ileal digestibility was increased when feeding the 1390 and 1700 μm compared with the 540 and 690 μm D_{gw} corn diets for protein (86.2% & 86.2% vs. 83.8% & 84.4%; $p = 0.001$) and apparent energy digestibility (78.1% & 78.8% vs. 76.6% & 76.4%; $p = 0.029$). Apparent ileal digestibility of energy ($p = 0.029$) and OM digestibility ($p = 0.007$) were found to be decreased in birds fed the xylanase diet. Total tract digestibility showed that birds fed 540 μm D_{gw} corn diet had inferior protein digestibility compared to birds fed the 1390 and 1700 μm D_{gw} corn diets (66.8% vs. 70.2% & 70.1%; $p = 0.001$). Apparent total tract energy and DM digestibility were both reduced ($p = 0.025$ and $p = 0.001$, respectively) with xylanase supplementation.

The estimation of total intake of digestible nutrients showed a significant increment in the ileal samples on protein and energy for coarse particle diets (1700 and 1390 μm D_{gw} corn diets) compared to the fine diets (540 and 660 μm D_{gw} corn diets). Additionally, total intake of digestible nutrients showed a significant increment in the fecal samples on protein, energy, and OM for 1700, 1390, and 660 μm D_{gw} corn diets compared to the 540 μm D_{gw} corn diet.

Table 6. Effects of particle size and xylanase inclusion on 21-day apparent ileal digestibility and intake of digestible nutrients [1,2].

Main Effects		Ileal Digestibility (%)				Intake of Digestible Nutrients			
		Protein	Energy	OM	Starch	Protein g	Energy kcal	OM g	Starch G
Particle size	540	83.8 [b]	76.6 [b]	74.1	96.8	220 [b]	3848 [b]	776 [b]	319
	660	84.4 [b]	76.4 [b]	73.5	96.1	221 [b]	3839 [b]	770 [b]	320
	1390	86.2 [a]	78.1 [a]	75.1	96.5	235 [a]	4045 [a]	811 [a]	328
	1700	86.2 [a]	78.8 [a]	76.2	96.5	233 [a]	4033 [a]	810 [a]	326
SEM		0.47	0.66	0.78	0.31	3.7	53.1	10.9	3.6
Xylanase	-	85.4	78.2 [a]	75.8 [a]	96.7	225	3968	797	322
	+	84.9	76.8 [b]	73.7 [b]	96.3	229	3914	786	324
SEM[2]		0.35	0.47	0.55	0.22	2.9	36.6	7.9	2.5
p-value [3]									
Particle Size		0.001	0.029	0.086	0.525	0.011	0.003	0.013	0.135
Xylanase		0.29	0.037	0.007	0.139	0.429	0.277	0.311	0.605
Particle size x Xylanase		0.515	0.775	0.867	0.099	0.537	0.645	0.486	0.511

[1] Data are a pool of 3 birds ileal contend per pen with 8 pen per treatment. [2] Protein, energy, dry matter (DM), organic matter (OM) and starch. [2] Standard error of the mean. [3ab] Values in the same column with different letters are statistically different ($p < 0.05$).

Table 7. Effects of particle size and xylanase inclusion on 21-day apparent total tract digestibility and intake of digestible nutrients [1,2].

Main Effects		Total Tract Digestibility (%)			Intake of Digestible Nutrients		
		Protein	Energy	OM	Protein g	Energy kcal	OM g
Particle size	540	66.8 [b]	77.6	74.6	171 [c]	3862 [b]	794 [b]
	660	69.5 [ab]	78.5	74.9	182 [b]	4016 [ab]	822 [a]
	1390	70.2 [a]	78.1	74.9	189 [a]	4066 [a]	836 [a]
	1700	70.1 [a]	78.7	74.9	185 [ab]	4128 [a]	827
SEM		0.61	0.44	0.55	2.2	64.1	9.4
Xylanase	-	69.5	78.7 [a]	75.3	181	3987	818
	+	68.9	77.7 [b]	74.3	183	4050	821
SEM [3]		0.41	0.3	0.37	1.5	42.6	6.5
p-value [4]							
Particle Size		0.001	0.296	0.961	<0.0001	0.031	0.016
Xylanase		0.367	0.025	0.058	0.434	0.317	0.700
Particle size x Xylanase		0.788	0.633	0.765	0.857	0.293	0.485

[1] Data are a pool of 8 birds feces samples per pen with 8 pen per treatment. [2] Protein, energy, dry matter (DM) and organic matter (OM) [3] Standard error of the mean. [4abc] Values in the same column with different letters are statistically different ($p < 0.05$).

3.5. Relative Organ Weights

There were no interactions between particle size and xylanase inclusion for relative organ weights, and the relative weights of duodenum, jejunum, ileum, and small intestine were not influenced by the main factors (Table 8). Coarse diets (1390 and 1700 μm D_{gw}) resulted in heavier ($p < 0.001$) gizzards compared with the 540 μm D_{gw} corn diet (1.78 & 1.91 vs. 1.54 g/100 g BW), whilst the caecum was heavier ($p = 0.004$) when birds were fed the 540 μm D_{gw} corn diet compared with the 1390 μm D_{gw} corn diet (0.42 vs. 0.38 g/100 g BW).

Table 8. Effects of particle size and xylanase inclusion on 21-day relative organs weight [1].

Main Effects		Gizzard	Duodenum	Jejunum	Ileum	Ceca	Small Intestine [2]
		g/100 g of Body Weight					
Particle size	540	1.54 [c]	1.11	1.41	1.29	0.42 [a]	3.82
	660	1.66 [bc]	1.14	1.38	1.28	0.40 [ab]	3.83
	1390	1.78 [ab]	1.15	1.36	1.22	0.38 [b]	3.75
	1700	1.91 [a]	1.13	1.27	1.29	0.40 [ab]	3.71
SEM [3]		0.051	0.033	0.042	0.032	0.011	0.089
Xylanase	-	1.75	1.15	1.35	1.28	0.39	3.80
	+	1.69	1.11	1.37	1.25	0.39	3.76
SEM		0.035	0.023	0.029	0.024	0.007	0.063
p-value [4]							
Particle Size		<0.0001	0.826	0.102	0.353	0.004	0.767
Xylanase		0.288	0.227	0.599	0.247	0.831	0.407
Particle size x Xylanase		0.721	0.646	0.805	0.353	0.314	0.917

[1] Data are means of 3 birds per cage with 8 pens per treatment. [2] Small intestine = Duodenum + Jejunum + Ileum. [3] Standard error of the mean. [4abc] Values in the same column with different letters are statistically different ($p < 0.05$).

3.6. Microbial Diversity Analysis

No interactions were observed in the analysis of alpha and beta diversity. Three alpha-diversity estimators were performed (Simpson, Shannon and Inverse Simpson). Birds fed the 1700 μm D_{gw} corn diet showed greater diversity than birds fed the 540 μm D_{gw} ($p < 0.05$; Table 9) corn diet in Shannon and Inverse Simpson estimators. There were no effects of xylanase supplementation ($p > 0.10$) on any estimate of diversity. For the beta-diversity, 1700 μm D_{gw} corn diet had a different ($p = 0.04$) diversity composition compared to the 540 μm D_{gw} corn diet (Figure 4).

Table 9. Effects particle size [1] and xylanase inclusion in alpha diversity on 21-day cecal microbiota [2].

Main Effects		Shannon	Simpson	Inverse-Simpson
Particle size	540	4.22 [b]	0.96	31.0 [b]
	1700	4.44 [a]	0.97	44.1 [a]
SEM [3]		0.068	0.004	4.49
Xylanase	-	4.28	0.96	35.4
	+	4.38	0.96	39.4
SEM		0.068	0.004	4.49
p-value [4]				
Particle size		0.034	0.146	0.048
Xylanase		0.282	0.471	0.566
Particle size x Xylanase		0.092	0.155	0.290

[1] Only 1700 and 540 μm D_{gw} diets, with and without xylanase, were analyzed. [2] Data are a mean of 3 birds per pen with 8 pen per treatment. [3] Standard error of the mean. [4ab] Values in the same column with different letters are statistically different ($p < 0.05$).

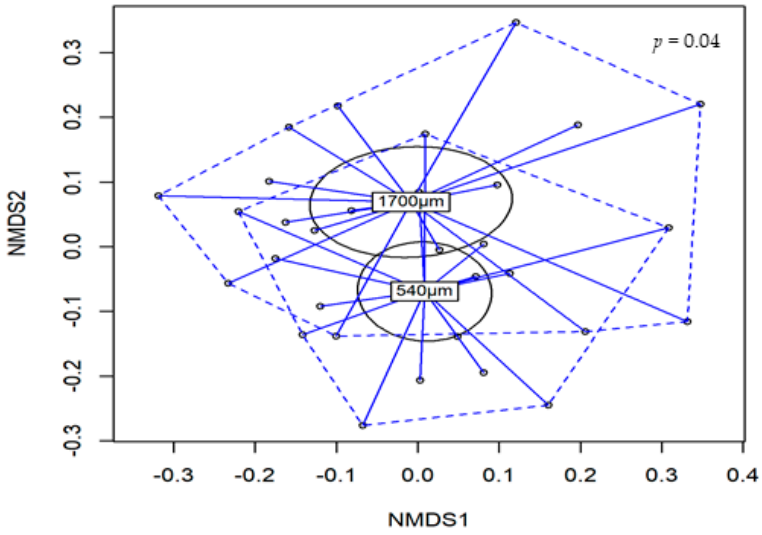


Figure 4. Comparison of beta-diversity of caecum microbiota between 540 and 1700 µm D_{gw} corn diets. The nonmetric-multidimensional scaling (NMDS) plots was generated using Bray-Curtis distances. *p*-value was obtained from adonis analysis.

3.7. Composition of Gut Microbiota

At the phylum level, 7 phyla were determined, with Firmicutes predominant with a total abundance >83%, followed by Bacteroidetes, Tenericutes, Proteobacteria, and Actinobacteria and other less abundant phyla. No effects were observed at phylum, class, and order levels ($p < 0.10$; data not shown). Some family members were influenced with xylanase supplementation in the 540 and 1700 µm D_{gw} corn diets (Figure 5). Defluviitaleaceae, Bacteroidaceae, Bacillaceae, Eggerthellaceae, Streptococcaceae, Lachnospiraceae, and Clostridiales vadinBB60 group were increased ($p < 0.05$) in the 1700 µm diet when the xylanase was added. On the other hand, Peptococcaceae, Peptostreptococcaceae and Lactobacillaceae families were reduced when the enzyme was supplemented in this diet. In the 540 µm D_{gw} corn diet, Christensenellaceae, Lachnospiraceae, and Peptococcaceae ($p < 0.10$) were increased when xylanase was added. In contrast, the relative abundance of Enterobacteriaceae was reduced in xylanase supplemented birds ($p < 0.05$). Additionally, particle size and xylanase inclusion as a main effect showed significant changes (Figures 6 and 7) at family (f.) and genus (g.) levels, individually (Figures 6 and 7). Therefore, f. Christensenellaceae, f. Eubacteriaceae, g. *Ruminiclostridium* 1, g. *Lachnospira*, g. *Ruminococcus* 1, g. *Hydrogenoanaerobacterium*, g. *Christensenellaceae R-7 group*, g. *Fournierella*, g. *Merdibacter*, g. *Eubacterium coprostanoligenes group*, g. *Anaerofustis* and g. *Ruminococcaceae UCG-009* showed a greater presence in birds fed with the 1700 µm D_{gw} corn diet compared to birds fed the 540 µm D_{gw} corn diet (p-adjust ≤ 0.05). Other changes at the family level were observed due to particle size such as the increase in 1700 µm D_{gw} corn diet of Christensenellaceae, Eubacteriaceae, Peptococcaceae, Defluviitaleaceae, Lactobacillaceae, and Bacillaceae, and a reduction of Peptostreptococcaceae in 1700 µm D_{gw} corn diet compared to the 540 µm D_{gw} corn diet ($p < 0.15$). Xylanase inclusion showed a significant increment in f. Lachnospiraceae, g. *Gordonibacter,* and g. *Pseudomonas*, and a reduction of f. Eubacteriaceae, g. *Eubacterium hallii group*, g. *Tyzzerella 3*, and g. *Intestinimonas* ($p < 0.05$).

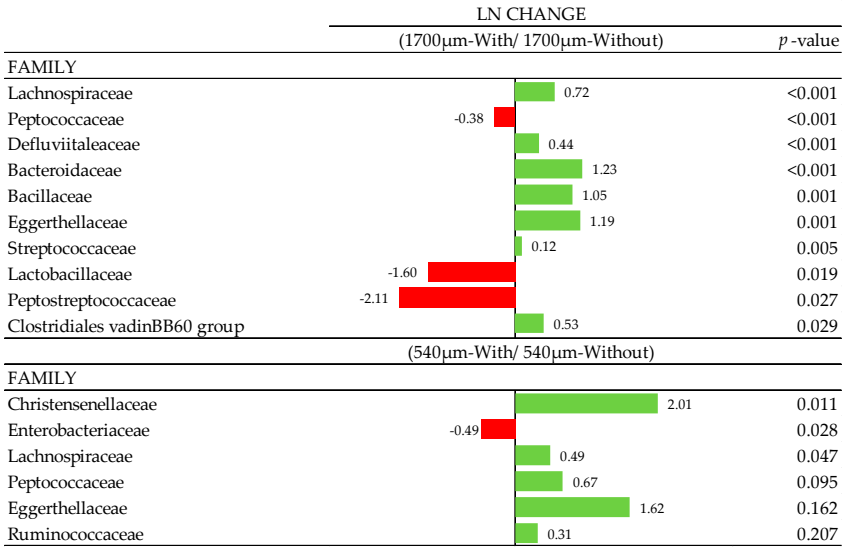


Figure 5. Ln changes promoted by xylanase inclusion in 540 and 1700 µm D_{gw} corn diets (fold discovery rate *p*-adjusted < 0.20) in taxa. Positive values ( ) and negative values ( ) indicate greater and lower abundance. Taxa are sorted by level of significance (from higher to lower). Differences presented are based on all taxa detected in samples per diet.

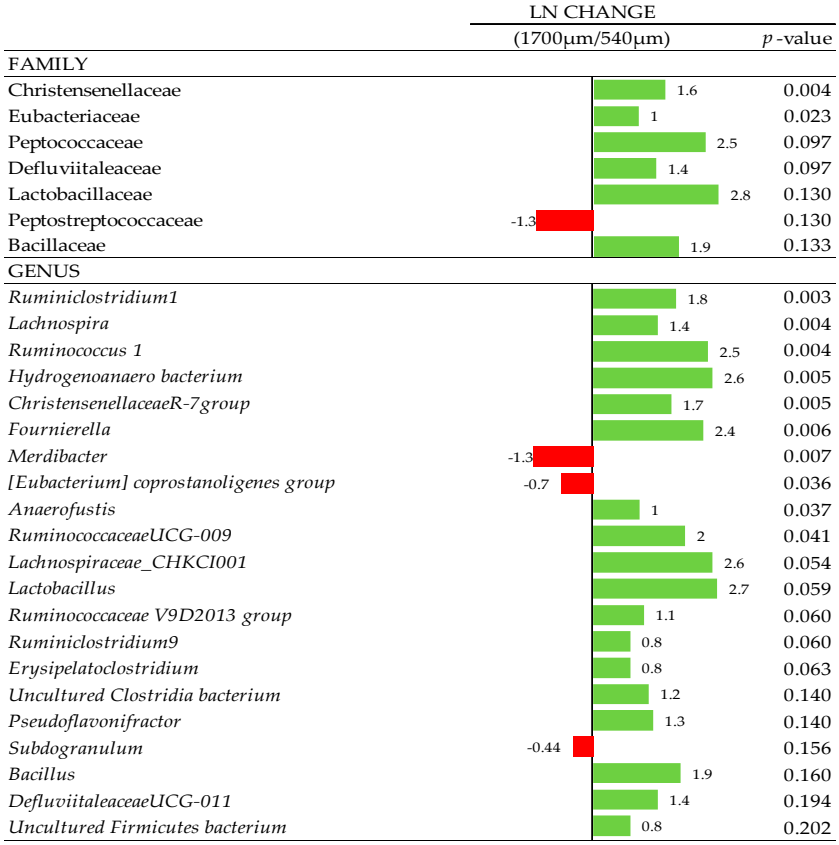


Figure 6. Ln changes promoted by the particle size (1700 and 540, µm) of D_{gw} corn diet (fold discovery rate *p*-adjusted < 0.20) in taxa. Positive values ( ) and negative values ( ) indicate greater and lower abundance. Taxa are sorted by level of significance (from higher to lower). Differences presented are based on all taxa detected in samples per diet.

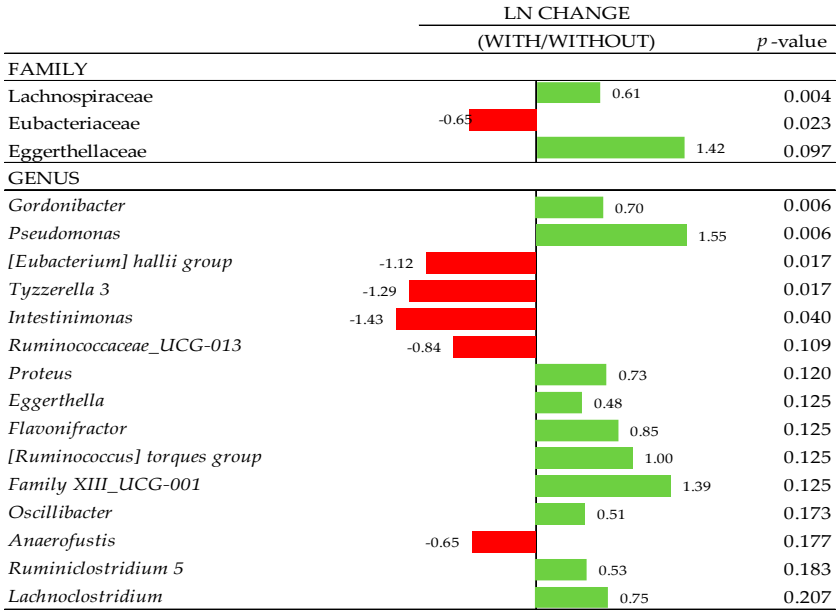


Figure 7. Ln changes promoted by xylanase inclusion (fold discovery rate *p*-adjusted < 0.20) in taxa. Positive values ( ) and negative values ( ) indicate greater and lower abundance. Taxa are sorted by level of significance (from higher to lower). Differences presented are based on all taxa detected in samples per diet.

3.8. Determination of Short-Chain Fatty Acids

For SCFA, a significant interaction between particle size and xylanase inclusion was observed for cecal digesta propionic acid ($p = 0.027$; Table 10). Xylanase supplementation in the 1700 µm D_{gw} corn diet reduced propionic acid concentration compared to the same diet without the enzyme (3.8% vs. 5.0%), but this effect was not observed in the other particle size diets. Lactic acid concentration was increased in birds fed 540 µm D_{gw} corn diet than those fed the coarse 1700 µm D_{gw} corn diet (9.4% vs. 7.3%; $p = 0.025$) and total volatile fatty acid (VFA) was increased with the 1700 µm D_{gw} corn diet compared to the 540 µm D_{gw} corn diet (92.8% vs. 90.6%; $p = 0.029$).

Table 10. Effect of particle size [1] and xylanase inclusion on total short-chain fatty acid [2,3].

					Acid				
Particle Size	**Xylanase**	**SCFA mM**	**VFA**	**BCFA**	**Acetic**	**Propionic %**	**Butyric**	**Valeric**	**Lactic**
540	-	97.3	90.5	1.93	71.3	4.1 ^{bc}	12.1	1.07	9.5
	+	98.5	90.7	1.80	71.4	4.7 ^{abc}	11.7	0.97	9.3
660	-	96.9	91.5	1.73	72.3	4.4 ^{abc}	12.0	0.95	8.5
	+	93.0	92.1	1.95	71.5	5.3 ^{a}	12.3	1.01	7.8
1700	-	93.1	92.5	1.85	71.7	5.0 ^{ab}	12.9	1.03	7.5
	+	91.8	92.9	1.81	74.7	3.8 ^{c}	11.7	0.83	7.0
SEM [5]		4.33	0.802	0.159	1.43	0.40	0.88	0.090	0.80
Main effect									
Particle size	540	97.9	90.6 ^{b}	1.86	71.3	4.5	11.9	1.03	9.4 ^{a}
	660	95.0	91.8 ^{ab}	1.84	71.9	4.8	12.2	0.98	8.1 ^{ab}
	1700	92.5	92.8 ^{a}	1.83	73.2	4.4	12.3	0.94	7.3 ^{b}
SEM		3.06	0.56	0.112	1.01	0.28	0.62	0.063	0.56
Xylanase	-	95.8	87.7	1.83	71.8	4.5	12.4	1.02	8.48
	+	94.4	86.9	1.85	72.5	4.6	11.9	0.94	8.03
SEM[4]		2.50	2.52	0.092	0.82	0.23	0.51	0.052	0.462
p-value [5]									
Particle Size		0.459	0.039	0.981	0.416	0.519	0.911	0.628	0.039
Xylanase		0.711	0.565	0.898	0.527	0.697	0.552	0.288	0.507
Particle size*Xylanase		0.836	0.947	0.552	0.403	0.028	0.721	0.349	0.954

[1] Only 540, 660 and 1700 μm D_{gw} corn diets, with and without the enzyme were analyzed. [2] Data are a mean of 5 birds per pen with 8 pen per treatment. [3] Volatile fatty acid (VFA), branched-chain fatty acid (BCFA) and lactic acid concentrations in ceca content are expressed in percentage of the total SCFA. [4] Standard error of the mean. [5abc] Values in the same column with different letters are statistically different ($p < 0.05$).

4. Discussion

The broiler chickens in the current study performed above breed standards (959 g vs. 1055 g at day 21). This result may reflect optimum management and environmental conditions produced by the cage allocation and feeding with well-equilibrated corn soybean diets. This sets the experimental results in context.

4.1. Particle Size

In the present study, the effect of the second grinding of pelletizing clearly reduces the particle size of the coarse and medium diets, narrowing the gap with the fine diets. Thus, the differences in particle size distribution post-pelletizing, added to the good management and environmental conditions, were not sufficiently large to cause any differences in bird performance. However, significant changes were observed due to the particle size on the in-vitro SolDM and WRC, CV of body weight, nutrient digestibility, organ relative weight and changes in the microbiota profile. Thus, the 1700 um Dgw corn diet was associated with heavier relative gizzard weights, greater WRC and ileal digestibility of protein, energy, DM and OM, and greater alpha and beta microbiota diversity with many changes in the relative abundance of taxonomic bacteria composition. The benefits of gizzard development are described in the literature and include a prevention of pathogenic bacteria entering the small intestine [32–34], increased gizzard contractions, HCl production and gastrointestinal reflux [33,35,36]. Moreover, the increase of HCl secretion and exposure time of nutrients to digestive enzymes may improve nutrient digestibility [37,38] and enhance GIT motility [33,35,36]. Lastly, it has been reported that a lower pH of gizzard contents may increase pepsin activity [35] and improve protein digestion, confirming the important influence coarse particles have in poultry gut function. In this way, several studies in poultry with different cereals showed an improvement in the size of the gizzard and the nutrient utilization when coarse diets or post-pelleting

of whole grain were used [6,7,18]. Therefore, Sing and Ravindran [39] indicated that 115 g/kg of ground corn can be replaced by whole corn in broiler starter diets without adverse effects on growth performance; however, the increase in the relative weight of the gizzard at 21 days was lower than in the present study (15,4 vs. 19.1, g/kg, respectively). Alternatively, Vukmirovic et al. [9] reported that the use of hammer mill with a sieve of 9 mm and a roller pellet press of 2 mm was the best option to conserve coarse particles with higher PDI and reduce the cost of pelletizing. In the present study, a similar configuration was used to obtain the coarsest corn treatments (1390 and 1700 μm) with similar results preserving coarse particles and PDI.

On the other hand, although SolDM at pH 5 was increased in the 540 μm D_{gw} corn diet, no positive changes were observed in the nutrient digestibility. Contrary, the apparent ileal digestibility of protein and energy were significantly decreased in this diet. Thus, the negative effect on the gizzard development, added to the decrease in PDI, could explain the inferior digestibility and increased CV observed in this treatment group. Thus, a positive correlation between pellet durability and feed efficiency has been described [40].

As we noticed previously, important changes in microbiota composition, such as the greater alpha and beta diversity, were observed when birds were fed with 1700 μm D_{gw} corn diet. This suggests that intestinal microbiota diversity could be improved when the proximal digestive tract is developed with coarse particles. Moreover, coarse particles increased the relative abundance of *Ruminoclostridium1, Lachnospira and Ruminococcus1*, and numerically increased families like Lactobacillaceae and Bacillaceae, suggesting a greater relative abundance of butyric, propionic, and acetic producers [41–43]. Some of these bacteria are related to a better performance in broilers [44,45]. Thus, it seems that an improvement in the development of proximal GIT due to feeding coarser particle pelleted diets promoted positive changes in the intestinal microbiome, with significant increases in nutrient digestibility and VFA production. Although no significant effects were observed on performance, the increased BW homogeneity observed when birds were fed the 1700 μm D_{gw} corn diet may suggest a better GIT development with positive effects on gut functions.

4.2. Xylanase

In-vitro results showed that xylanase increased the DM solubility at pH 2, and interacted with particle size at pH 5, showing a greater solubility in coarse diets. These results suggest a release of encapsulated nutrients or/and the production of XOS or AXOS from the plant cell walls, as suggested by some authors [46–48]. Thus, several studies have reported benefits when XOS were included in pig and poultry diets [49–52]. Therefore, the XOS obtained when xylanase was added could improve the relative abundance of Lachnospiraceae family, and several members of this family are recognized as butyric-producer bacteria [51]. Consequently, the significant increment of these families suggest that these specific bacterial communities, fiber degrading bacteria, could improve the production of SCFA that are correlated with gut health improvements, but are also a good source of energy for the intestine [53]. On the contrary, the current study did not show differences in SCFA when xylanase was added, which might be related with the extremely fast turnover rate of SCFA from the intestine into the blood [54], and/or the preference for this VFA over others in the intestinal epithelial cells and the liver [55]. Thus, the GIT is not only a site for digestion and absorption of nutrients but also experiences enormous interactions with the microbiota, and functions as a metabolic and immunological organ [56]. Thus, xylanase supplementation could have a role in reducing the immunological stress in the gut, probably through microbiota modulation and subsequent reduction in the metabolic yield of an innate immune system, which increases the availability of nutrients for growth rather than maintenance. In the present study, although there were no changes in performance, xylanase improved BW homogeneity, which is intriguing, given the digestibility data. Likewise, predicting long-term effects of a diet on performance parameters, such as BW gain or FCR, based on nutrient digestibility responses to xylanase also appeared to be incongruous in previous studies [49]. However, when estimating the total intake of digestible nutrients, using digestibility coefficients, the low digestibility associated with the xylanase supplementation disappears, showing a better adjusted estimation on digestibility effects.

4.3. Particle Size and Xylanase Interaction

No interaction between particle size and xylanase supplementation was observed for any productive parameters in the present study. In general, the agreement is that smaller particles increase the specific surface of feed particles allowing better contact with the enzymes. However, significant benefits on performance and digestibility have been reported when exogenous enzymes supplementation was accompanied by coarse particles or whole grain in the diets [4,18]. Therefore, it is reasonable to suggest that the increasing gizzard size improves exogenous enzyme response due to increased peristalsis, mixing, pH, and gut stimulation. On the latter, Kheravii et al. [57] demonstrated that the coarse particles modulate expression of genes encoding important digestive enzymes and nutrient transporters, with consequent benefits in the performance of birds [57]. Although, no significant interactions on digestibility or performance were observed in the present study, there were significant changes in the microbiota profile of both 1700 and 540 μm D_{gw} corn diets when xylanase was added. Xylanase addition supported the growth of bacteria producing SCFA from polysaccharides, such as the Defluviitaleaceae, Bacillaceae, and Lachnospiraceae families [58,59] in the 1700 μm D_{gw} corn diet, and Christensenellaceae and Lachnospiraceae families [60,61] in the 540 μm D_{gw} corn diet. Specifically, the Lachnospiraceae family, described as butyric acid producers, have genes coding for enzymes (xylanases, cellulases) to degrade a wide variety of polysaccharides [62], and as a result, may have protective properties against digestive disorders in pigs [63]. It is important to highlight that the Enterobacteriaceae family was reduced when xylanase was included in 540 μm D_{gw} corn diet, which could have a role in explaining the reduction in the BW CV observed with this diet. Furthermore, inhibitory effects of SCFA on pathogenic bacteria, such as *Salmonella*, a zoonotic agent belonging to the Enterobacteriaceae family, have also been described [64,65]. Therefore, the promotion of specific bacterial communities that can degrade complex substrates, such as non-starch polysaccharides, leading to better growth performance of the broiler chicken, is certainly the most important mechanism that is discussed when xylanase is proposed as a feed additive in corn based diets for poultry.

Overall, particle size determined the development and functionality of the GIT, which instead, clearly influence the gut microbiota, showing different responses to the dietary supplementation of xylanase.

5. Conclusions

It is concluded that xylanase supplementation and particle size of corn interact in the intestinal environment, producing changes in microbial composition. Coarse diets and xylanase supplementation improved body weight homogeneity, gut development, and microbiota modulation.

Author Contributions: Conceptualization and Methodology: J.F.P., D.S.-O., G.G.-O., and D.M.-D.; Investigation: D.M.-D., S.V.-E., J.F., and D.S.-O.; Data Curation and Software: D.M.-D.; Validation: J.F.P., D.S.-O., and G.G.-O.; Formal Analysis: D.M.-D. and S.V.-E.; Writing—Original Draft Preparation: D.M.-D., J.F.P., and D.S.-O.; Writing—Review and Editing: R.S., M.B, H.G., and G.G.-O.; Supervision: J.F.P., D.S.-O., and G.G.-O. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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CHAPTER IV

GENERAL DISCUSSION

Livestock production faces several challenges related to the use of natural resources, such as water and land. It is the poultry sector that needs the most land for cereal production, an estimated 44% of the total area demanded by the global livestock sector (Mottet et al., 2017). Consequently, efficiency in the use of feed is one of the most important issues that the poultry industry needs to improve. One of the main concerns that could affect the efficiency of animal diet is the presence of anti-nutrients, which can produce a decrease in the birds' digestibility and performance, with the consequent excretion of nutrients into the environment. A key technology that can help to address these challenges is the suitable use of exogenous enzymes (Choct, 2006). These are currently added to animal diets in order to enhance growth performance and feed conversion, and ameliorate environmental problems, since fewer undigested nutrients are excreted. The most widely used groups of enzymes in animal diets include carbohydrases, including xylanase, which is commonly added to cause degradation of arabinoxylans within viscous grains (e.g. barley, oats, and wheat), hence improving their nutritional value (Gonzalez-Ortiz et al., 2017; González-Ortiz et al., 2020). However, the use of xylanase has also been tested in corn-based diets with inconsistent animal performance results (Gehring et al., 2013; Stefanello et al., 2016).

The present thesis aims to address the possible factors that could alter the response of xylanase in corn-based diets, as well as to improve knowledge about its mechanisms of action.

The mechanisms of action of xylanase allow the replacement of antibiotics in animal production.

Currently, the restrictions on the use of antibiotics as growth promoters and the need to produce antibiotic-free animal protein mean it is crucial to look for alternative strategies. Regarding this concern, the present dissertation has suggested that the

mechanisms of action of carbohydrases, especially xylanase, not only improve the digestibility of nutrients but also improve intestinal health and modulate microbiota, thus allowing them to be considered part of the replacement scheme for the use of antibiotics such as growth promoters in animal production.

Therefore, in **Articles II, III and IV** the results of the in vitro analysis showed that xylanase supplementation is capable of increasing soluble compounds in corn and final diets, indicating that the enzyme could release nutrients trapped between arabinoxylans chains and/or produce xylo-oligosaccharides. Regarding the latter, **Article II** reports that xylanase enzyme supplementation leads to a 42 % increase in the production of xylotriose from the arabinoxylans of the corn grain, after the "in vitro" process. Supplementation with xylo-oligosaccharides in broiler diets has shown an improvement in performance, which can potentially be explained by the stimulation of butyrate-producing bacteria (De Maesschalck et al., 2015). Indeed, in **Article IV**, it was reported that this microbiota stimulation was observed when chickens were fed diets supplemented with xylanase. Thus, the metagenome sequence of the intestinal microbiota allowed us to observe an increase in the relative abundance of Lachnospiraceae, Bacillaceae and Ruminococcaceae and a reduction in Enterobacteriaceae families when xylanase was included. The negative relationship between butyrate-producing species and the Enterobacteriaceae family has been described previously (Van Immerseel, 2003; Vermeulen et al., 2017), and could be an important strategy to control several bacteria pathogens. It has also been reported that xylanase and fermented polysaccharides ameliorate pathogen infection by increasing the antioxidant and anti-inflammatory activities of broilers via an increase in probiotics (Zhang et al., 2018).

Therefore, the production of xylo-oligosaccharides through xylanase supplementation in corn-based diets is presented as one of the important mechanisms by which performance and health benefits can be obtained. However, changes in performance are inconsistent in the literature when xylanase is included in corn-based diets (Gehring et al., 2013; Stefanello et al., 2016). Although no changes in weight gain and feed efficiency were observed in **Article IV**, an improvement in body weight homogeneity due to xylanase supplementation was reported, showing the enzyme's ability to help animals that may be lagging due to different circumstances. Consequently, the release of trapped nutrients and the promotion of specific bacterial communities that can degrade complex substrates, such as non-starch polysaccharides, are ways to maintain the balance of nutrients and microbiota in the gut even in adverse or stressful situations.

In this context, it is difficult to conclude that enzymes per se could replace antibiotics in efficient animal production, but it is plausible to believe that any strategy to reduce the negative impact of antinutritional factors as challenging compounds for both intestinal health and efficiency is welcome. Hence, the use of enzymes, such as xylanase, could be part of a holistic approach to nutritional strategies as an alternative to the use of antibiotics in animal production.

The variability of corn physicochemical composition affects the xylanase response.

Regarding the inconsistent response in the productive behavior of chickens when xylanase is added to corn-based diets, the present dissertation proposes that variability in the physicochemical composition of corn can generate negative interactions between macronutrients and fiber components, and that these changes could be one of the main factors influencing the xylanase response. On this issue, **Articles II and III** evaluated genotype and kernel size, as two of the less studied factors that may cause variability in

the physicochemical composition of corn. **Article II** reported that both the genetic background and the position of the kernels on the cob significantly affected most physicochemical components. However, it is noted that most of these components showed an interaction between both factors, including for apparent metabolizable energy (AME), crude fat, crude fiber, neutral detergent fiber, acid detergent fiber, total and soluble non-starch polysaccharides (NSP), total and soluble arabinoxylans, phytic phosphorus, protein solubility index, vitreous, and total arabinose: xylose ratio. These interactions showed that variability in nutrient composition between apical and basal grains on the cob strongly depends on the genetic variety of corn.

Regarding kernel size, our data revealed that apical kernels can amount to 23.3% of the total grains on the cob, with less AME (258 kJ/kg) and crude protein (3 g/kg) content than their homologues at basal position. Furthermore, an interaction was observed for AME between kernel position and genotype, the most extreme variation being a difference between portions of 432 kJ/kg. To our knowledge, this is the first study to report the potential effect that grain position on the cob has on nutritional composition. The present results will enable further studies evaluating the effect that grain position has on the variability of final diets and consequently on animal performance. Similarly, genetic background shows a difference in AME and protein of up to 1392 kJ/kg and 19 g/kg, respectively, among 16 corn varieties. These energy and protein values may directly affect the nutritional value of poultry diets if the matrix of corn nutrient composition is not adjusted (Latham et al., 2016).

Regarding fiber components, our results show an influence of genetics and kernel cob position. In terms of total non-starch polysaccharides, a difference of 5.2 g/kg is observed between kernel cob positions, apical grains having the highest concentration.

Moreover, the genotype interacted with kernel cob position, with a higher total NSP difference in some hybrids, including a difference of up to 15.6 g/kg between apical and basal kernels. However, the highest differences were observed among corn varieties, with a difference of up to 25.9 g/kg of total NSP, suggesting that some genotypes could have more negative effects due to these anti-nutritional factors.

In this context, one of our objectives was to provide new information regarding the possible negative interactions between these nutritional components. A negative correlation between total NSP content and dry matter solubility was observed in corn and final diets, suggesting that higher NSP levels could trap some nutrients. Such trapping has been observed through in vitro microscopic studies in viscous cereals (Jha et al., 2015; Ravn et al., 2016), and evaluated in chickens fed corn-based diets without conclusive results (Khadem et al., 2016). Our results also show that protein solubility index is negatively correlated with total NSP and soluble arabinoxylans. However, the chemical composition of the chains could also play an important role in nutrient trapping, for the soluble arabinose:xylose ratio showed a positive correlation with protein solubility, suggesting that arabinoxylans with less arabinose side branches could have a greater trapping effect.

Considering these findings, it is plausible to suggest that negative interactions between macronutrients and fiber components are involved in the variability in physicochemical composition, which may affect the final availability of these nutrients. However, it is worth considering that physicochemical variation in commercial corn batches is difficult to evaluate and not completely viable under current commercial conditions. Therefore, one of the strategies to control the physicochemical variation of corn due to genetic background and kernel cob position could be the genetic selection of

corn hybrids with less variation. On the other hand, the variability associated with the smallest apical grains could be controlled using sieving strategies in feed mills. However, considering that commercial corn batches often come from different farmers or from unknown sowing conditions, enzyme supplementation could be an important method to control or smooth these negative effects by enhancing the nutrient value of corn-based poultry diets.

Regarding the effects of corn nutrient variability on xylanase response, in **Article II** we observed an increase in soluble compounds after an in vitro process when xylanase was included, regardless of kernel size or genotype. This disruption to arabinoxylans chains may expose the stored starch and protein granules to endogenous enzymes and microbial fermentation (Bedford & Schulze, 1998; De Lange et al., 2010), and/or increase xylo-oligosaccharides production. Regarding this last issue, xylotriose production showed an interaction for both the position of kernels on the cob and genotype, showing that corn composition can affect enzyme response, at least in the production of xylo-oligosaccharides.

In this context, it is reasonable to consider that xylanase response and the consequent benefits for animal performance may be dependent on the concentration and nature of corn NSP.

The interaction between nutrients and fiber components of corn affect the broiler performance.

Adjustment of the nutritional value of raw materials prior to feed formulation is a fundamental practice nowadays. In fact, such adjustments to the main macronutrients significantly reduce performance deficiencies due to nutrient imbalance (Latham et al., 2016). However, as mentioned earlier, there are many factors that can affect the

physicochemical composition of corn, and the vast majority cannot be detected in commercial batches. Furthermore, this variation could produce negative interactions in the availability of nutrients, as observed in **Article II.** Regarding this issue, **Article III** used variation in nutrient physicochemicals due to genotype to test the effects of these interactions between NSP and macronutrients on broiler performance.

Our results showed that body weight gain and feed intake were significantly affected by corn genotype on day 21. This, added to the fact that the composition of the matrix for protein and AME were adjusted by the values predicted by near infrared spectroscopy, suggests that this loss in yield is due to differences in the availability of nutrients, perhaps due to encapsulation produced by the fibrous components of corn. These reductions in performance were accompanied by a decrease in dry matter digestibility and energy utilization, showing a loss of nutrients in the excreta.

The genetic impact on chemical composition and physical characteristics in cereals, including corn, has been reported (Rodehutscord et al., 2016). However, the variability in content and chemical nature of NSP chains have only been studied in wheat, barley, oats, and rice (Rodehutscord et al., 2016; Shewry et al., 2013), and not in corn. However, our results in **Article II** show that the corn genotype can produce variability in NSP content (range: 56 - 77, g / kg) and the total arabinose:xylose ratio (range: 0.68 and 0.74, g/g) of corn, suggesting that this variability in the content and nature of NSP could be behind the changes observed in performance and digestibility in **Article III**. On this matter, the regression values obtained in **Article III** for total NSP and total AX increase the usefulness of near infrared spectroscopy for predicting these values, which could help to improve the feed formulation system and prevent impaired performance.

It is therefore plausible to believe that variation in the content and nature of NSP in corn could produce detrimental performance in broilers mainly due to nutrient trapping.

The corn particle size affects the xylanase response.

The technological process for chicken feed can include milling, mixing, crumbling or pelleting. The final distribution of particle size in the diet is affected by both grinding intensity and pelleting (Amerah et al., 2007b). The effects of coarse particles in the gastrointestinal tract of broilers are well documented. The main effect of using large particles in broiler chicken diet is improved gizzard size, which has been related with several digestive benefits. These include the prevention of pathogenic bacteria entering the small intestine due to low pH, increased gizzard contractions, HCl production, and exposure time of nutrients to digestive enzymes, especially pepsin, whereby a lower pH increases their activity thus improving protein digestion (Ferket & Gernat, 2006; Pacheco et al., 2013; Zang et al., 2009). Particle size distribution has also been related with xylanase response in wheat-based diets (Amerah et al., 2008a).

In this context, **Article IV** looked for variations in xylanase response when used in different PSDs of pelleted corn-based broiler diets. Our results were in accordance with previous broiler studies in which coarse particle diets increased gizzard weight, WRC and ileal digestibility of protein, energy, dry matter and organic matter (Abdollahi et al., 2019; Córdova-Noboa et al., 2020; Naderinejad et al., 2016). Despite no differences in performance being observed, these results confirm that the effects on gizzard development, as well as its associated benefits, persist after pelleting and may be a method to improve the conditions of smaller animals. A 2.5 % increase in body weight homogeneity was observed in birds fed with coarse pelleted diets.

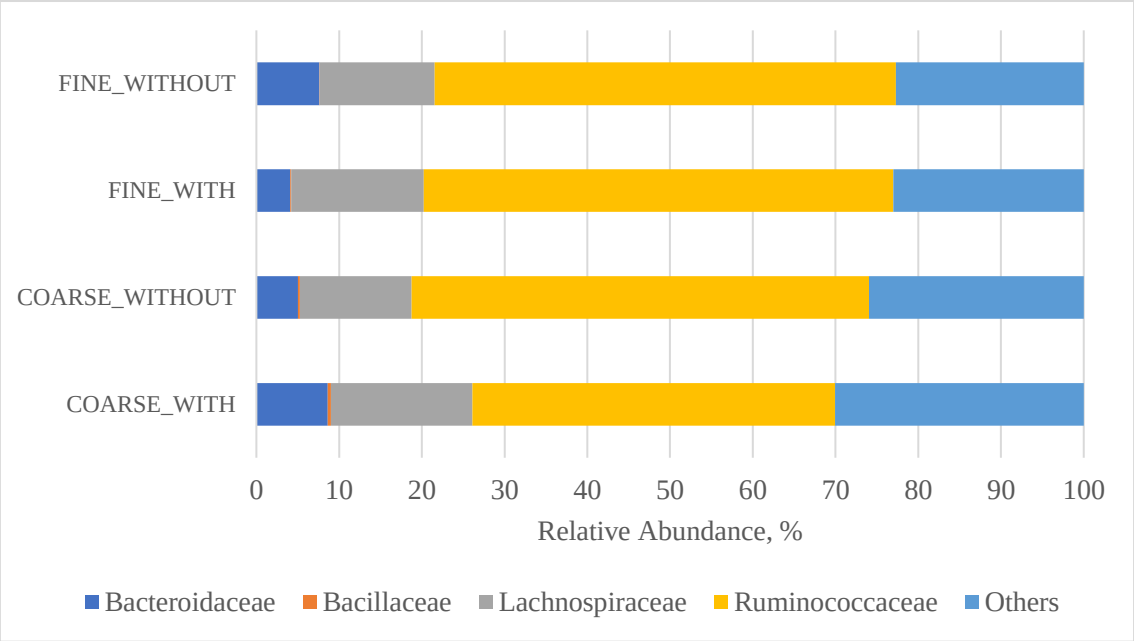

Figure 2. Relative abundance of the main families of bacteria affected by the interaction between corn particle size distribution (Fine: 540 vs. Coarse: 1,700, µm) and xylanase supplementation (Without: 0 vs. With: 16,000, BXU).

Regarding xylanase response in different distributions of corn particle size, the inclusion of the enzyme showed a greater increase in soluble components in coarse corn distributions versus fine distributions (20 % vs. 5 %, respectively), after the in vitro process at pH 5. Xylanase supplementation also increased the soluble components (12.5 %) of corn regardless of particle size distribution at pH 2.5. This increase was not accompanied by changes in the digestibility of the nutrients or the productive parameters of the chickens, but included an increase in the homogeneity of body weight of 4.5 % in fine diets and 1.5 % in coarse diets. There is agreement in the literature that smaller particles increase the specific surface of food particles, thus enabling better contact with the enzymes, and this has been shown when the speed coefficients for starch digestion decreased as the size of the particles in different cereals increased (Al-Rabadi et al., 2009). However, it should be noted that birds need coarse particles in their diet in order to increase gizzard size. This development of the gizzard implies an improvement in aspects such as peristalsis, mixing, pH and modulation of the microbiota, as previously discussed.

On this last issue, a novel finding was the interaction between particle size and xylanase supplementation in modulating the microbiota. Thus, several changes in microbiota composition were detected when coarse diets with and without xylanase were contrasted. Coarse diets with xylanase presented the most beneficial changes in microbial profile, including an increase in the relative abundance of families such as Lachnospiraceae, Bacillaceae, and Eggethellaceae, which contain butyric acid-producing bacteria.

With this point in mind, the prebiotic mechanism of xylanase is clearly affected by particle size in corn-based pelleted diets. Thus, the effectiveness of enzyme and gizzard development could be improved by considering the use of a coarse particle size distribution in the diet.

CHAPTER V

CONCLUSIONS

Taking together the results obtained by the present PhD thesis dissertation four blocks with its conclusions can be drawn:

The mechanisms of action of xylanase allow the replacement of antibiotics in animal production.

1. There are limited literature on carbohydrase as an alternative to antimicrobials. However, the supplementation with xylanase in corn kernels and corn-based diets increases soluble compounds under in vitro conditions that emulate the pH of the gizzard (pH = 2.5), part of this increase is in the production of xylotriose. This increase in xylotriose production added to the modulation of the microbiota and the improvement in body weight homogeneity due to xylanase supplementation support the theory of including xylanase as part of the holistic antibiotic replacement plan in animal production.

The variability of corn physicochemical composition affect the xylanase response.

2. The genotype and the position of the kernel on the cob interact in most of the physicochemical components except moisture, apparent metabolizable energy, crude protein and starch, with higher content of these nutrients in basal grain. The apical kernels of the cob have a higher content of non-starch polysaccharides, total arabinoxylans and soluble arabinoxylans than basal kernels; however, the magnitude of the difference depends on the genetic variety. Finally, the apparent metabolizable energy, starch content, and protein solubility are lower when corn has a higher non-starch polysaccharide content.

3. In vitro supplementation of xylanase increases the soluble compounds regardless of the variation in the physical-chemical composition of corn. Meanwhile, the production of xylotriose depended on arabinoxylan content, the levels of these xylo-oligosaccharides

being higher in some varieties and in the apical grains with higher levels of total arabinoxylans.

The variability of corn physicochemical composition affect the xylanase response.

4. The physicochemical variability of corn affects broiler body gain weight at day 21 despite the previous adjustment of the main corn macronutrients during feed formulation. The content and nature of non-starch polysaccharides are revealed to be one of the main factors affecting the solubility and availability of nutrients in corn with the consequent effect on bird performance.

5. Near infrared spectroscopy is shown to be more efficacious in the prediction of total non-starch polysaccharides and arabinoxylans. However, further studies with a higher number of samples are needed to validate the efficacy of this important method for predicting the content and nature of these fiber components.

The corn particle size affect the xylanase response.

6. The use of corn with coarse particles (geometric mean diameter: 1,700 μm) increases gizzard size and improves nutrient digestibility, microbiota modulation and body weight homogeneity despite the pelletizing process of poultry diets. Corn particle size affects the prebiotic mechanism of xylanase, the most beneficial changes in microbial composition being observed in the distribution of coarse particles in corn when including xylanase.

CHAPTER VI

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ANNEX I

CURRICULUM VITAE

Personal Information

Surname, Name: Melo Durán Diego **Nationality:** Ecuadorian

Email: diego.melo.d@outlook.com **Date of birth:** 13/07/1989

Education

2017-present **Ph.D. Student in Animal Science**

Universitat Autonòma de Barcelona

2016-2017 **M.Sc. in Animal Production and Health**

Universidad Politécnica de Madrid / Universidad Complutense de Madrid

2007-2013 **B.Sc. in Veterinary Medicine**

Universidad Central del Ecuador

Post-graduate Courses

2018 **Training course on statistical techniques with R**

Servei de Estadística Aplicada, UAB

2018 **Biological Agents: Risk and Preventive Measures**

Public Health Agency of Canada

2019 **English course (B2.1)**

Servei de Llengues, UAB

2019 ***English course (B2.2)***

Servei de Llengues, UAB

2020 **Statistics applied with R-Studio**

U*niversitat Autònoma de Barcelona*

Professional Experience

2017-present **Member of the Animal Nutrition and Welfare Service**

Universitat Autònoma de Barcelona (Bellaterra)

- Collaboration in several research projects (experimental design, farm controls, laboratory analyses, statistical analyses and writing technical reports)

2020 **Member of the Faculty of Veterinary of UCE (6 months)**
Universidad Central del Ecuador

- Collaboration in research projects and teaching activities.

2016 **M.Sc. Practice (2 months)**

Universidad Politécnica de Madrid

- Collaboration in a research project.

2016 **M.Sc. Practice (5 months)**

Universidad Complutense de Madrid

- Collaboration in microbiology research (laboratory analyses, isolation and identification of zoonotic pathogens)

2015 **Veterinary (5 months)**

Poultry farms of Corporation "la Favorita" (Ecuador)

- Responsible for the management and health of poultry farms.

2013 **Teaching Assistant (1 year 6 months)**

Universidad Central del Ecuador

- *Collaboration in teaching activities in the avian pathology class.*

Fellowships

2018-present **Pre-doctoral research grant (CZ02-000816-2018)**

Secretaria Nacional de Educación Ciencia y Tecnología del Ecuador

2017 **Grant for practice of M.Sc. in Animal Production and Health.**

Fundación Premio Arce / Universidad Politécnica de Madrid

Scientific Publications

Abdelli, N., Pérez, J. F., Vilarrasa, E., Cabeza Luna, I., **Melo-Durán**, D., D'Angelo, M., & Solà-Oriol, D. (2020). Targeted-Release Organic Acids and Essential Oils Improve Performance and Digestive Function in Broilers under a Necrotic Enteritis Challenge. *Animals*, *10*(2). https://doi.org/10.3390/ani10020259

Cho, H. M., González-Ortiz, G., **Melo-Durán**, D., Heo, J. M., Cordero, G., Bedford, M. R., & Kim, J. C. (2020). Stimbiotic supplementation improved performance and reduced inflammatory response via stimulating fiber fermenting microbiome in weaner pigs housed in a poor sanitary environment and fed an antibiotic-free low zinc oxide diet. *PLOS ONE*, *15*(11), 1–21. https://doi.org/10.1371/journal.pone.0240264

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González-Ortiz, G., Callegari, M. A., Wilcock, P., **Melo-Durán**, D., Bedford, M. R., Oliveira, H. R. V, da Silva, M. A. A., Pierozan, C. R., & da Silva, C. A. (2020). Dietary xylanase and live yeast supplementation influence intestinal bacterial populations and growth performance of piglets fed a sorghum-based diet. *Animal Nutrition*, *6*(4), 457–466. https://doi.org/https://doi.org/10.1016/j.aninu.2020.05.005

Melo-Durán, D., Gonzalez-Ortiz, G., Sola-Oriol, D., Martinez-Mora, M., Perez, J. F., & Bedford, M. R. (2019). Relationship between peptide YY, cholecystokinin and fermentation products in fasted, re-fed and ad libitum fed broiler chickens. *Animal Feed Science and Technology*, *247*. https://doi.org/10.1016/j.anifeedsci.2018.11.007

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Melo-Durán, Diego, Pérez, J. F., González-Ortiz, G., Villagómez-Estrada, S., Bedford, M. R., Graham, H., & Sola-Oriol, D. (2021). Maize nutrient composition and the influence of xylanase addition. *Journal of Cereal Science*, *97*, 103155. https://doi.org/https://doi.org/10.1016/j.jcs.2020.103155

Villagómez-Estrada, S., Blanco, J. L., **Melo-Durán**, D., Martín, C., Harmanus, C., Kuijper, E. J., & García, M. E. (2019). Detection of Clostridium difficile in the environment in a veterinary teaching hospital. *Anaerobe*, *57*. https://doi.org/10.1016/j.anaerobe.2019.03.011

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Villagómez-Estrada, Sandra, Pérez, J. F., van Kuijk, S., **Melo-Durán**, D., Forouzandeh, A., Gonzalez-Solè, F., D'Angelo, M., Pérez-Cano, F. J., & Solà-Oriol, D. (2021). Strategies of inorganic and organic trace mineral supplementation in gestating hyperprolific sow diets: effects on the offspring performance and fetal programming. *Journal of Animal Science*, *99*(7). https://doi.org/10.1093/jas/skab178

Villagómez-Estrada, Sandra, Pérez, J. F., van Kuijk, S., **Melo-Durán**, D., Karimirad, R., & Solà-Oriol, D. (2020). Dietary Preference of Newly Weaned Pigs and Nutrient Interactions According to Copper Levels and Sources with Different Solubility Characteristics. *Animals*, *10*(7). https://doi.org/10.3390/ani10071133

Villagómez-Estrada, Sandra, Pérez, J. F., van Kuijk, S., **Melo-Durán**, D., Karimirad, R., & Solà-Oriol, D. (2021). Effects of two zinc supplementation levels and two zinc and copper sources with different solubility characteristics on the growth performance, carcass characteristics and digestibility of growing-finishing pigs. *Journal of Animal Physiology and Animal Nutrition*, *105*(1), 59–71. https://doi.org/https://doi.org/10.1111/jpn.13447

Skills and Competences

Language		**Software**	
Spanish	+++++	*Microsoft Office*	+++++
English	++++	*SAS*	++++
Portuguese	++++	*Rstudio*	++++