Universitat Autònoma de Barcelona

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DOCTORAL THESIS – 2017

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AOS MEUS PAIS

“There is a driving force more powerful than steam,

electricity and atomic energy:

the will.”

A. Einstein

ACKNOWLEDGEMENT

O meu verdadeiro pai foi aquele que sempre motivou os meus sonhos, minhas viagens e minha ânsia de conhecer o mundo. Ele me dizia: “minha filha, se tu queres algo, se realmente queres algo, enfrenta sem medo, com a cara e com a coragem, porque essa vida é muito curta para deixar de fazer as coisas pensando no amanhã ou no que os outros vão dizer sobre ti. Na vida, a gente tem que fazer aquilo que a gente gosta”. A minha mãe me ensinou a ver sempre o lado positivo das coisas, que por mais que a situação pareça insuperável, tens que enfrentar o problema, dar a volta por cima e vencer. Ambos não ensinaram apenas com palavras, mas predicaram com o exemplo: meu pai saindo muito jovem de uma cidadezinha do interior do estado para estudar na Itália, morando com os índios na Amazônia e depois formando uma família atípica numa sociedade conservadora. A mãe é aquela mãe coragem que lutou e trabalhou muito a vida inteira porque a prioridade sempre foi os nossos estudos. Ela também lutou muito contra um mundo machista e contra a sua própria educação para entender uma filha que não pensava como os outros. Tenho orgulho de dizer que a minha mãe é a minha melhor amiga. É normal que uns pais assim, que se esforçaram tanto pela educação acadêmica e ética dos filhos, pensassem e dissessem: “minha filha, eu não entendo, tu que sempre conseguistes tudo o que te propusestes nesta vida porque não terminas este doutorado?”. Na verdade, nem eu tenho a verdadeira resposta. Só sei que esta tese eu dedico a vocês.

Basada en las enseñanzas de estos padres maravillosos, voy a pensar en los aspectos positivos de haber tardado en presentar esta tesis. Al estar más

tiempo en contacto con el CReSA, me hizo conocer a personas excepcionales durante estos años, aunque tengo que admitir que tengo un especial cariño por los antiguos, como Nilsa, Lana, Carolina, Aida, Cristina y mi gran amiga Bibiana, a quien admiro y quiero mucho. Entre estas paredes construí grandes amistades: mis monstruos – Àlex, Marta y Judith – amenizaron los problemas, hicieron que la vida fuera más divertida y algunas veces muy dulce (literalmente hablando). No puedo dejar de pensar que fue también durante este recorrido que encontré a una de las personas más pacientes y buenas que he conocido en mi vida, mi gran amigo, mi petardo favorito, la persona que conoció mi peor versión y aun así siempre estuvo a mi lado, quien me prestó a su familia para que las Navidades aquí fueran más amenas. Gracias, Dani.

Otro aspecto positivo, es que al dejar la escritura de la tesis en *stand-by* para trabajar mientras además hacía un postgrado creo haber madurado profesional y personalmente. Donde, además de desarrollar la capacidad analítica y estratégica, fue donde aprendí a gestionar conflictos de la mejor manera posible y la importancia de la empatía tanto en las relaciones profesionales como personales. Aun me queda mucho por aprender y mejorar, pero he de admitir que fue una de las decisiones más acertadas que tomé en mi vida. Así como también fue una buena decisión dejar aquello para terminar la tesis. Una tesis con muchos altibajos, muchos conflictos y muchos errores por mi parte, en la que he tenido muchísimas dificultades para acabarla, hasta el punto de depositarla sin correcciones. Un doctorado donde más de uno de mi entorno me dijo que desistiera de ello y que me dedicara a otra cosa. A estos me olvidé

explicarles que amo lo que hago y que además me encantan los retos. Aunque tengo que admitir que esta vez hubiera deseado que fuera un poco más fácil.

Como decía al principio sobre lo de aprender de las dificultades… no lamento mi decisión. Porque aunque haya sido difícil, en 2007 prometí a cierto catedrático que no me arrepentiría de ello. He de admitir que durante el desarrollo de la tesis eché muchísimo de menos al departamento de nutrición animal de la UAB. Además del compañerismo, la dinámica de trabajo que tienen es admirable. Tuve muchos compañeros allí a quienes tengo mucho aprecio, desde la que me llamaba guapa y que ahora vive en Italia, la que decía aquellos animados buenos días por el pasillo que te cambiaban el día, los que se casaron y fueron a vivir a la República Dominicana o la que se jubiló antes de poder hacerme una tortilla de patatas. Y eché muchísimo de menos a mi directora de master, a mi modo de ver puso el listón muy alto tanto como persona como orientadora. Os agradezco muchísimo a todas las enseñanzas, la paciencia y el cariño.

També em sento molt agraïda a la gent del CReSA, hi ha molts que van col·laborar ensenyant-me petites coses al laboratori però que juntes van marcar la diferència. Al laboratori vaig aprendre diverses tècniques amb els investigadors, els tècnics i els becaris. També vaig passar bons moments i em vaig riure molt amb vosaltres. Encara m'enrecordo del nostre equip de futbol femení i de quan vam guanyar la lliga de la UAB. Recordo també els sopars a vila per veure el partit del Barça amb en Gerard, Martí i Iván Diaz, on ens ho passavem molt bé i per unes hores ens oblidàvem de tot.. Em sento molt agraïda a qui, des de la clandestinitat, em va ensenyar des de com clonar fins a

com presentar millor els meus resultats. Tampoc tinc paraules per agrair a qui, sense cap mena d'obligació, em va prestar el nom del seu fill per poder llogar una habitació a la vila perquè sovint em quedava fins massa tard al laboratori. I agraeixo molt a qui em va fer una boníssima carta de recomanació; que a més d'ajudar-me a aconseguir la feina que tant desitjava, va servir d'incentiu per creure en mi mateixa. Moltes gràcies a tots!

This PhD Thesis could not be realized without the appreciation of my thesis directors, PhD David Torrallardona and PhD Ignacio Badiola. I really acknowledge them to give me the opportunity to come back in order to finish this stage. I would like to thank ADISSEO Company for the financial support to realize this project and, specially, PhD. Estelle Devillard.

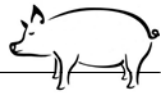
A la familia Lerones-Chorén-Medina-Roig-Rico, gracias por la paciencia de estos años, prometo que en breve volveré a ser persona. En especial a ti, Aitor, por apoyar siempre mis decisiones y haber soportado esta novela durante seis años que parecía no acabar nunca. Agradezco mucho tu apoyo, creo que no puedes llegar ni a imaginar lo que realmente has hecho por esta tesis, va mucho más allá de ayudarme con las tablas dinámicas del Excel, con los gráficos o con la corrección de los espacios dobles y los puntos. Va de tener paciencia, va de dejarme mi espacio, va de no dejar que yo flaqueara y volviera a trabajar, diciéndome: primero acaba la tesis y después piensa en eso, ya nos apañaremos".

Aos Willamil e agregados, essa família tão maravilhosa que já nem sei mais como agradecer, muito obrigada por todo amor recebido. Tive sorte de crescer numa família que está unida para tudo, nos momentos bons e nos difíceis.

Talvez tenha tias que me mimam demais, mas contra isso eu não posso lutar. Aos de casa, meus pais, meus irmãos e sobrinho, a distancia às vezes é cruel demais, a saudade é enorme e confesso que algumas vezes foi difícil gerir este sentimento de culpa por estar longe. Sou eternamente grata pela educação recebida, pelos valores que me transmitiram e pela força que recebi em todas as metas que me propus.

Puede parecer una tontería tener tantos agradecimientos personales en una tesis doctoral. Para mí, unos fueron mi combustible, otros, mi válvula de escape para llegar a meta y mi madre fue la persona que vigilaba la luz al final del túnel para que no se apagara. Hoy tengo la sensación de haber cumplido no solo lo que me propuse hace muchos años, sino también lo que prometí a una persona muy especial.

A única coisa que lamento é que já não estejas aqui para participar desse momento e poder compartilhar contigo a alegria que sinto. Pai, hoje posso riscar a última tarefa pendente de todas aquelas coisas que prometi naquela tarde do dia 02 de dezembro. Obrigada!



ABSTRACT

The gut health concept is based on the equilibrium and the interaction among gut mucosa, diet and gut microbiota. Prebiotics, probiotics, organic acids, dietary fiber and enzymes are being reported to improve the intestinal health of pigs. The purpose of these products is to affect beneficially the gut microbial composition and its activities, thus they can have a direct or indirect effect on gut immune response. The main objective of this PhD Thesis was to investigate the effect of an enzyme preparation on performance parameters and gut health in growing pigs fed with diets based on wheat-barley-rye (WBR) or corn (C). To achieve this objective an *in vivo* experiment was designed.

Thirty-six pigs (22 kg of BW) were used to evaluate a carbohydrase preparation, with xylanase and β-glucanase as main activities, added to either wheat-barley-rye- (WBR) or corn-based (C) diets on performance, intestinal environment, and nutrient digestibility. Pigs were offered for 27 d one of four different dietary treatments distributed according to a factorial arrangement of treatments (2 × 2) with 2 cereal types (WBR or C) and 2 levels of supplemental carbohydrase (0 or 0.01%). Pig growth and feed intake were individually measured every week until the end of the experiment when pigs were slaughtered to obtain samples of digesta and tissues.

Relative to WBR, C diets increased the number of crypt intraepithelial lymphocytes, the *Staphylococcus* spp and the formate concentration and decreased lactate concentration in the ileum. In the caecum, C diets also increased increased ammonia concentration and the bacterial populations of

Clostridiaceae and Veillonellaceae. On the other hand, WBR resulted in an increased population of Ruminococcaceae in the caecum and a decrease of the OTUS of Clostridia, Peptostreptococcaceae, Clostridiaceae and *Clostridium* spp. in the ileum. Only in absence of enzyme, C diets resulted in an important increase of IEL in the villi of the ileum, and a decreased concentration of acetate, propionate and total SCFA in the caecum, compared to the other three diets. The enzymes increased the VH in the ileum and the caecal digestibility of starch and mannose-NSP. Supplementation with enzymes also increased *Leuconostoc* spp. in the ileum and decreased Clostridiaceae in the caecum. The carbohydrase supplementation enhanced ADG and ADFI for the pigs fed the WBR diet, but not for those fed the C diet. Moreover, supplementation of the WBR diet increased the VH:CD ratio, the caecal dry matter digestibility also the OTUs of Cyanobacteria and *Roseburia* spp. On the other hand, supplementation of the C diet increased the population of *Megasphaera* spp., *Coprococcus* spp., *Lachnospira* spp. and *Veillonella* spp..

In conclusion, the changes in the nature of the non-digested substrate resulting from enzyme supplementation lead to changes in the composition of the microbiota and the fermentation metabolism, which in the case of WBR appear to enhance performance. In corn diets, however, the supplementation decreased the number of intraepithelial lymphocytes in the ileum. The changes in the microbiota and the immune response suggest that the carbohydrase supplementation may enhances performance by improving gut health.

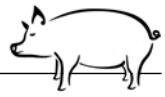
RESUMEN

El concepto salud intestinal se basa en el equilibrio y la interacción entre la mucosa intestinal, la dieta y la microbiota intestinal. Los prebióticos, los probióticos, los ácidos orgánicos, la fibra dietaria y las enzimas están adquiriendo relevancia como mejoradores de la salud intestinal en cerdos. El propósito de dichos productos es el de afectar de forma beneficiosa la composición de la microbiota intestinal y su actividad, de manera que pueden tener un efecto directo o indirecto en la respuesta inmunitaria del intestino. El principal objetivo de esta tesis doctoral era investigar el efecto de una preparación enzimática sobre los parámetros productivos y la salud intestinal en cerdos de engorde alimentados con dietas a base de trigo-cebada-centeno (WBR) o maíz (C). Para conseguir dicho objetivo se diseñó un experimento *in vivo*.

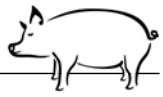
Se utilizaron treinta y seis cerdos (22 kg de PV) para evaluar una preparación carbohidrasa, con actividades principales xilanasa y β-glucanasa, añadidas bien a dietas a base trigo-cebada-centeno (WBR) o maíz (C) sobre la productividad, el ambiente intestinal y la digestibilidad de nutrientes. A cada cerdo se le ofreció, durante 27 días de ensayo, uno de los cuatro tratamientos experimentales distribuidos de acuerdo a un diseño factorial (2 x 2), con 2 fuentres de cereal (WBR o C) y 2 niveles de suplementación de carbohidrasa (0 o 0.01%). El crecimiento y consumo de pienso de los cerdos fueron controlados individualmente cada semana hasta el final del ensayo, cuando los cerdos fueron sacrificados para la obtención de muestras de contenido digestivo y tejidos.

En comparación con los animales alimentados con WBR, los animales alimentados con C presentaron un incremento de los linfocitos intraepiteliales en las criptas de las vellosidades, de la concentración del ácido fórmico y de la población de *Staphylococcus* spp. y una disminución de ácido láctico en el íleon. En ciego, las dietas C también aumentaron la concentración de amonio, y las poblaciones de de Clostridiaceae and Veillonellaceae. Por otro lado, WBR aumentó la población de Ruminococcaceae en ciego y disminuyó de las de Clostridia, Peptostreptococcaceae, Clostridiaceae and *Clostridium* spp. en íleon. En ausencia de enzimas, las dietas de maíz conllevó un importante incremento de los linfocitos intraepiteliales en las vellosidades del íleon y a una disminución de la concentración de los ácidos acético y propiónico y de los SCFA totales en el ciego.

Las enzimas aumentaron la altura de las vellosidades del íleon y la digestibilidad del almidón y de los PNA-manosa en ciego. La suplementación con enzimas también aumentó la población de *Leuconostoc* spp. en íleon y disminuyó la de Clostridiaceae en ciego. El uso de enzimas mejoró el crecimiento y consumo de los cerdos alimentados con WBR, pero no para los cerdos alimentados con C. Además, la suplementación de dietas WBR con carbohidrasa aumentó la ratio VH:CD, la digestibilidad cecal de la materia seca y poblaciones bacterianas de Cyanobacteria y de *Roseburia* spp.. Por otro lado, la suplementación de la dieta de maíz aumentó las poblaciones de *Megasphaera* spp., *Coprococcus* spp., *Lachnospira* spp. y *Veillonella* spp..



En conclusión, los cambios en la naturaleza del sustrato no digerido, resultantes de la suplementación enzimática llevó a cambios en la composición de la microbiota y el metabolismo fermentativo que, en el caso de WBR, parece mejorar la productividad de los animales. En dietas de maíz, la suplementación enzimática disminuyó el número de linfocitos intraepiteliales en el íleon. Los cambios en la microbiota y la respuesta inmunitaria sugieren que la suplementación con carbohidrasas puede mejorar la productividad a través de la mejora de la salud intestinal.



ABBREVIATIONS USED

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Chapter 1.

General introduction

Chapter 1. General introduction

1.1. Introduction

The swine industry has undergone changes and growth over the last 20 years. This sector has to face-off an increasing in production and productivity and, at the same time, has to maintain or decrease the production costs without lost the product quality. This challenge together with changes in the European Union legislation in the last years, banning the use of antibiotics as growth promoter on January 2006, increased the needs to find solutions in order to produce healthy animals with competitiveness costs. One of these new approaches was the gut health concept; it is based on the equilibrium and the interaction among gut mucosa, diet and gut microbiota (Montagne et al., 2003). From nutrition side, many strategies are used to improve the intestinal health of pigs, as prebiotics, probiotics, organic acids, dietary fiber (DF) and enzymes. The aim of these products is to affect beneficially the gut microbial composition and its activities, thus can have a direct or indirectly effect on gut immune response. The DF of cereals can be exploited in different ways leading to the design of new cereal ingredients that can target specific microbial populations. Cereals can be used as fermentable substrates for the growth of probiotic microorganisms. Additionally, cereals can be used as sources of non-digestible carbohydrates that besides promoting several beneficial physiological effects can also selectively stimulate the growth of lactobacilli and bifidobacteria present in the colon and act as prebiotics (Charalampopoulos et al., 2002). However, the utilization of the cereals rich on non-starch polysaccharides (NSP; i.e.: wheat, barley, oats and rye) is frequently correlated with a decrease on

productive parameters limiting the use of this cereals in animal feed formulation. Nevertheless, the higher interest to use these feedstuffs on poultry and pig industry promoted a development of carbohydrases complex to approach the carbohydrates in these cereals. During several years, the carbohydrases have been used in animal nutrition to increase the digestibility of the cereal nutrients, hydrolyzing the NSP of the cereals (Bedford and Schulze, 1998) and reduce the costs of diets formulation.

In conclusion, pigs undergo a dramatic physical development of the gastro-intestinal tract (GIT) throughout their productive lives. The intestinal colonization and gut immune response may be affected by some factors, as diet formulations. Diet components, such as NSP, are potential modulators of the intestinal environment. Also, enzymes have been shown to influence the intestinal microbial ecosystem towards a healthier state. Therefore, it should be possible to manipulate the gut microbiota by dietary enzyme supplementation and dietary ingredients.

1.2. Dissertation organization

The following review of the scientific literature will focus on the pig GIT, gut immune response and microbiota with emphasis on the nutritional strategies to promote gut health. Although it is not the purpose of this dissertation to review the extensive literature on enzyme supplementation, a general review is necessary to further understand of how NSP and enzyme supplementation may affect the gut immune response and microbiota of pigs and broilers.

Based on the work to achieve the objectives of this dissertation, two manuscripts were written for submission. Each of manuscript is presented as a separate chapter in the format prepared for the journal we submitted then for publication. A review of literature as background for this research is outlined in chapter 2. In chapter 3, are described the objectives. The manuscripts are in the chapter 4, which is divided in two subchapter level (one by manuscript). In the subchapter 4.1, the effect of carbohydrase supplementation of wheat-barley-rye and corn diets on performance of growing pigs was studied. This article was published in Journal of Animal Science. Subchapter 4.2 examines the effects of carbohydrase supplementation of wheat-barley-rye and corn diets on the immune response and gut microbiota of growing pigs. This article was also submitted to Journal of Animal Science. The chapter 5 includes an overall discussion of the research. Conclusions are presented in the chapter 6. In the chapter 7 are related all bibliography references used in the chapter 1, 2 and 5.

Chapter 2.

Literature review

2.1. Gastro-Intestinal Tract

2.2. Intestinal microbiology

2.3. Modulation on intestinal equilibrium through the feed

Chapter 2. Literature review

2.1. Gastro-intestinal tract

The main function of GIT is to assimilate nutrients that supply requirements for maintenance, growth and reproduction of the organism. It is achieved through the digestion which consists of a number of physical and chemical processes where carbohydrates, protein and lipids are hydrolyzed into a smaller compounds suitable for absorption in the intestine (McDonald, 2002). During these processes, digestive secretions from saliva, stomach, pancreas, bile and intestine provide mucus for protection and lubrication of tract, enzymes that help in digestion, watery medium and optimal pH required for digestion.

2.1.1. Intestinal histomorphology of pig

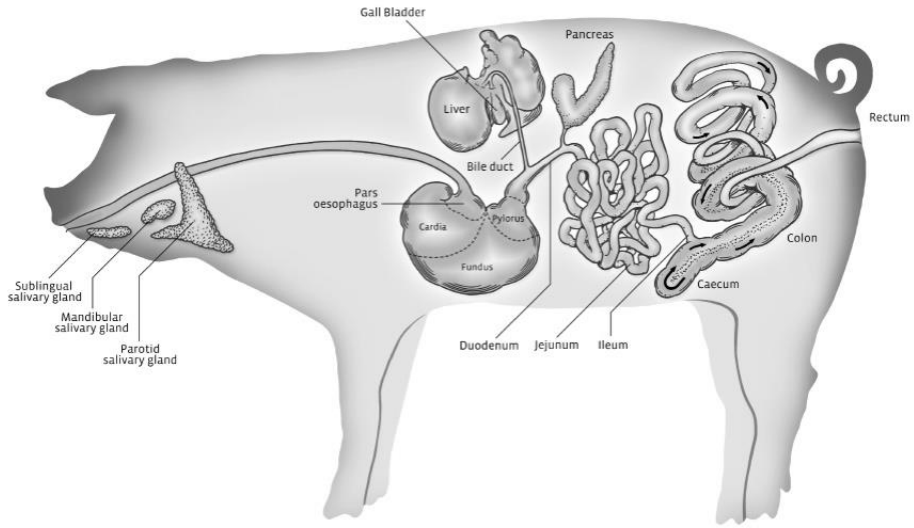
2.1.1.1. Macroscopic features

The small intestine is divided into duodenum, jejunum and ileum. The small intestine of fully grown pigs is 16-21 m, weighs 2-2.5 kg and has a capacity of >20 L (Lærke and Hedemann, 2012). The large intestine or hindgut encompasses three main sections: caecum, colon and rectum (Figure 1). The jejunum involves both the further breakdown of nutrients as well as the beginning of absorption of nutrients. Nutrient absorption continues into the final section of the small intestine, called ileum (Kararli, 1995).

Adult pigs have a large intestine weighing approx. 2.8 kg, with a length of 7.5 m and a capacity of 25 L (Lærke and Hedemann, 2012). The cells of the large intestine have no digestive enzymes as in the small intestine, but the large

intestine is the major site of absorption of fluids and electrolytes (Klasing, 1998). However, microbial enzymes activity occurs in the large intestine, which forms volatile fatty acids (VFAs) (Argenzio and Southworth, 1975).

Figure 1. Outline of the porcine digestive system.

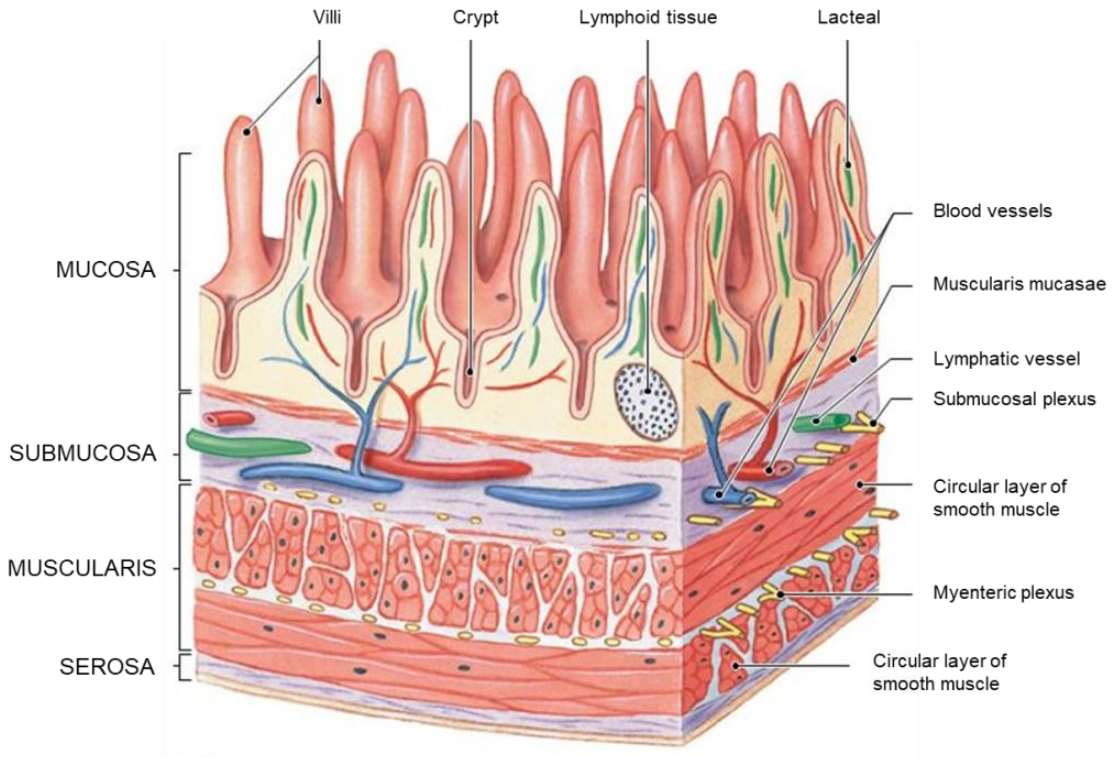


Source: Illustration by Mads Salicath from Lærke & Hedemann (2012)

2.1.1.2. Microscopic features

The small and large intestine share certain histologic characteristics. The wall of the small intestine and colon is composed of four layers: mucosa, submucosa, muscularis and serosa (Rao and Wang, 2010) (Figure 2) .

Figure 2. Architecture and organization of intestine wall.



Source: Illustration adapted from (Martini et al., 2015)

Mucosa or mucous membrane

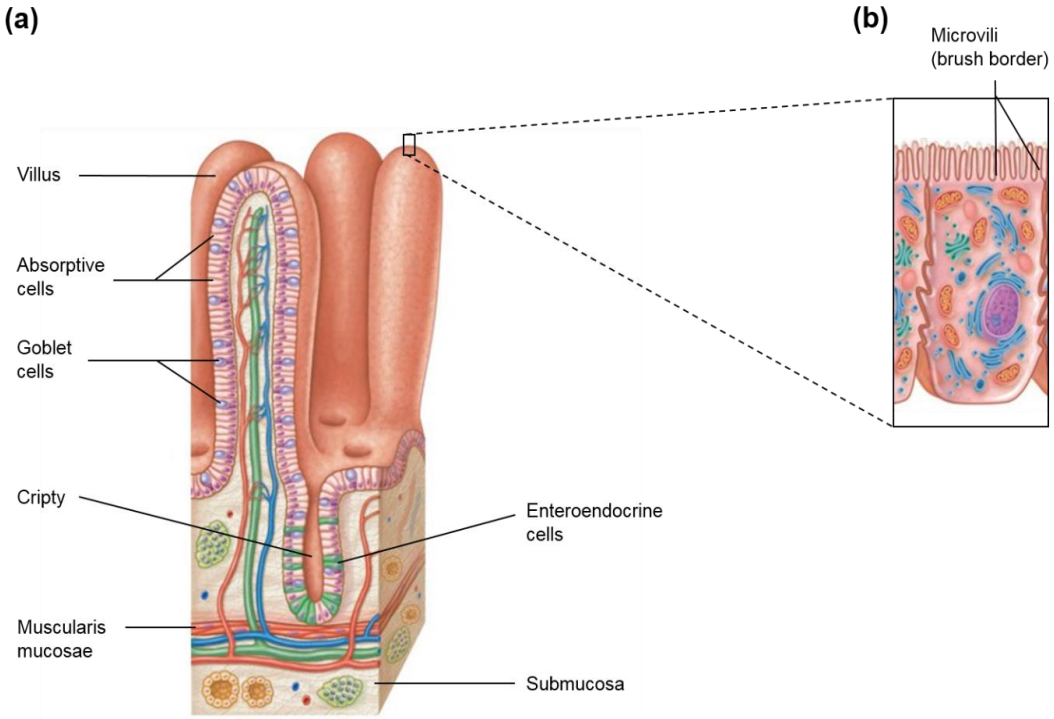
The mucosa is the innermost layer composed of three layers: i) a single-cell-thick layer of epithelial cells lining the intestinal lumen; ii) mesenchymal cells or the lamina propria; iii) muscularis mucosa. The cells of mucosa act as a selective permeable barrier allowing nutrient, electrolyte and water absorption, while excluding pathogens, toxins and other antigens.

The mucosa of small intestine is arranged into two fundamental structures: villi and crypts (Figure 3a). **Villi** are projections into the lumen and there are main 3 types of epithelial cells on the villus surface: enterocytes (absorptive cells), goblet cells and enteroendocrine cells. The enterocytes live only for a few days, die and are shed into the lumen to become part of the ingesta to be digested

and absorbed. Villi are mucosal folds and the length of villi increases from the duodenum to the mid-jejunum and then decreases again towards the terminal ileum. There are of different shapes in the various segments of the small intestine of pigs: they may be broad, short, or leaf-like in the duodenum, tongue-like in the jejunum, and finger-like more distally. Villous atrophy is generally assumed to be associated with reduced mucosal enzyme activity (Hedemann et al., 2006). **Crypts** are moat-like invaginations of the epithelium around the villi, and are lined largely with undifferentiated or younger epithelial cells which are involved primarily in secretion and microfold (M) cells (Zachary and McGavin, 2011). Toward the base of the crypts are stem cells, which continually divide and provide the source of all the epithelial cells in crypts and villi. The villous / crypt depth ratio is a useful criterion for estimating the digestive capacity in the small intestine (Montagne et al., 2003).

The apical part of enterocytes presents a cellular differentiation where the luminal surface appears similar to velvet due to its being covered by millions of small projections which extend about 1 mm into the lumen (Figure 3b). In this area termed ‘brush border’ occurs the absorption of nutrients in the jejunum and the ileum.

Figure 3. Structure of villus and crypt (a) and brush border (b)



Source: Illustration

The mucosa surface is covered by mucus layer which presents protective, lubricant and transport functions (Forstner, 2009). This mucus is secreted by **Goblet cell** (GC) and is compound by water (95%) but its main characteristics is due to glycoproteins called mucins (Fontaine et al., 1996). Mucin glycoproteins can be classified into three groups on the basis of their histochemical staining: neutral mucins, sialic acid-containing mucins and estersulphate containing mucins (Banks, 1993). The composition and quantity of mucus layer depends on animal age (Turck et al., 1993; Brown et al., 2006), diet (Montagne et al., 2003; Oswald, 2006), gut microbiota composition (Fontaine et al., 1996) and others factors. Many dietary factors including fiber, protein and anti-nutritional factors have been shown to influence secretion of mucin and/or erosion of the mucus in the pig intestine (Nyachoti et al., 1997).

Fiber increase endogenous protein synthesis as well as excretion of mucin at the terminal ileum (Montagne et al., 2003).

Villus and crypt together with mucus compound the villus-crypt complex. The mucus layer, together with antimicrobial peptides such as α-defensins released by Paneth cells, colectivelly forms the glycocalyx which traps invading microorganisms and enables their expulsion (Stokes, 2017). Generally speaking, shortly after birth, there are increases in the intestinal mucosal surface area, brush border membrane enzymes, and carrier-mediated transport. These adaptive changes occur as a result of the genetic endowment of the animal, but may be modified by environmental factors, particularly nutrient intake (Thomson and Keelan, 1986).

In pigs, the most obvious histological difference between small and large intestinal is that the mucosa of the large intestine is devoid of villi. In the large intestine, it has numerous crypts which extend deeply and open onto a flat lumenal surface. The stem cells which support rapid and continuous renewal of the epithelium are located either at the bottom or midway down the crypts. These cells divide to populate the cryptal and surface epithelium. Mucus-secreting goblet cells are also much more abundant in the colonic epithelium than in the small gut.

The **lamina propria**, which supports the epithelium, is a layer of reticular connective tissue with elastin, reticulin, and collagen fibers, lymphocytes, plasma cells and eosinophilic granulocytes, as well as lymphatics and capillaries. The muscularis mucosae consists of a thin layer of smooth muscle at the boundary of the mucosa and submucosa.

Submucosa

The submucosa, between the muscularis mucosae and the muscularis propria, is a fibrous connective tissue layer that contains fibroblasts, mast cells, blood and lymphatic vessels, and a nerve fiber plexus - Meissner's plexus - composed of nonmyelinated, postganglionic sympathetic fibers, and parasympathetic ganglion cells.

Muscularis or muscularis propria

The muscularis, mainly responsible for contractility, consists of two layers of smooth muscle: an inner circular coat and an outer longitudinal coat arranged in a helicoidal pattern. A prominent nerve fiber plexus called the myenteric plexus, or Auerbach's plexus, is found between these two muscle layers.

Adventitia or serosa

The adventitia is the outermost layer of connective tissue. When covered by a single layer of mesothelial cells, it is called the serosa.

2.1.2. Gut immune system

2.1.2.1. Short description of immune system of pig

The immune response is classified in innate (inborn components of the immune system) or acquired (adaptive) (Calder and Kew, 2002). The first line of protection against pathogen invasion is innate immune defense (Flajnik and Dupasquier, 2004). However, the adaptive immune system has evolved the wondrous machinery that relies on lymphocytes with diverse receptors capable

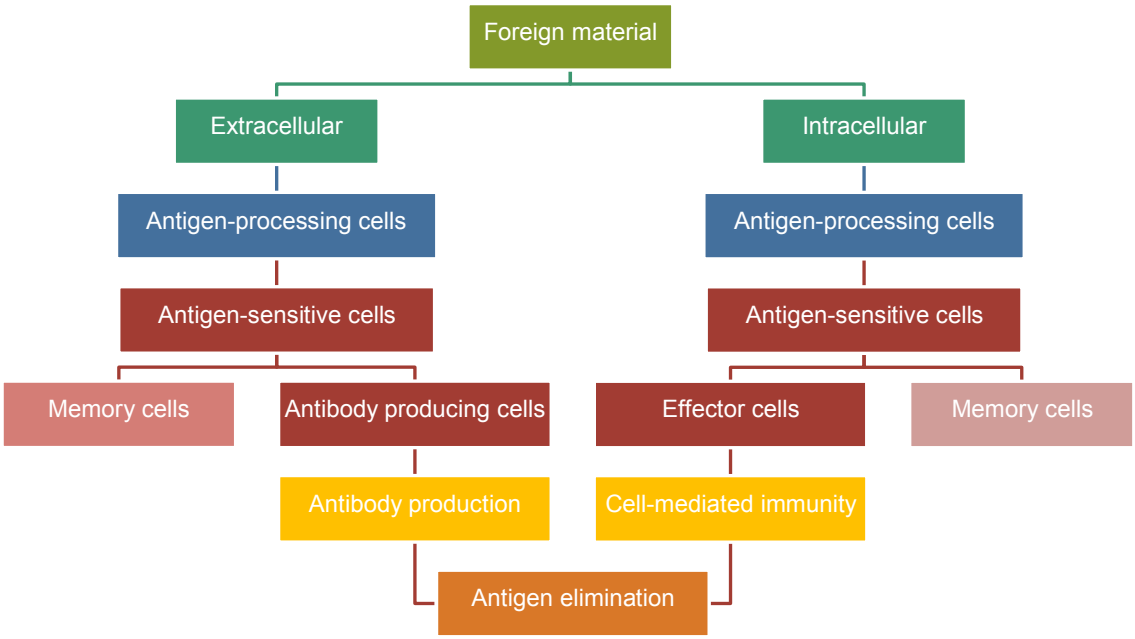
of recognizing and removing potential pathogens. The components and cells that comprise these two arms of the immune system are presented in Table 1. The **innate immune system** comprises the cells and mechanisms that defend the host from infection by other organisms, in a non-specific way (skin, mucosa, phagocytes, inflammation cells, natural killer cells, dendritic cells, cytokines and acute phase protein response) (Tizard, 2004).

The **acquired immune system** therefore consists of two major branches that defend against invaders: humoral immune response and cell-mediated immune response. The Figure 4 shows the four major components of the acquired immune system includes: i) antigen-processing cells - cells that can trap and process antigen and them present it for recognition to the cells of the immune system; ii) antigen-sensitive cells - cells that have receptors for the processed antigens (these cells can thus bind and respond to the antigen); iii) antibodies producing cells - cells that, once activated by antigen will produce specific antibodies or effector cells – cells that will participate in the cell mediated immune response against the antigen; iv) memory cells - cells that will retain the memory of the event and react rapidly to the specific antigen if it is encountered at a later time (Delves and Roitt, 2000; Tizard, 2004).

Table 1. Components of immune system (adapted from Schley and Field (2002).

	Defenses	Components	Functions
Innate immunity	Physical barriers	Skin, Mucous membranes	Act as first layer of defense, preventing the antigens entrance into systemic circulation.
	Cell-mediated barriers	Phagocytic cells (e.g. neutrophils, macrophages)	Engulf foreign antigens
		Inflammatory cells (e.g. basophils, mast cells)	Release inflammatory mediators (e.g. histamine, prostaglandins)
		Natural killers cells	Destroy infected or malignant cells
		Dendritic cells	Present antigens to lymphocytes
	Soluble factors	Cytokines	Activate/recruit other cells
		Complement	Enhance phagocytosis
		Acute-phase proteins	Promote repair of damaged tissue
Acquired immunity	B-lymphocytes	Plasma cells	Secrete antibody
	T-lymphocytes	CD4+ T-cells	Induce activation of lymphocytes
		Th1 cells	Promote cell-mediated responses
		Th2 cells	Promote humoral (antibody) responses
		CD8+ T-cells	
		Cytotoxic T-cells	Destroy infected or malignant cells
		Suppressor T-cells	Suppress activity of lymphocytes

Figure 4. Essential features of acquired immune system.



Acquired responses involve the proliferation of antigen-specific B and T cells, which occurs when the surface receptors of these cells bind to antigen. Specialized cells, called antigen-presenting cells, display the antigen to lymphocytes and collaborate with them in the response to the antigen. B cells secrete immunoglobulins, the antigen-specific antibodies responsible for eliminating extracellular microorganisms. T cells help B cells to make antibody and can also eradicate intracellular pathogens by activating macrophages and by killing virally infected cells (Delves and Roitt, 2000). Innate and acquired responses usually work together to eliminate pathogens.

The cells that compound the immune system are very different regarding the function and structure. In the follow paragraphs, the main cells will be described shortly. **Macrophages** are important in the regulation of immune responses. Besides their role in phagocytosis (Elhelu, 1983), they may function as antigen-presenting cells (APCs) because they ingest foreign materials and present

these antigens to other cells of the immune system such as T-cells and B-cells (Tizard, 1971). This is one of the important first steps in the initiation of an immunological response. Macrophages, stimulated by certain lymphokines, exhibit increased levels of phagocytosis and are also secrete cytokines that modulate immune responses (Hume, 2008). **Dendritic cells** also work as APCs. In fact, the dendritic cells are more efficient APCs than macrophages. These cells are usually found in structural compartments of the lymphoid organs such as the thymus, lymph nodes and spleen. However, they are also found in the bloodstream and other tissues of the body. They are the only antigen-presenting cell capable of stimulating naive T cells, and hence they are pivotal in the generation of adaptive immunity (Howard et al., 2004).

The **T cells** provide cell-mediated immunity. They get their name because they develop in the thymus, a specialized immune organ needed for T cell growth. The T-cells control the specific immune response, and they recognize the major histocompatibility complex (MHC) associated antigens of class I and II (Godfrey et al., 2015). T-cells are usually divided into two groups that are functionally and phenotypically different — T helper (Th) and T cytotoxic (Tc) cells. The **T helper cells**, also called CD4+ T cells, are involved in coordination and regulation of immunological responses. The main function, when they "recognize" or see their specific antigen, is to produce cytokines to help the other T and B cells grow and divide, and to grow and divide themselves to produce more cells to fight future infections (MacLeod et al., 2009; MacLeod et al., 2010). The second subset type of T lymphocytes are **cytotoxic T cells** or CD8+ T cells. These cells are involved in directly killing certain tumor cells, virus-infected cells,

transplant cells, and sometimes eucaryotic parasites (Cantor and Boyse, 1975). CD8+ T cells are also important in down-regulation of immune responses. Both types of T cells can be found throughout the body, most conspicuously in lymphoid organs (lymph nodes and spleen) but also the liver, lung, blood, and the intestinal tract (Koretzky, 2010). **Natural killer cells**, known as NK cells, are similar to CD8+ T cells. They function as effector cells that directly kill certain tumors such as melanomas, lymphomas and virus-infected cells (Vivier et al., 2008). However, NK cells, unlike the Tc cells, kill their target cells without need for recognition of antigen in association with MHC molecules.

The **B-cells** generate immunoglobulin (Ig) that can associate to the B-cell membrane or work as antibody-secreting plasma. B cells, in contrast to T cells, mature in different organs depending on species. These include bursa of Fabricius in birds and intestinal lymphoid tissues in pigs. In addition to producing antibodies (Alberts et al., 2002), B cells also express MHC class II molecules and can act as an antigen-presenting cell to CD4+ T cells (Strugnell and Wijburg, 2010).

Antibodies (Ab) are specialized glicoproteins called immunoglobulins that specifically recognize and bind to specific antigens that caused their stimulation. In mammals, there are five different classes (or isotypes) of Ig: IgG, IgM, IgA, IgD, IgE (Urich, 1994).

Immunoglobulin A is the main element of the humoral immune response that has been selected through evolution, together with innate mucosal defences, to provide protection against microbial antigens at mucosal surfaces. IgA responses are initiated in organized inductive structures, such as Peyer's

patches and nasal-associated lymphoid tissues, as well as diffuse effector tissues, such as gut lamina propria and nasal mucosa. The finding that IgA is the most abundant Ig isotype in mucosal secretions led immunologists to search for the origin of IgA plasma cell precursors, the sites and mechanisms of their induction and migration, and the role of IgA at mucosal surfaces.

The **lymphoid organs** are classified in primary and secondary. The primary lymphoid organs are developed in early fetal life and they are responsible for maturation of lymphocytes. In contrast, the secondary lymphoid organs arise late in fetal life and persist in adults. Spleen, lymphoid nodes, tonsils and other lymphoid tissues in the intestinal, respiratory and urogenital tract are examples of secondary lymphoid organs.

As in mammals, the swine immune system is compound of cells, tissues, organs and soluble factors. However, pigs present some morphological and functional particularities like the inverted lymph node structure, the low number of lymphoid cells in the efferent lymph vessels and high number of these cells in blood (Rothkötter, 2009).

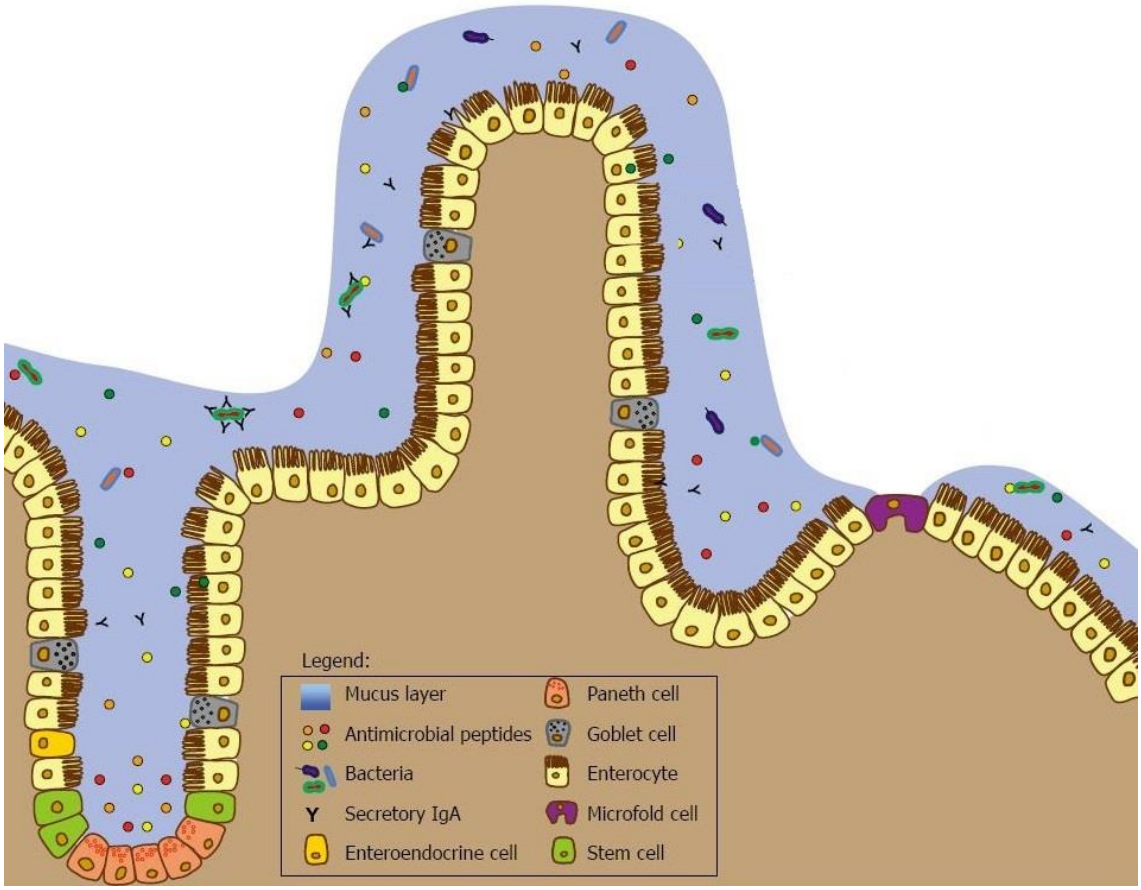
2.1.2.2. Gut Immune system of pigs

Not only is the gut the major organ for nutrient digestion and absorption, it is also the largest immunological organ in the body, containing more lymphocytes and plasma cells than the spleen, bone marrow and lymph nodes combined (Stokes and Bailey, 2000). The GIT has a lot of defense mechanisms, as non-immunological or immunological. The non-specific defenses are the gastric acid production, the peristaltic movements, the mucosa layer, the epithelial

renovation, the proteolysis, the resistance against microbial colonization and the reticulo-endothelial systems. The specific immunity defenses are the antibody production and the function developed by M cells, the lymphocytes and other cells of the immune system.

In mammals, the gastrointestinal tract harbors an extraordinarily dense and complex community of microorganisms. The gut microbiota provide strong selective pressure to the host to evolve adaptive immune responses required for the maintenance of local and systemic homeostasis. During the process of digestion and absorption of the nutrients, the gut is exposed to a lot of antigens and pathogen bacteria (from the feed and from the microbiota). Thus, it is often implied that a more robust gut will make a healthier animal, which, in turn, digests and utilizes nutrients more efficiently. Intestinal epithelial cells (IECs) form a biochemical and physical barrier that maintains segregation between luminal contents and the mucosal immune system (Peterson and Artis, 2014). The gut works as the first protective mechanism to exogenous pathogens which can colonize and/or enter the host cells and tissues (Choct, 2009). Because of this fact, the gut mucosa develops important defense and protector barrier functions. Mucosal “barrier function” is central to mucosal defense and is made up of a number of elements, such as the enterocytes renewing with their “tight junctions”, mucus secreted by goblet cells, antimicrobial peptides (AMP), together with secreted soluble IgA (Antoni, 2014)(Stokes, 2017) (Figure 5). Any alteration that affects this first line of defense against commensals and pathogens can result in inflammation or tissue aggression.

Figure 5. Main components of potector barrier of gut mucosa.



Source: Adapted from (Antoni, 2014)

Experiments have demonstrated a crucial role of microbial colonization in establishing and regulating the epithelial barrier (Hooper et al., 2001). At least in mice, the beneficial effects of commensal bacteria on the barrier function are largely mediated via pattern recognition receptors expressed by the gut epithelium, particularly toll-like receptors (TLR) (Rakoff-Nahoum et al., 2004). The continuous antigenic presence in the gut imposes a dynamic remodeling of gut-associated lymphoid tissues (GALT) and the selection of multiple layered strategies for immunoglobulin (Ig) A production (Fagarasan et al., 2010).

Gut Associated Lymphoid Tissue (GALT)

The GALT is composed of lymphatic tissue arranged in tonsils, Peyer's patches, diffusely distributed lymphoid cells in the lamina propria and the epithelium of the gut (Fagarasan and Honjo, 2004) . The organized GALT is responsible for the immunity induction response, and difuse GALT also is the place of the immunity action response. The intraepithelial lymphocytes (IEL) represented 50% of gut lymphocytes and are the major pool of immune-competent cells of the organism. The IEL are composed basically of CD3+ T cells, mainly CD8+ (50-80%). The Natural Killers cells are also part of the IEL lymphocytes (up to 25%) and generally these NK cells express IL-2 receptor (indicator of cellular activation). The localization of the IEL (in contact with the luminal gut) suggests that the IEL can be the first compartment of gut immune system against the intestinal antigens.

Peyer's patches (PP) are large lymphoid structures built on a stromal scaffold, composed of several B cell follicles separated by areas containing T cells and dendritic cells (DCs) that develop before birth. PP organogenesis was originally described by Nishikawa and colleagues (Nishikawa et al., 2000). These patches have a sub-epithelial layer and the lymph tubes that enable the lymphocyte circulation and cellular migration. The epithelium follicle associated is a modification of the gut epithelial tissue with a lot of M cells; that cells have a higher capacity of antigen trans-epithelial transport by endocytosis until the sub-epithelial area, in this space the antigens are captured and processed by (APC). Just below are the dendritic cells (MCH II+), macrophages, plasmatic cells and B and T lymphocytes. The follicular zone has mainly B cells IgM+ and

some lymphocytes CD4+ that are responsible for the change to IgA isotype. The inter-follicular zone has T cells (mainly CD4+, but also CD8+, CD4+CD8+ and CD25). The PPs are the major site of intestinal antigen-attracting cells and have a higher IgA specific response (Craig and Cebra, 1971). The B and T specific lymphocytes against the antigen migrate to lymph node where they proliferate and maturate and finally enter into blood circulation (through come back to gut).

In relation to the lamina propria leucocytes, they presented many plasmatic and B cells (90% secreted IgA) on the basal zone of the crypts, and T lymphocytes on the apical zone (mainly CD4+, and some CD8+ near the epithelium). Besides the immune production response against antigens, the lamina propria, mainly the T cells, is responsible for regulation and maintenance of the mucosa homeostasis. The presence of the B cells, the cytokines and the higher apoptosis susceptibility suggested a prevalence of antigenic tolerance avoiding the overreaction against the gut luminal antigens.

Gut Mucosal Immune Response

The mucosal immune system that is associated with the GIT is essential both for protection from enteric infection and for many of the other physiological roles required of the gut for the maintenance of health and development (Stokes, 2017). It is not well developed in the newborn pig and gradually develops over the first six weeks of life. Gut epithelial cells are thus the first cells to be exposed to nutrients and the microbiota, with complementary functions between the small intestine aiming at digestion and nutrient absorption and the large intestine specialized in the fermentation of undigested materials (Lallès, 2016).

This system includes not only immune cells, but the epithelial cells of the mucosa that help with antigen recognition and immune modulation. The epithelial cells – enterocytes and colonocytes - are coated with a mucous-glycocalyx layer that helps protects the cells, while at the same time the epithelial cells of the mucosa are continually in contact with commensal and pathogenic organisms. The mucosal immune system provides the first immune defense barrier for more than 90% of potential pathogens. This system alone contains more than a trillion lymphocytes and has a greater concentration of antibodies than other tissues in the body. It protects against harmful pathogens, but also induces tolerance the immune system to dietary antigens and normal microbiota (Figure 6).

In the mucosal lymphoid tissues, mature T-cells and B-cells that have been stimulated by antigen and induced to switch to produce IgA will leave the submucosal lymphoid tissue and reenter the bloodstream. These lymphocytes will exit the bloodstream and locate near mucosal surfaces. The B cells will differentiate into plasma cells that will secrete IgA. Many of these cells will return to the same mucosal surface from which they originated, but others will be found at different mucosal surfaces throughout the body. This homing of lymphocytes to other mucosal sites throughout the body is referred to as the "common immune system."

The intestinal immune response has two important factors: i) the development of B cells with capacity to produce immunoglobulin and ii) the activation/differentiation of the T cells. The antigens enter in the gut mucosa across the M cells or the enterocytes; they migrate to inter-follicular zone and/or

follicular zone where they are presented to immune-competent lymphocytes. Immediately, the stimulated T cells secrete TGF-β that inhibit the IgM and IgG production and induce the isotype change on B cells to IgA production. The antigen specific T cells and the precursor B cells of the plasmatic cells that produce IgA leave the Peyer patch's and migrate to lymph node where undergoing multiplication and maturation. These cells enter in the blood and are back to gut mucosa. The B cells arrive to lamina propria and complete their differentiation in plasmatic IgA producing cells, like responding to an antigen re-introduction. These Ig conferred protection avoids the toxins and bacteria adherence on the epithelium and are mediating of cellular cytotoxicity.

Whereas the innate immune system provides protection via the mucus layer, antimicrobial peptides (AMPs) and innate lymphoid cells (ILCs) to indiscriminately control microbial composition and penetration of the epithelium, the adaptive immune system provides an additional layer of protection. This is mediated by the production of IgA, which acts as a link between these two arms of the immune system. Whereas nonspecific IgA binds to microbial surface glycans causing bacterial agglutination (Mestecky and Russell, 2009), microbe-specific IgA is the main adaptive immune response controlling the microbiota. Production of IgA results from stimulation of B cells in Peyer's Patches by dendritic cells, which sample the small number of bacteria penetrating through the mucus layer (Macpherson and Uhr, 2004a).

The IgA is crucial for the regulation of the intestinal bacterial community, for the maintenance of an appropriate geographical distribution of bacteria in intestinal segments, and for immune homeostasis generally. Development of mucosal

lymphoid tissues and production of SIg requires colonization with commensal flora, as shown by the underdeveloped Peyer's patches and absence of IgA in neonates and in animals raised under germ-free conditions (Shroff et al., 1995). Maturation of IgA-secreting plasma cells requires specific signals and may occur in the presence or absence of CD4+ T cell help in Peyer's patches and isolated lymphoid follicles (Cerutti and Rescigno, 2008).

In a study by (Peterson et al., 2007) provides further clues to how secretory IgA might limit in vivo the inflammatory reactions in the gut. In their system, germ-free mice lacking B and T cells were monoassociated with Bacteroides thetaiotaomicron in the presence or absence of specific IgA for a capsular polysaccharide. Clearly, in the presence of specific IgAs, bacteria elicited fewer proinflammatory signals in the host. Interestingly, however, the effect is not restricted to the host. Indeed, bacteria adapted to the presence of specific IgAs by switching the expression of the target epitope to another capsular polysaccharide and by decreasing the expression of genes involved in nitric oxide metabolism. Thus, not only is IgA required to downmodulate or block bacterial-mediated inflammation, but also our adaptive immune system apparently drives the diversification of bacterial surface structures by exposing commensal bacteria to IgA (Fagarasan et al., 2010).

Gut health concept

The gut health concept is based on the equilibrium and the interaction among gut mucosa, diet and gut microbiota (Montagne et al., 2003). The diet factor includes macronutrients, micronutrients, additives, anti-nutritional and indigestible factors. The gut mucosa concept is composed of mucus layer,

epithelium and GALT and the gut microbiota is composed of commensal and transient bacteria.

The control of enteric pathogen bacteria can be realized across antibiotic use or dietary manipulation of the hindgut fermentation. This change in the fermentation can be promoted by the choice of dietary raw materials or by the use of additives. Both rules improve the colonization resistance exerted by the commensal microbiota to exclude enteric pathogens and thereby improve “gut health”.

2.2. Intestinal microbiology

The mammalian intestine is colonized by trillions of microorganisms, estimated to comprise ~15,000 – 36,000 species and 1,800 genera (Gill et al., 2006; Frank et al., 2007). Most of which are bacteria that have co-evolved with the host in a symbiotic relationship. This collection of microbial populations that reside on and in the host is commonly referred to as the microbiota. A principal function of the microbiota is to protect the intestine against colonization by exogenous pathogens and potentially harmful indigenous microorganisms via several mechanisms, which include direct competition for limited nutrients and the modulation of host immune responses.

Culture methods were used to describe the composition of the gastrointestinal microbiome and most studies were focused more on function. However, a high percentage of all species of bacteria present in the mammalian gastrointestinal tract have not been cultured in vitro (Isaacson and Kim, 2012). In addition, there have few studies with culture methods that have estimated concentration of

bacteria at different sites within the same animal. The development of the current molecular tools helps the investigations and the understanding of the microbiome of the GIT. Methods to characterize the pig microbiome have been divided into three categories: i) physical methods based on 16S rRNA gene sequence differences (i.e. denaturing gradient gel electrophoresis (DGGE), RFLP) , ii) PCR amplification of the 16S rRNA gene followed by cloning and full-length sequencing, and iii) PCR amplification of specific variable regions of the 16S rRNA gene sequence followed by high throughput next generation DNA sequencing (Isaacson and Kim, 2012). Recent studies have been published on the microbiome of the pig using sequencing of PCR-amplified variable regions of the 16S rRNA gene coupled with pyrosequencing. Because of the nature of the sequencing technology, the depth of coverage is much greater compared to all of the previous studies combined.

2.2.1. Composition and succession

The majority of mammalian gut microbiota is composed of strict anaerobes (Harris et al., 1976). There is composed largely of Bacteria, but Archaea, Protozoa, viruses, and fungi are also present (Leser and Mølbak, 2009; Sommer and Bäckhed, 2013). The species variability among individuals has important implications for understanding intestinal immune system function, as it indicates that the mucosal immune system must be able to flexibly and rapidly adapt to a microbiota, the composition of which may change in unpredictable ways as a function of host diet or other interactions with the external environment. Nine main bacterial phyla are represented in the mammalian gut microbiota: Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria,

Verrucomicrobia, Cyanobacteria, Fusobacteria, Spirochaetes and TM7 (Brown et al., 2013).

Most of the bacterial species found in the mammalian intestine are from the phyla Firmicutes and Bacteroidetes (Ley et al., 2008; Holman and Chénier, 2014) and accounted for approximately 90% of all bacteria present (Kim et al., 2011). The remaining intestinal bacteria, accounting for less than 10% of the total population, belong to the Proteobacteria, Fusobacteria, Actinobacteria, Verrucomicrobia and Spirochaetes phyla and a bacterial group that is closely related to Cyanobacteria (Eckburg et al., 2005; Ley et al., 2008). At the genus level, Prevotella, Lactobacillus, Treponema, Roseburia, and Streptococcus are among the most abundant (Kim et al., 2011; Lamendella et al., 2011; Holman and Chénier, 2014; Park et al., 2014). Archaeal and eukaryotic microorganisms also can colonize the intestine in low abundance (Sekirov et al., 2010).

The most abundant microbial genes in the pig microbiome were related to carbohydrate metabolism. The intestinal Firmicutes are Gram-positive bacteria, dominated by species belonging to the Clostridia class, but also include Enterococcaceae and Lactobacillaceae families and Lactococcus spp. (Eckburg et al., 2005). Intestinal Bacteroidetes are Gram-negative bacteria comprised of several Bacteroides species.

The bacterial composition (quantity and proportion) is species specific and varies depending on age, physiological state, gut site, diet composition (especially the presence and nature of DF). In the gastrointestinal tract (GIT) there are 3 microhabitats: i) the lumen of GIT. ii) the mucus layer; iii) the mucosal surface. Due to normal mucus excretion, epithelial turnover and

peristaltic movements in the GIT, it is generally assumed that the mucus and the mucosa associated microbiota are a subset of the luminal microbiota (Leser et al., 2002).

The quantity and composition of microbial species differs over time and along the intestinal tract. (Leser et al., 2000) have reported that different bacterial groups are enriched at different sites of gut, with significantly more microbial diversity and higher concentrations of bacteria in the colon (10^{10} to 10^{12} CFU·(g digesta)$^{-1}$) than in the ileum (10^{8} to 10^{9} CFU·(g digesta)$^{-1}$), and in the ileum than in the stomach and proximal small intestine (10^{3} to 10^{5} CFU·(g digesta)$^{-1}$). (Frank et al., 2007) showed that samples from the small intestine were enriched for the Bacilli class of the Firmicutes and Actinobacteria. On the other hand, Bacteroidetes and the Lachnospiraceae family of the Firmicutes were more prevalent in colonic samples.

Over time, the proportion of bacteria in the phylum Firmicutes increased, while the proportion of bacteria in the phylum Bacteroidetes decreased (Kim et al., 2011). In addition, over the same time frame, the number of bacteria that fell into the non-classified group increased (Kim et al., 2011). The non-classified group consists of sequences that do not show sufficient homology to other sequences to allow for taxonomic classification.

In addition to the longitudinal heterogeneity, the microbiota present in the intestinal lumen differs significantly from the microbiota attached and embedded in this mucus layer as well as the microbiota present in the immediate proximity of the epithelium (Sekirov et al., 2010). Some studies have found that many bacterial species present in the intestinal lumen did not access the mucus layer

and epithelial crypts. In a study with mice, (Swidsinski et al., 2005) reported that Bacteroides, Bifidobacterium, Streptococcus, members of Enterobacteriacea, Enterococcus, Clostridium, Lactobacillus, and Ruminococcus were all found in feces, whereas only Clostridium, Lactobacillus, and Enterococcus were detected in the mucus layer and epithelial crypts of the small intestine.

2.2.2. Factors affecting microbiota

A key element of the mammalian intestinal strategy for maintaining homeostasis with the microbiota is to minimize contact between luminal microorganisms and the intestinal epithelial cell surface. This is accomplished by enhancing the physical barrier through the production of mucus, antimicrobial proteins and IgA (Hooper et al., 2001). Goblet cells secrete mucin glycoproteins that assemble into a thick, stratified mucus layer. Bacteria are abundant in the outer mucus layer, whereas the inner layer is resistant to bacterial penetration. Epithelial cells (such as enterocytes, Paneth cells and goblet cells) secrete antimicrobial proteins that further help to eliminate bacteria that penetrate the mucus layer (Hooper et al., 2010). Plasma cells secrete IgA that is transcytosed across the epithelial cell layer and secreted from the apical surface of epithelial cells, limiting numbers of mucosa-associated bacteria (Suzuki et al., 2004) and preventing bacterial penetration of host tissues (Macpherson and Uhr, 2004b)

Commensal gut microorganisms also provide resistance against the colonization of pathogens by competing for nutrients and binding sites on the host intestinal epithelium and by altering the local intestinal environment with the production of volatile fatty acids, modified bile acids, and antimicrobial compounds (Lallès et al., 2007; Allen and Stanton, 2014). The host immune

system largely tolerates these commensal gut bacteria, which also help stimulate normal immunological development and homeostasis (Kelly and King, 2001; Zoetendal et al., 2004; Sommer and Bäckhed, 2013).

However the feed plays a crucial role on the pig gut microbiome, and the gut microbiota is a vital component of a healthy animal. Bacteria in the colon metabolize undigested carbohydrates into short-chain fatty acids, which are then absorbed by the host. In this way, intestinal bacteria help recover nutrients that would otherwise be lost through excretion (Cummings and Macfarlane, 1997; Laparra and Sanz, 2010).

Changes in diet should result in differences in the composition of the microbiome, such that the proper metabolic capacities are provided by the bacteria to digest different substrates (Lamendella et al., 2011). The texture of the feed and granulometry (Mølbak et al., 2008), as well as the sanitary conditions of the housing environment, can also alter the gut microbiota, with pigs raised under poor sanitation having been shown to shed more Lactobacillus (increase of 0.9 log10 CFU·(g feces)−1) and Enterobacteriaceae (increase of 1.0 log10 CFU·(g feces)−1) in their feces (Montagne et al., 2012) (Montagne et al. 2010).

2.2.3. Host response to bioactive fermentation products

The control of enteric pathogen bacteria can be realized across antibiotic use or dietary manipulation of the hindgut fermentation. This change in the fermentation can be promoted by the choice of dietary raw materials or by the

use of additives. Both rules improve the colonization resistance exerted by the commensal flora to include enteric pathogens and thereby improve “gut health”.

2.2.3.1. Bioactive amines and peptides

2.2.3.2. Short chain fatty acids

The short chain fatty acids (SCFA) and the lactate are the main product of DF fermentation, predominantly acetate, propionate, butyrate and succinate. The SCFA produced are rapidly absorbed from the gut lumen; especially when the luminal pH is low or when there is a higher SCFA concentration in the lumen. The acetate is transported to the liver where it is used as energy substrate for muscle tissue while the propionate is converted to glucose in the liver.

The butyrate is used primarily by the colonocytes and provides a major source of energy for its metabolic activities; the butyrate stimulates the development and growth of the large and small intestine, by stimulation of the epithelial cell proliferation. In the large intestine, the SCFA stimulate the reabsorption of water and sodium; limiting the risk of diarrhoea and they are capable of inhibiting the growth of some intestinal bacterial pathogens (as *Escherichia coli* and *Clostridium difficile* in pigs for instance).

In humans, during inflammatory bowel disease (IBD), decreased colonization with Lachnospiraceae and Bacteroidales spp. are linked to reductions in SCFAs (Treem et al., 1994; Frank et al., 2007). Patients with diversion colitis were found to have negligible levels of SCFAs, and instillation of SCFAs (acetate, propionate, butyrate) resulted in the disappearance of symptoms (Maslowski et al., 2009).

2.3. Modulation on intestinal equilibrium through the feed

The feed ingestion per se and the feed intake level affect the intestinal structure and its function (Spreeuwenberg et al., 2001), but also the functional properties that can have the feed. The strategies to improve the pig gut function are i) changes in the feed process or structure (thermal process, pellets, liquid fermented feed); ii) feed components change (cereal type, proteins source, types and fiber level); iii) use of additives (enzyme, organic acids, prebiotics, probiotics, plasma and others) (Pluske et al., 1998; Hedemann et al., 2005; Jensen et al., 2011).

2.3.1. Macro-ingredients

2.3.1.1. Proteins and aminoacids

2.3.1.2. Carbohydrates

The carbohydrates can be classified by physiology or by chemistry (ASP). The first classification divider nutritionally into two groups the carbohydrate fractions of plants incorporated into animal feed: i) those that are hydrolysable by the intestinal enzymes of the animal (these sugars are located predominantly within the plant cell); and ii) those that are hydrolysable only by enzymes produced by the microbiota or added in the diet (these are mainly cell wall polysaccharides (BLAS AND GINDENNE). The chemistry classification considers the molecular weight of the dietary carbohydrates (Table 1). The carbohydrates with low-molecular weight are classified are mono-, di- and oligosaccharides and often referred to as ‘sugars’ (ASP); whereas the polysaccharides are defined as having 10 or more monomeric residues.

The dietary fiber (DF) is the main substrate for the bacterial fermentation; the DF term includes any polysaccharide reaching the hindgut and so includes resistant starch (RS) and soluble and insoluble non-starch polysaccharide (NSP). The starch is compound by amylose (α-(1-4)-glucosidic linkage) and amylopectin (α-(1-6)-glucosidic linkage). These linkages are susceptible to hydrolysis by the salivary and pancreatic α-amylase (small intestine). However, the hydrolysis is not always complete and this resistant starch (RS) escapes the digestion in the small intestine. A higher proportion of the NSP leaves of the small intestine nearly intact, and is fermented by the microbiota in the large intestine.

The DF concept includes any polysaccharides reaching in the hindgut and so includes resistant starch (RS), some oligosaccharides, soluble and insoluble non-starch polysaccharides (NSP).(Englyst and Cummings, 1985)

The role of dietary fiber

The type and composition of dietary fiber (DF) influence the digestion process on the gastro-intestinal tract (GIT), the same way as physical-chemical digesta characteristics (Anguita et al., 2007), gut morphology (Nofrarías et al.; Jørgensen et al., 1996) and intestinal function (Correa-Matos et al., 2003). The factors that influence the fermentescibility of DF are the fiber source, solubility, degree of lignification, feed processing, level of inclusion in the diet, intestinal transit time, age and weight of the animals and microbial composition. The fiber source is a decisive factor for fermentation and digestibility. There is wide variation in the composition and content of DF among feed ingredients, thereby their physico-chemical properties in the GIT of swine. These variations affect

the digestion and fermentation characteristics in the GIT of animals. The soluble fibers increase the intestinal transit time and delays the gastric emptying. This process delays the glucose absorption, increases the pancreatic secretion and slows down absorption. Whereas, the insoluble fibers decrease the intestinal transit time and enhance water holding capacity, this assists fecal bulking. The soluble DF is more easily, rapidly and completely fermented once it arrives within the large intestine. The soluble DF increases the number and the activity of the bacteria in the large intestine, and even in the ileum. The insoluble DF fermentation happens along the large intestine.

The fiber from oats, wheat and barley are considerate to be protective against the proliferation of E. coli and the occurrence of post-weaning colibacillosis in piglets, but is not always true, and depends on the part of the grain used. Unfortunately, in pigs, higher levels of insoluble fiber such as wheat straw or cellulose have been associated with the depression of weight gain.

Non-starch polysaccharides

The cell wall polysaccharides of barley and wheat may be responsible for the nutrient-encapsulating effect (Emiola et al., 2009). Consequently, starch may be surrounded by cell wall structure and thus unavailable for digestion in the ileum. The NSP fermentation is higher for cell material from non-lignified compared to lignified materials.

The NSP composition on each cereal is different; the corn presents 9% of NSP, mainly arabinoxylanos (Dierick and Decuypere, 1996). The wheat contains from 8.3 to 9.8% of total NSP (Slominski et al., 2004); although arabinoxylans are the

main polysaccharides of wheat (Henry, 1987), significant amounts of β-glucan and cellulose are also present (Steenfeldt et al., 1995). Whereas the barley have 4.2% and 6.6% of β-glucan and pentosan, and the rye contain 50-60% of NSP, mainly arabinoxylans (Evers et al., 1999). Because of difference on NSP composition between cereals, more authors suggested that the combination of various enzymes is required for complete degradation of complex NSP and improves the nutrient utilization (Chesson, 1993).

2.3.2. Micro-ingredients and in-feed additives

2.3.2.1. Acidifiers

2.3.2.2. Probiotics

2.3.2.3. Prebiotics

2.3.2.4. Enzymes

Phosphatases

Carbohydrases

The NSP form the endosperm wall of the all cereals and it restrict access to nutrients found in the endosperm (Simon, 1998). Enzymes able to break down the cell wall matrix, especially the insoluble components, may facilitate the release of nutrients encapsulated in cell walls or incorporated into the cell wall itself, resulting in an easier access of digestive enzymes (Bedford and Schulze, 1998).

The primary rationale for the use of enzyme technology on animal feed is to improve the nutritive value of the feedstuffs. All animals use enzymes in the digestion of feed, those produced either by the animal itself or microbes present in the digestive tract. However, the digestive process in nowhere near 100% efficient; for example, swine are unable to digest 15-25% of the food they eat. Therefore, the supplementation of animals feed with enzymes to increase the efficiency of digestion can be seen as an extension of the animal's own digestive process.

According to , the enzymes are used in animal feed by some reasons: i) to break down anti-nutritional factors that are present in many feed ingredients. These substances, many of which are not susceptible to digestion by the animal's endogenous enzymes, can interfere with normal digestion, causing poor performance and digestive upsets. ii) to increase the availability of starches , proteins and minerals that are either enclosed within fiber-rich cell walls and, therefore not as accessible to the animals own digestive enzymes. iii) to break down specific chemical bonds in raw materials that are not usually broken down by the endogenous enzymes, thus realizing more nutrients. iv) to supplement the enzymes produced by young animals where, because of the immaturity of their own digestive system, endogenous enzyme production may be inadequate. Additionally, enzyme addition can reduce the variability in nutritive value between feedstuffs, improving then accuracy of feed formulations.

The use of carbohydrases, targeted to hydrolyze NSP, is widely and successfully implemented when wheat, barley, rye, oats, or triticale are used on

poultry (Francesch and Geraert, 2009). But the application of supplementary enzymes to pig diets hasn't reported the same clear-cut results obtained in poultry. In one hand, many pigs trials have demonstrated the value of enzyme addition to grower and finisher diets in terms of performance parameters improving (Kiarie et al., 2007; Omogbenigun et al., 2004) One the other hand, others similar trials failed to show great benefit (Mavromichalis et al., 2000) , being the lack of adequate enzymatic preparations a possible explanation on this fact.

The negative influence of the DF are often more important in the DM flow and endogenous losses, leading to a decrease in the ileal and fecal digestibilities of energy and nutrients including starch, protein and lipid. The solution for this problem can be the cooking, the dehulling of legume seeds or the microbial enzyme supplementation.

The carbohydrases supplementation of cereals based diets promoted a more proximal digestion of the NSP, increasing the nutrients digestibility (Omogbenigun et al., 2004; Ji et al., 2008) and, consequently, the productive parameters (Emiola et al., 2009). Some studies found an enhance on performance parameters and ileal digestibility with carbohydrase supplementation of barley (Bedford, 2000), wheat/barley/rye and corn diets (Bedford and Schulze, 1998).

The change on small intestine substrate can modify the mucosa morphometry. As the cereal NSP composition of corn/soybean and wheat/barley/rye diet is different, the products of the exougenous enzyme action in each diet also are different. (Hedemann et al., 2006) showed that the disacchararidases exhibited

the greatest activities at 50% of the small intestine, whereas we have analyzed the final of the small intestine.

Therefore, the enzymatic supplementation of the NSP rich diets breaks down these NSP in oligosaccharides. This change in the substrate entails a change in the microbiota and fermentation profile.

Chapter 3.

Objectives

Chapter 3. Objectives

The research work of this PhD Thesis is framed in a bigger project entitled "Effect of a non-starch polysaccharides degrading enzymes (Rovabio®) in cereal-based diets on the performance, the intestinal microbiota and intestinal health in swine and poultry" and funded by Adisseo France SAS, (Antony, France). This project has as main objectives: i) to test the efficacy of economically affordable doses of a multi-enzyme preparation on performance of growing pigs and broilers and compare the effect based on the type of cereal source; ii) to clarify mode of action with which enzymes act in different cereal sources and to clarify how these carbohydrases can influence gut health in growing pigs and broilers; iii) to evaluate the intestinal microbial characteristics, and different markers of intestinal health of growing pigs and broilers in response to NSP enzyme supplementation; iv) to design feeding strategies that enhance gut health of swine and poultry.

The work of this PhD Thesis was mostly related to the first three main objectives stated above and aimed to investigate the role of non-starch polysaccharides degrading enzymes included in diets with different cereal composition (corn-soybean or wheat/barley/rye) on the performance and the gut health of growing pigs. This general objective was split in tree specific ones:

To evaluate the effect of a non-starch polysaccharides degrading enzymes on productive and nutritional parameters.

To evaluate the ability of non-starch polysaccharides degrading enzymes to modify the intestinal ecosystem and to assess if varies depending on cereal composition of the diet.

To study the effect of these two types of cereals in the feed and the supplementation of the diets on gut mucosa architecture and the immune response of growing pigs

To assess these objectives, one trial was designed:

An *in vivo* experiment was designed to evaluate the capacity of non-starch polysaccharides degrading enzymes in cereal-based diets added to either corn- or wheat-rye-barley- based diets during four weeks on growth performance, intestinal environment, energy and nutrient digestibility, profile and composition of gut microbiota, intestinal mucosa architecture, and gut mucosa immune response.

Chapter 4.

Manuscripts

Chapter 4. Manuscripts

4.1. Wheat-barley-rye- or corn-fed growing pigs respond differently to dietary supplementation with a carbohydrase complex

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Wheat-barley-rye- or corn-fed growing pigs respond differently to dietary supplementation with a carbohydrase complex

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ABSTRACT: Thirty-six pigs (22 kg of BW) were used to evaluate a carbohydrase preparation, with xylanase and β-glucanase as main activities, added to either wheat-barley-rye- (WBR) or corn-based diets on performance, intestinal environment, and nutrient digestibility. Pigs were offered 1 of 4 different dietary treatments for 27 d according to a factorial arrangement of treatments (a 2 × 2) with 2 cereal types (WBR or corn) and 2 levels of supplemental carbohydrase (0 or 0.01%). Pig growth and feed intake were individually measured every week until the end of the experiment when pigs were slaughtered to obtain samples of digesta and tissues. Cereal type affected performance only during wk 1, in which WBR improved ADG (590 vs. 440 g/d; $P = 0.008$) and G:F (0.61 vs. 0.43; $P = 0.045$) compared with corn. The WBR also increased the viscosity of the digestive contents in stomach (1.95 vs. 1.23 mPa·s; $P = 0.001$) and ileum (6.53 vs. 2.80 mPa·s; $P = 0.001$) and resulted in greater cecal starch digestibility (95.7 vs. 93.9%; $P = 0.012$). However, trends for a reduction in digestibility were observed for glucose in the nonstarch polysaccharide (NSP) fraction in the ileum (64.4 vs. 75.8%; $P = 0.074$) and galactose in the NSP fraction in the cecum (1.4 vs. 1.8%; $P = 0.055$). The use of the enzyme preparation increased ADFI during wk 2 (1,328 vs. 1,215 g/d; $P = 0.028$), and increased villus height (423 vs. 390 μm; $P = 0.045$) and tended to reduce relative pancreas weight (0.16 vs. 0.17% BW; $P = 0.079$) at d 27. The enzyme also improved cecal starch digestibility (95.5 vs. 94.1%; $P = 0.043$) and tended to improve ileal energy digestibility (61.3 vs. 53.7%; $P = 0.090$) and cecal glucose digestibility in the NSP fraction (76.0 vs. 54.5%; $P = 0.055$). However, it reduced the cecal digestibility of mannose in the NSP fraction (27.0 vs. 50.5%; $P = 0.016$). Interactions ($P < 0.05$) between cereal type and enzyme supplementation were observed for ADG and G:F during wk 2, BW and ADG during wk 3, and BW and ADFI over the whole trial; and also for villus-height-to-crypt-depth ratio and for cecal DM digestibility. In all instances, whereas the added enzyme had no effect in the case of the corn diet, improvements were observed with WBR. In conclusion, the multi-enzyme tested had different effects depending on the type of cereal present in the diet.

Key words: digestibility, β-glucanase, nonstarch polysaccharide, swine, villus height, xylanase

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INTRODUCTION

Swine diets are mainly composed of cereals with variable compositions in nonstarch polysaccharides (**NSP**). Those may interfere with the digestion of other nutrients (Simon, 1998). The NSP content ranges from 7 to 9% in corn, to 11% in wheat and rye, and 16% in barley (Chesson, 1993; Dierick and Decuypere, 1996). In addition, whereas NSP in corn mainly consist of insoluble arabinoxylans (Summers, 2001), wheat and rye contain arabinoxylans (both soluble and insoluble) and insoluble β-glucans (Henry, 1987; Evers et al., 1999) and barley contains arabinoxylans (mainly insoluble) and β-glucans (mainly soluble; Chesson, 1993; Partridge, 2001). Although the use of NSP-degrading carbohydrases has been widely and successfully implemented in poultry fed NSP-rich diets (Francesch and Geraert, 2009), the results are not as clear in swine. The efficacy of carbohydrase in pigs might not be due to improvements in nutrient digestibility alone (Omogbenigun et al., 2004; Ji et al., 2008) but also to changes in digestive content characteristics, which can indirectly affect gut mucosa integrity (Jakob et al., 2005b; Nofrarías et al., 2006). Although some authors report improvements in

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pig performance with enzymes (Omogbenigun et al., 2004; Jakob et al., 2005a; Kiarie et al., 2007), others do not (Mavromichalis et al., 2000). The differences in the efficacy observed may be explained by the differences in the NSP fraction of the diets or in tested carbohydrase activities.

Carbohydrase efficacy in growing pigs is hypothesized to be dependent on enzyme activities, as well as the nature (and concentration) of the dietary NSP fraction and how well they match each other. The aim of this study was to test the efficacy of a multi-enzyme preparation (mainly xylanase and β-glucanase) added to either corn- (low-NSP content, mostly insoluble) or wheat-rye-barley- (**WBR**; high-NSP content, mostly soluble) based diets on growth performance, intestinal environment, and energy and nutrient digestibility in growing pigs.

MATERIALS AND METHODS

This experiment was conducted at the Experimental Farm of the Institut de Recerca i Tecnologia Agroalimentària (**IRTA**) following approval by IRTA's Ethical Committee on Animal Experimentation. The management, housing, husbandry, and slaughtering conditions were conducted according to the European Union Guidelines (VICH-GL9, 2000).

Animals, Housing, and Dietary Treatments

Thirty-six growing pigs (Landrace × Pietrain; mixed entire males and females; BW 22.0 ± 0.4 kg) from 9 different litters (4 pigs per litter) of the IRTA farm herd were used. At weaning, piglets were identified by ear tags and maintained on a common pre-experimental diet formulated to meet or exceed the nutrient requirements for weaning pigs until the start of the experiment (NRC, 1998; Table 1). Pigs were kept individually in 36 pens (2 × 1 m). The facilities were provided with forced ventilation for thermal regulation, and each pen had 1 feeder and 1 water nipple to allow for ad libitum access to feed and water.

At the start of the experiment, pigs from the same litter were randomly assigned to the 4 experimental treatments, providing a total of 9 replicates per treatment. Treatments were designed as a 2 × 2 factorial arrangement using 2 sources of cereal (corn or WBR), and with or without supplementation with NSP-degrading enzymes (0 or 0.01%). All experimental diets were formulated to be isonutritive (i.e., 3,125 kcal of ME/kg, 16% CP, 10 g/kg of Lys, 9 g/kg of Ca, and 6 g/kg of P) and to meet or exceed the nutrient requirements for growing pigs (NRC, 1998; Table 2), and were provided in mash form. The enzyme preparation tested was a multi-enzyme complex obtained from *Penicillium funiculosum* (Rovabio Excel AP, Adisseo France SAS, Antony, France). This product is guaranteed to provide 22,000 U/g of endo-β-1,4-xylanase and 2,000 U/g of endo-β-1,3(4)-glucanase. Units are defined as the amount of enzyme, which hydrolyzes wheat arabinoxylans, reducing the viscosity of the solution to give a change in relative fluidity of 1 arbitrary unit·min^{-1} under the assay conditions (pH 5.5 and 30°C) for xylanase activity, and the quantity of enzyme, which hydrolyzes barley β-glucan (bound to a chromophore), releasing ethanol-soluble oligomers to give an absorbance of 0.820 units at 590 nm for endo-β-1,3(4)-glucanase activity. In addition to endo-β-1,4-xylanase and endo-β-1,3(4)-glucanase activities, other enzymes (cellulases, pectinases, mannanase, and others) are also active in the preparation (Karboune et al., 2008, 2009). The presence of the enzyme preparation in the supplemented diets was confirmed by analyzing the corresponding β-glucanase and xylanase activities (Cosson et al., 1999). Titanium dioxide was added to all the experimental diets as an indigestible marker.

Experimental Procedures and Sampling

The animals were fed the experimental diets for 27 d. Individual BW and feed intake were recorded weekly at d 0, 7, 14, 21, and 27 of the trial. At the end of the experiment, all 36 pigs were euthanized by exsanguination under isofluorane anesthesia. The abdominal cavity was immediately opened, and the whole gastrointestinal tract (**GIT**) from cardia to rectum was removed. The GIT was tied to retain the digestive contents in the different sections (stomach, small intestine, and cecum). The pH of the digesta in stomach, ileum (100 cm cranial to the ileocecal valve), and cecum were measured with a unipolar electrode pH meter (HI83141 pH meter, Hanna Instruments, Eibar, Spain). Samples of digestive contents, GIT tissues, and mucosa were collected immediately to perform the different analyses. Finally, the weights of the dissected pancreas and the emptied stomach were also recorded.

Table 1. Ingredient composition of the common pre-experimental diet, as-fed basis

Ingredient, %	Content
Barley	28.94
Whey, acid, dehydrated	16.67
Corn	15.46
Soybean, whole extruded	14.64
Soybean meal, 48% CP	11.42
Fish meal	8.00
Soybean oil	4.03
Vitamin-mineral premix[1]	0.40
Calcium carbonate	0.17
L-Lys·HCl	0.13
DL-Met	0.11
L-Thr	0.04

[1]Providing per kilogram of diet: vitamin A, 1.5 mg; vitamin D_3, 0.025 mg; vitamin E, 15 mg; thiamine, 1.3 mg; riboflavin, 3.5 mg; vitamin B_{12}, 0.025 mg; vitamin B_6, 1.5 mg; calcium pantothenate, 10 mg; nicotinic acid, 15 mg; biotin, 0.1 mg; folic acid, 0.6 mg; vitamin K_3, 2 mg; Fe, 80 mg as iron sulfate; Cu, 6 mg as copper sulfate; Co, 0.75 mg as cobalt sulfate; Zn, 60 mg as zinc oxide; Mn, 30 mg as manganese sulfate; I, 0.75 mg as potassium iodate; Se, 0.10 mg as sodium selenite; and ethoxiquin, 150 mg.

Table 2. Ingredient composition and chemical analysis of the experimental diets, as-fed basis

	Corn		Wheat-barley-rye	
Item	0% enzyme[1]	0.01% enzyme	0% enzyme	0.01% enzyme
Ingredient, %				
Corn	71.11	71.11	0	0
Wheat	0	0	24.58	24.58
Barley	0	0	24.58	24.58
Rye	0	0	22.72	22.72
Soybean meal, 44% CP	17.83	17.83	13.55	13.55
Sunflower meal	6.88	6.88	6.88	6.88
Lard	0.03	0.03	3.47	3.47
Calcium carbonate	1.15	1.15	1.19	1.19
Dicalcium phosphate	1.28	1.28	1.17	1.17
Sodium chloride	0.37	0.37	0.50	0.50
Vitamin/mineral premix[2]	0.40	0.40	0.40	0.40
L-Lys·HCl	0.34	0.34	0.36	0.36
L-Thr	0.04	0.04	0.07	0.07
DL-Met	0.03	0.03	0.04	0.04
L-Trp	0.04	0.04	0.01	0.01
Titanium dioxide	0.50	0.50	0.50	0.50
Analyzed composition				
DM, %	86.82	86.89	88.78	88.96
Ash, %	5.32	5.46	5.62	5.55
GE, kcal/kg	3,888	3,858	4,031	4,045
Ether extract, %	3.23	3.13	4.56	4.7
CP, %	15.17	15.23	16.33	15.88
Crude fiber, %	4.21	3.72	4.76	4.63
NSP,[3] %	9.25	9.27	13.68	14.05
Arabinose, %	3.22	3.29	5.37	5.18
Xylose, %	1.38	1.31	1.86	1.29
Mannose, %	0.32	0.34	0.51	0.26
Glucose, %	3.08	3.04	4.86	6.29
Galactose, %	1.25	1.29	1.08	1.04
Enzyme recovery, %				
β-Glucanase	—	92	—	92
Xylanase	—	106	—	103

[1]Rovabio Excel AP (Adisseo France SAS, Antony, France).

[2]Providing per kilogram of diet: vitamin A, 1.5 mg; vitamin D_3, 0.025 mg; vitamin E, 15 mg; thiamine, 1.3 mg; riboflavin, 3.5 mg; vitamin B_{12}, 0.025 mg; vitamin B_6, 1.5 mg; calcium pantothenate, 10 mg; nicotinic acid, 15 mg; biotin, 0.1 mg; folic acid, 0.6 mg; vitamin K_3, 2 mg; Fe, 80 mg as iron sulfate; Cu, 6 mg as copper sulfate; Co, 0.75 mg as cobalt sulfate; Zn, 60 mg as zinc oxide; Mn, 30 mg as manganese sulfate; I, 0.75 mg as potassium iodate; Se, 0.10 mg as sodium selenite; and ethoxiquin, 150 mg.

[3]Nonstarch polysaccharides; total neutral sugars.

Viscosity, Enzyme Activity, and Histology Analysis

Fresh samples (approximately 10 mL) of digesta from stomach, ileum (20 cm cranial from the ileocecal valve), and cecum were immediately refrigerated on ice. The liquid fraction of digesta was obtained by centrifugation at 3,500 × *g* for 10 min at 10°C, and viscosity of the supernatant (0.5 mL) was immediately measured at a shear rate of 12 s^{-1} and 30°C (Digital DV-II cone/plate viscometer, Brookfield Engineering Laboratories, Stoughton, MA) as described by Steenfeldt et al. (1998).

A section of the ileum (10 cm) was opened lengthwise along the mesenteric attachment, rinsed carefully with ice-cold 0.9% NaCl, and blot dried. Then the mucosa layer was scraped with a sterile cell scraper, placed in capped tubes (2 to 3 g), and immediately snap-frozen in liquid N and stored at −80°C until enzyme activities were determined. To analyze saccharase and maltase activities of the ileal mucosa, the samples were homogenized and diluted in ice-cold 0.9% NaCl (100 mg/mL for saccharase activity and 5 mg/mL for maltase activity). The samples were then incubated with a solution of either saccharose or maltose in maleate buffer 0.2 *M* (pH 6.5) at 2% for 60 min at 37°C, followed by 10 min at 95°C in a thermocycler. The glucose concentration was then measured (in duplicate) with an assay kit (Amplex Red Glucose/Glucose Oxidase Assay Kit, Invitrogen, Eugene, OR) and read by a spectrophotometer (SpectraMax Plus 384, MDS Analytical Technologies Inc., Sunnyvale, CA) with a software (SoftMax Pro 5.3 Ink Software, MDS Analytical Technologies Inc.). The enzyme activities were adjusted for protein content, which was measured with an assay kit and fluorometer (Quant-iT Protein Assay Kit and Qubit fluorometer, Invitrogen, Eugene, OR).

Another 5 cm section of the ileum (20 cm cranial to the ileocecal valve) was obtained for the histological study of ileal mucosa. Samples were partially opened

(about 3/4 of length) along the mesenteric attachment and fixed by immersion in 10% (vol/vol) formaldehyde (3.7%, pH 7.0, stabilized with methanol). Tissue samples were dehydrated and embedded in paraffin, sectioned at 3 μm, and stained with hematoxylin and eosin. Morphometric measurements were performed with a light microscope (BHS, Olympus, Barcelona, Spain) using a linear ocular micrometer (Olympus, Ref. 209-35040, Microplanet, Barcelona, Spain). Villus height (**VH**) and crypt depth (**CD**) were measured on 10 well-oriented villi and crypts per animal, and the VH:CD ratio was calculated.

Digestibility Determination

The remaining ileal and cecal digestive contents were stored at −20°C in hermetic plastic bags until digestibility was determined using TiO_2 as an indigestible marker. Feed and digesta were analyzed for DM, NSP, starch, CP, and energy according to standard procedures (AOAC, 1995). Crude protein (N × 6.25) was determined by the Dumas method (model TruSpec N, Leco, St. Joseph, MI). Gross energy was determined by combustion under high pressure of oxygen in an adiabatic bomb (model C4000 adiabatic calorimeter, IKA Werke GmbH & Co. KG, Staufen, Germany). Total starch was determined colorimetrically, as glucose liberated after enzymatic incubation of 0.2 g of sample with 0.1 mL of thermostable α-amylase (Ref. A-4551, Sigma, Madrid, Spain) diluted 1/10 with distilled water for 1 h at 100°C, and amyloglucosidase (Ref. A-3514, Sigma) for 6 h at 60°C, according to the method of Theander (1991). Nonstarch polysaccharides were analyzed by gas chromatography according to the method described by Englyst and Cummings (1984). The concentrations of TiO_2 were measured as described by Short et al. (1996). The digestibility coefficients were calculated using the index method (Adeola, 2001).

Statistical Analyses

Data were analyzed using the SAS MIXED procedure (SAS Inst. Inc., Cary, NC) using the animal as the experimental unit. The model included cereal type (corn or WBR), enzyme (0 or 0.01%), and their interaction as fixed effects, and litter as a random effect. In addition, for the analysis of the performance data (Table 3), BW at the start of the corresponding experimental period was used as the covariable. Results are presented as least squares means. The level of significance was set at $P < 0.05$, and trends were discussed at $P < 0.1$.

RESULTS

The presence of the enzyme preparation in the supplemented diets was confirmed (Table 2). Recovery rates were 92 and 92% (β-glucanase activity) and 106 and 103% (xylanase activity) for the supplemented corn and WBR diets, respectively.

Growth Performance

The growth performance of the growing pigs over the whole experimental period is presented in Table 3. Pigs fed the WBR-based diets had greater BW (26.3 vs. 24.9 kg; $P = 0.008$), ADG (590 vs. 440 g/d; $P = 0.008$), and G:F (0.61 vs. 0.43; $P = 0.045$) than the corn diets during the first week of trial and also BW during the second week (31.1 vs. 29.3 kg; $P = 0.006$). No effects of cereal type were observed during wk 3 and 4, or over the whole experimental period. Diet supplementation with the enzyme preparation resulted in greater ADFI during the second week of trial (1,328 vs. 1,215 g/d; $P = 0.028$). Interactions between cereal type and enzyme supplementation were observed for ADG ($P = 0.045$) and G:F ($P = 0.037$) during the second week of trial. Whereas the addition of enzyme improved ADG for the WBR-based diet, it had no effect for the corn diet. Similarly, there were no differences in G:F between the 2 nonsupplemented diets, but adding the enzyme preparation to the WBR diet resulted in greater G:F than the corn diet. During the third week, interactions were observed for BW ($P = 0.031$), ADG ($P = 0.031$), ADFI ($P = 0.081$), and G:F ($P = 0.086$). Similar interactions were observed for ADG ($P = 0.056$) and G:F ($P = 0.075$) during the last week of trial and for BW ($P = 0.013$) and ADFI ($P = 0.035$) over the whole trial.

Characteristics of Digesta and Gut Morphology

Digestive organ weight, digestive content properties, and ileal mucosa enzymatic activity and histology are shown in Table 4. Viscosities of the digestive contents were greater for the WBR than the corn diets in the stomach (1.95 vs. 1.23 mPa·s; $P = 0.001$) and the ileum (6.53 vs. 2.80 mPa·s; $P = 0.001$), but no difference was observed in the cecum. Supplementation of diets with the enzyme preparation increased villus height (423 vs. 390 μm; $P = 0.045$) and tended to reduce relative pancreas weight (0.16 vs. 0.17%; $P = 0.079$) at the end of the trial. An interaction between cereal nature and enzyme supplementation was observed for the VH:CD ratio ($P = 0.031$) so that the ratio increased with the enzyme for the WBR diet, but there was no difference for the corn diet.

Nutrient Digestibility

Nutrient digestibilities measured in the ileum and cecum are presented in Table 5. Cecal starch digestibility was greater for the WBR-based than for the C diets (95.7 vs. 93.9%; $P = 0.012$). However, the digestibility of the corn-based diets tended to be greater for glucose in the NSP fraction in the ileum (75.8 vs. 64.4%; $P = 0.074$) and for galactose in the NSP fraction in the cecum (1.8 vs. 1.4%; $P = 0.055$). The enzyme preparation improved starch digestibility in the cecum (95.5 vs. 94.1%; $P = 0.043$), and tended to improve energy di-

Table 3. Effect of carbohydrase supplementation to corn- or wheat-barley-rye-based diets on the performance of growing pigs

	Corn		Wheat-barley-rye			*P*-value		
Item	0% enzyme[1]	0.01% enzyme	0% enzyme	0.01% enzyme	SED	Cereal	Enzyme	Cereal × enzyme
BW, kg								
d 0	21.7	22.0	22.3	22.1	0.69	0.466	0.973	0.644
d 7[2]	25.2	25.0	26.1	26.2	0.51	0.008	0.831	0.692
d 14[2]	29.6	29.4	30.3	31.5	0.66	0.006	0.315	0.133
d 21[2]	35.4	34.0	34.8	36.9	1.09	0.145	0.626	0.031
d 27[2]	41.4	38.5	39.3	42.3	1.58	0.475	0.998	0.013
ADG, kg/d								
0 to 7 d[2]	0.46	0.42	0.59	0.60	0.073	0.008	0.832	0.694
8 to 14 d[3]	0.63	0.63	0.59	0.75	0.055	0.303	0.048	0.045
15 to 21 d[4]	0.84	0.68	0.64	0.75	0.083	0.295	0.699	0.031
22 to 27 d[5]	1.01	0.78	0.76	0.84	0.106	0.213	0.342	0.056
0 to 27 d[2]	0.72	0.61	0.64	0.75	0.058	0.475	0.998	0.013
ADFI, kg/d								
0 to 7 d[2]	1.02	1.04	0.96	1.15	0.087	0.661	0.104	0.179
8 to 14 d[3]	1.25	1.32	1.18	1.34	0.068	0.628	0.028	0.314
15 to 21 d[4]	1.65	1.58	1.35	1.45	0.068	0.001	0.700	0.081
22 to 27 d[5]	1.89	1.69	1.64	1.65	0.122	0.114	0.277	0.273
0 to 27 d[2]	1.43	1.38	1.27	1.42	0.062	0.201	0.265	0.035
G:F								
0 to 7 d[2]	0.44	0.42	0.70	0.52	0.117	0.045	0.278	0.349
8 to 14 d[3]	0.50	0.48	0.50	0.56	0.029	0.081	0.337	0.037
15 to 21 d[4]	0.51	0.46	0.46	0.52	0.041	0.761	0.866	0.086
22 to 27 d[5]	0.54	0.43	0.47	0.51	0.056	0.944	0.442	0.075
0 to 27 d[2]	0.50	0.46	0.51	0.53	0.034	0.152	0.668	0.179

[1]Rovabio Excel AP (Adisseo France SAS, Antony, France).
[2]BW at d 0 used as covariable.
[3]BW at d 7 used as covariable.
[4]BW at d 14 used as covariable.
[5]BW at d 21 used as covariable.

Table 4. Effect of carbohydrase supplementation to corn- or wheat-barley-rye-based diets on the weight of digestive organs, the characteristics of the digestive contents, and the enzymatic activity and histology of ileal mucosa of growing pigs

	Corn		Wheat-barley-rye			*P*-value		
Item	0% enzyme[1]	0.01% enzyme	0% enzyme	0.01% enzyme	SED	Cereal	Enzyme	Cereal × enzyme
Organ weight, % of BW								
Stomach	0.71	0.67	0.70	0.66	0.033	0.664	0.101	0.861
Pancreas	0.17	0.16	0.17	0.16	0.009	0.386	0.079	0.932
Digesta pH								
Stomach	4.05	3.81	3.31	3.37	0.538	0.133	0.803	0.698
Ileum	6.61	6.50	6.52	6.48	0.149	0.617	0.467	0.738
Cecum	6.08	5.91	5.95	5.96	0.111	0.599	0.319	0.258
Digesta viscosity, mPa·s								
Stomach	1.20	1.26	1.90	2.01	0.236	0.001	0.894	0.659
Ileum	2.07	3.53	5.52	6.08	1.177	0.001	0.243	0.600
Cecum	2.44	2.08	2.40	2.58	0.541	0.560	0.826	0.494
Enzyme activity ileal mucosa, U/mg of protein								
Sucrase	15	15	12	14	2.1	0.221	0.513	0.468
Maltase	204	185	170	188	21.3	0.319	0.959	0.224
Morphometry ileal mucosa, µm								
VH[2]	410	417	370	430	22.7	0.396	0.045	0.115
CD[3]	318	316	328	297	18.7	0.733	0.245	0.283
VH:CD ratio	1.29	1.32	1.16	1.46	0.085	0.949	0.008	0.031

[1]Rovabio Excel AP (Adisseo France SAS, Antony, France).
[2]Villus height.
[3]Crypt depth.

Table 5. Effect of carbohydrase supplementation to corn- or wheat-barley-rye-based diets on the ileal and cecal digestibility of nutrients in growing pigs

	Corn		Wheat-barley-rye			*P*-value		
Item	0% enzyme[1]	0.01% enzyme	0% enzyme	0.01% enzyme	SED	Cereal	Enzyme	Cereal × enzyme
Ileal digestibility, %								
DM	60.1	53.8	46.8	60.9	7.29	0.557	0.463	0.060
Starch	90.1	88.2	89.9	93.5	2.94	0.242	0.690	0.211
Energy	60.4	60.6	46.9	62.0	5.85	0.176	0.090	0.100
CP	67.5	63.2	63.2	70.9	5.14	0.649	0.646	0.118
NSP[2]	38.1	35.3	13.5	42.4	17.78	0.517	0.337	0.243
Arabinose	23.2	−0.8	−9.4	28.6	27.52	0.939	0.736	0.144
Xylose	12.8	5.4	−7.5	32.0	26.21	0.873	0.420	0.242
Mannose	7.3	−3.6	−9.7	−51.0	30.11	0.165	0.257	0.505
Glucose	77.5	74.0	56.9	71.9	8.18	0.074	0.356	0.142
Galactose	15.3	17.3	−20.9	2.2	20.63	0.108	0.425	0.500
Cecal digestibility, %								
DM	70.2	68.3	61.4	71.3	3.69	0.287	0.143	0.037
Starch	93.4	94.4	94.8	96.6	0.94	0.012	0.043	0.548
Energy	68.9	66.6	64.0	70.3	2.98	0.785	0.370	0.066
NSP[2]	21.8	21.4	22.7	22.4	3.12	0.691	0.863	0.999
Arabinose	22.6	8.9	11.7	45.8	18.73	0.362	0.475	0.127
Xylose	29.9	−7.8	43.0	45.4	26.58	0.116	0.392	0.360
Mannose	39.5	31.8	61.6	22.1	11.57	0.480	0.016	0.105
Glucose	61.6	69.8	47.4	82.2	13.87	0.932	0.055	0.239
Galactose	2.0	1.7	1.4	1.4	0.31	0.055	0.373	0.499

[1]Rovabio Excel AP (Adisseo France SAS, Antony, France).

[2]Nonstarch polysaccharides; total neutral sugars.

gestibility in the ileum (61.3 vs. 53.7%; $P = 0.090$) and glucose digestibility in the NSP fraction in the cecum (76.0 vs. 54.5%; $P = 0.055$). On the other hand, the digestibility of mannose in the NSP fraction in the cecum was reduced with the enzyme (27.0 vs. 50.5%; $P = 0.016$). An interaction between cereal and enzyme was found for DM digestibility in the cecum ($P = 0.037$), whereas trends were observed for DM in the ileum ($P = 0.060$) and energy in the cecum ($P = 0.066$).

DISCUSSION

The type and composition of dietary fiber influence the digestion process in the GIT (Cummings et al., 1986), the physicochemical characteristics of digesta (Anguita et al., 2007), the gut morphology (Jørgensen et al., 1996; Nofrarías et al., 2007), and the intestinal function (Correa-Matos et al., 2003). Dietary fiber includes any polysaccharides reaching the hindgut such as resistant starch, some oligosaccharides, and soluble and insoluble NSP (Englyst and Wiggins, 1979). Nonstarch polysaccharides are structural components of the endosperm cell walls in cereals, and they interfere with the access of the digestive enzymes to the nutrients inside the endosperm (Simon, 1998). Exogenous enzymes that are able to hydrolyze the plant cell wall matrix may facilitate the access of the digestive enzymes inside the endosperm and help release nutrients encapsulated in the cell walls or incorporated into the cell wall itself (Bedford and Schulze, 1998). The 2 cereal compositions used in the present study are representative of diets commonly used in the pig industry and vary substantially in their NSP content and nature. The corn diet was chosen for its low NSP content (mainly insoluble), whereas the WBR-based diet was selected for its high NSP content (mainly soluble). The aim of this experiment was to study possible interactions between enzyme supplementation and the type of cereals in the diet.

In our study, the pigs had a better growth and feed efficiency during the first week of trial for the WBR than the corn-based diets. In subsequent weeks, however, no differences in performance were observed between the 2 cereal compositions, indicating that the 2 types of diet were nutritionally equivalent, as intended. It is possible that the differences observed for the first week may be due to a better adaptation of the pigs to the WBR diet. The use of 3 different cereals at reduced inclusion, instead of a large proportion of corn as the only cereal source, may have facilitated the acceptance of the new diet at the start of the trial (Solà-Oriol et al., 2009). The viscosity of the stomach and ileum digestive contents for the WBR diets was greater than that of the corn diets. This may have impaired nutrient digestibility (Langhout et al., 2000) and, therefore, would not explain the aforementioned differences in performance during the first week. However, it can also be speculated that the greater proportion of soluble NSP in the digestive contents of pigs fed WBR may have delayed gastric emptying and digesta flow rate (Solà-Oriol et al., 2010), favoring nutrient digestibility.

Regarding the enzyme preparation tested, our data support the initial hypothesis for an interaction between the supplementation with carbohydrases and the nature of the dietary NSP fraction. We observed no responses to enzymes with corn-based diets, but positive effects with the WBR-based diets. The carbohydrase supplementation of the corn diet (based on corn and soybean meal) did not improve pig performance. Although it has been proposed that carbohydrase complexes can improve the energy digestibility of corn-soybean meal diets (Kim et al., 2003) in young pigs, older pigs were used in the current trial (20 to 40 kg of BW) and may have had a more mature digestive system. This may explain the different results. Nevertheless, the effects of enzyme supplementation on performance or nutrient digestibility of pigs fed corn-based diets, as reported in literature, are not consistent. Some studies show positive responses to enzyme supplementation (Jakob et al., 2005a; Fang et al., 2007; Ji et al., 2008), but others fail to show any effects (Li et al., 1999; Kim et al., 2004). Corn contains approximately 7 to 9% NSP, mainly insoluble arabinoxylans (Dierick and Decuypere, 1996; Summers, 2001), whereas soybean meal contains approximately 3% soluble NSP and 16% insoluble NSP (Irish and Balnave, 1993). It could be hypothesized that the enzyme complex modified the characteristics of the mainly insoluble NSP fraction of the corn diet, increasing the solubility of its NSP fraction and the viscosity of the digestive contents. This could impair nutrient digestion and absorption, counteracting possible positive effects of the enzyme and resulting in the absence of an overall response. Alternatively, changes in the intestinal bacterial population in response to the modification of fermentable carbohydrates may have also interfered. In this study, the intestinal microbiota was also studied, and it has been reported that enzymes dramatically change the composition of the intestinal microflora for both cereals (Willamil et al., 2009).

The NSP contents in wheat, rye, and barley are greater than corn, with around 11% in wheat and rye and 16% in barley (Chesson, 1993; Slominski et al., 2004). In addition, NSP in wheat and rye are present as a mixture of soluble and insoluble arabinoxylans, and insoluble β-glucans (Henry, 1987; Steenfeldt et al., 1995; Evers et al., 1999), and as a mixture of arabinoxylans (mainly insoluble) and β-glucans (mainly soluble; Chesson, 1993; Partridge, 2001) in barley. Therefore, in our study, a greater efficacy of the carbohydrases was expected for the WBR diet. Indeed, when WBR diets were supplemented with the enzyme complex, improvements in growth (17%) and feed efficiency (6%) were observed. This agrees with trials in which the use of enzymes improved the performance of piglets (Omogbenigun et al., 2004) and growing-finishing pigs (Schulze et al., 1996; Schulze and Campbell, 1998; Jakob et al., 2005a) fed similar cereal-based diets. The improved performance with the enzyme complex for the WBR diets in the present study agrees well with the observed improvements in nutrient digestibilities in the ileum and cecum. This is in line with the digestibility improvements reported for the enzyme supplementation of wheat-based diets in growing-finishing pigs (Barrera et al., 2004; Woyengo et al., 2008). Our digestibility results indicate that the multicarbohydrase cocktail may have exerted its beneficial effects on nutrient utilization, and thus growth performance, by eliminating the nutrient-encapsulating effect of plant cell wall structural polysaccharides (Bedford and Schulze, 1998). However, it must be considered that, to compensate for the decreased energy content, the WBR diet contained a greater proportion of added fat than the corn diet. This may have favored the efficacy of the enzyme in improving energy digestibility in the WBR diet because the NSP-degrading enzymes have consistently been reported to improve fat digestibility to a greater extent than that of any other nutrient (Bedford and Schulze, 1998). Carbohydrase supplementation of NSP-rich diets promotes a more proximal digestion of the polysaccharides (Omogbenigun et al., 2004; Ji et al., 2008) and changes the nutrients available for microbial growth and fermentation (Summers, 2001; Kiarie et al., 2007; Hanczakowska and Koczywas, 2008). In our study, the composition of the microbiota was also studied and has been reported previously (Willamil et al., 2009).

Saccharase and maltase are intestinal brush border glycoside hydrolases responsible for the final steps of carbohydrate digestion and release of absorbable monosaccharides (Hertel et al., 2000). The digestion and absorption of nutrients in the small intestine determines the amount of fermentable material reaching the hindgut and, therefore, may modulate the carbohydrolase activity at the ileal brush border, as suggested by Hedemann et al. (2006). The addition of β-glucanase and xylanase, likely to increase small intestine digestibility, enhances maltase and sucrose activities in the mucosa of jejunum and ileum of piglets (Fan et al., 2009). However, in our study, there was no difference between any of the treatments on the terminal ileum brush border enzyme activities. Similarly, Li et al. (2004) did not find any differences in maltase, saccharase, and lactase activities in the jejunum of piglets fed a barley-based diet supplemented with or without xylanase and β-glucanase. Brush border enzyme activities, however, not only depend on diet composition, but can also be influenced by pig age (Fan et al., 2002), section of the small intestine (Hedemann et al., 2006), and gut microbiota (Willing and Kessel, 2009). These factors might explain the differences among studies.

The viscosity of digesta was not reduced by enzyme supplementation in either corn or WBR diets, indicating that this may not be a key factor for the efficacy of carbohydrases in swine. Similarly, Kiarie et al. (2007) and Mavromichalis et al. (2000) did not find any effect of enzyme supplementation on the ileal digesta viscosity of pigs fed wheat-based diets. As stated by Bedford and Schulze (1998), the viscosity of digesta in pigs is considerably less than in poultry because of the greater concentration of water in pig digesta, and therefore, the

negative effects associated with digesta viscosity in pigs may be less relevant than in poultry.

The NSP amount and composition of the diet influence the epithelial morphology and cell turnover of the gut mucosa (Montagne et al., 2003). For example, pectin (soluble fiber) reduces VH and CD in piglets, whereas barley hulls (insoluble fiber) had no effect or even resulted in increased VH (Hedemann et al., 2006). In our study, enzyme supplementation did not have any effect on ileal morphometry in pigs fed the corn diet, in contrast to what has been reported previously in piglets (Kim et al., 2003; Jakob et al., 2005b). However, in pigs fed the WBR diet, we observed an increase in VH and VH:CD, and a reduction in CD in the ileum with the addition of carbohydrase. This agrees with previous observations in piglets fed enzyme-supplemented wheat-barley diets (Mori et al., 2007). The increased VH and VH:CD indicate improved absorption capacity, which, in turn, may contribute to the increased nutrient digestibilities observed.

In conclusion, substantially different effects of the multi-enzyme preparation (mainly xylanase and β-glucanase) were observed depending on the type of cereal in the diet. For the corn-based diet (low concentrations of primarily insoluble NSP), no effects were observed. The supplementation of the WBR-based diet (high NSP content and soluble), however, improved nutrient utilization and growth performance. Thus, the efficacy of exogenous NSP-degrading enzymes depends on the NSP composition of the diet.

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Chapter 4. Manuscripts

4.2. Changes in the gut immune response and microbiota of growing pigs fed with wheat-barley-rye or corn supplemented with carbohydrases complex.

Manuscript prepared in order to be submitted

Running Head: Carbohydrases in growing pigs

Changes in the gut microbiota and immune response of growing pigs fed wheat-barley-rye or corn supplemented with carbohydrase complex

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ABSTRACT: Thirty-six pigs (22 kg BW) were used to evaluate a carbohydrase preparation, with xylanase and β-glucanase as main activities, added to either wheat-barley-rye (WBR) or corn (C) based diets on gut microbiota and immune response. Pigs were offered 1 of 4 different dietary treatments for 27 d according to a factorial arrangement of treatments (2 × 2) with 2 cereal types (WBR or C) and 2 levels of supplemental carbohydrase (0 or 0.01%). At the end of the experiment pigs were slaughtered to obtain samples of bile, blood, digesta and tissues.

Relative to WBR, C diets increased the number of crypt intraepithelial lymphocytes, the *Staphylococcus* spp and the formate concentration and decreased lactate concentration in the ileum. In the caecum, C diets also increased increased ammonia concentration and the bacterial populations of Clostridiaceae and Veillonellaceae. On the other hand, WBR resulted in an increased population of Ruminococcaceae in the caecum and a decrease of the OTUS of Clostridia, Peptostreptococcaceae, Clostridiaceae and *Clostridium* spp. in the ileum. Only in absence of enzyme, C diets resulted in an important increase of IEL in the villi of the ileum, and a decreased concentration of acetate, propionate and total SCFA in the caecum, compared to the other three diets. Supplementation with enzymes also increased *Leuconostoc* spp. in the ileum and decreased Clostridiaceae in the caecum. The carbohydrase supplementation of the WBR diet increased the OTUs of Cyanobacteria and *Roseburia* spp. On the other hand, supplementation of the C diet increased the population of *Megasphaera* spp., *Coprococcus* spp., *Lachnospira* spp. and *Veillonella* spp.

Key words: enzyme, non-starch polysaccharides, swine, gut health, fermentation

INTRODUCTION

In last decade the research on gut health risen, in one hand due to its influences on feed digestion and nutrient absorption affecting growth performance of pigs. In the other hand, due to association of a poor gut health with animal infectious diseases and foodborne pathogen colonization (Patterson and Burkholder, 2003). The protector barrier functions of gastrointestinal tract (GIT) are crucial mechanisms of defense during the process of digestion and absorption of nutrients, when the gut is exposed to a lot of antigens and pathogenic bacteria from feed or microbiota (Wijtten et al., 2011; Vancamelbeke and Vermeire, 2017). Any alteration of the gut microbiota or mucosa may affects this barrier function and can result in inflammation or tissue aggression (Pluske, 2013; Pozuelo et al., 2015). The awareness of gut health is an essential objective for pig industry; based on the better knowledge of gut mucosa and microbiota physiologies and its interactions with nutrition, new uses for additives or new feed strategies are being developed in order to improve animal production, health and welfare.

The carbohydrases were widely used in animal nutrition to improve performance parameters by the hydrolysis of the non-starch polysaccharides (NSP) of feed (Bedford and Schulze, 1998). In addition to improved nutrient utilization, enzymes may increase the performance of young pigs through the production of a variety of polysaccharide hydrolysis products that have a direct effect on intestinal health by influencing, direct or indirectly, the growth of gastrointestinal microorganisms (Pluske, 2013). Indeed, studies with nursery pigs (Kim et al., 2003) and broilers (Mathlouthi et al., 2002) suggest that the use of feed enzymes may have a positive impact on gut health. Although, only a few studies has been conducted to determine the effect of carbohydrases on gut health.

The aim of this study was to test the efficacy of a multi-enzyme preparation (mainly xylanase and β-glucanase) added to either corn (**C**; low-NSP content, mostly insoluble) or wheat-rye-barley (**WBR**; high-NSP content, mostly soluble) based diets on microbiota profile and biodiversity, and gut immune response in growing pigs.

MATERIALS AND METHODS

This experiment was conducted at the Experimental Farm of the "Institut de Recerca i Tecnologia Agroalimentària – IRTA" following approval by IRTA's Ethical Committee on Animal Experimentation. The management, housing, husbandry and slaughtering conditions were conducted according to the European Union Guidelines (VICH-GL9, 2000).

Animals, Housing, and Dietary Treatments

Thirty-six growing pigs (Landrace × Pietrain; mixed entire males and females; BW 22.0 ± 0.4 kg) from 9 different litters (4 pigs per litter) of IRTA's farm herd were used. At weaning, piglets were identified by ear tags and maintained on a common pre-experimental diet formulated to meet or exceed the nutrient requirements for weaning pigs until the start of the experiment (NRC and Council, 1998) (Table 1). Pigs were kept individually in 36 pens (2 × 1 m). The facilities were provided with forced ventilation for thermal regulation and each pen had 1 feeder and 1 water nipple to allow for *ad libitum* access to feed and water.

At the start of the experiment, pigs from the same litter were randomly assigned to the 4 experimental treatments, providing a total of 9 replicates per treatment. Treatments were designed as a 2 × 2 factorial arrangement using 2 sources of cereal (C or WBR), and with or without supplementation with NSP-degrading enzymes (0 or

0.01%). All experimental diets were formulated to be iso-nutritive (i.e. 3,125 kcal ME/kg, 16% CP, 10 g/kg Lys, 9 g/kg Ca, and 6 g/kg P) and to meet or exceed the nutrient requirements for growing pigs (NRC and Council, 1998) (Table 2). Feed were provided in mash form. The enzyme preparation tested was a multi-enzyme complex obtained from *Penicillium funiculosum* (Rovabio Excel AP; Adisseo France SAS, Antony, France). This product is guaranteed to provide 22,000 U/g of endo-β-1,4-xylanase and 2,000 U/g of endo-β-1,3(4)-glucanase. Units are defined as the amount of enzyme, which hydrolyzes wheat arabinoxylans, reducing the viscosity of the solution, to give a change in relative fluidity of 1 arbitrary unit·min^{-1} under the assay conditions (pH 5.5 and 30°C) for xylanase activity, and the quantity of enzyme, which hydrolyzes barley β-glucan (bound to a chromophere), releasing ethanol-soluble oligomers, to give an absorbance of 0.820 units at 590 nm for endo-β-1,3(4)-glucanase activity. In addition to endo-β-1,4-xylanase and endo-β-1,3(4)-glucanase activities, other enzymes (cellulases, pectinases, mannanases and others) are also active in the preparation (Karboune et al., 2008; Karboune et al., 2009). The presence of the enzyme preparation in the supplemented diets was confirmed by analyzing the corresponding β-glucanase and xylanase activities (Cosson et al., 1999).

Experimental Procedures and Sampling

The animals were fed the experimental diets for 27 days. At the end of the experimental period, all 36 pigs were euthanized by exsanguination under isofluorane anesthesia. Before the euthanasia, blood samples were collected from the anterior vena cava. The abdominal cavity was immediately opened and the whole GIT (from cardia to rectum) was removed. The GIT was tied to retain the digestive contents in the different sections (small intestine and cecum). This was immediately followed by the sampling of

digestive contents, GIT tissues and mucosa to perform the different analysis as described below.

Immune Response Analysis

ELISA Tests

The serum and bile antibodies were measured using Pig IgA or IgG or IgM quantification Kit (Bethyl Laboratories Inc., Texas, USA). The analysis was conducted according to the kit instructions, but the coat, the conjugate and the sample concentrations (Table 3) were determinate to be optimal in preliminary determinations (not shown).

Gut histological study

A 5 cm section of the ileum (20 cm cranial to the ileocecal valve) was obtained for the histological study of ileal mucosa. Samples were partially opened (about ¾ of length) along the mesenteric attachment and fixed by immersion in 10% (vol/vol) formaldehyde (3.7%, pH 7.0, stabilized with methanol DC). Tissue samples were dehydrated and embedded in paraffin, and stained with haematoxylin and eosin and with Periodic Acid-Schiff (PAS) dye following (Law et al., 2007). Morphometric measurements were performed with a light microscope (BHS, Olympus, Barcelona, Spain) using a linear ocular micrometer (Olympus, Ref. 209-35040, Microplanet, Barcelona, Spain). Measurements were performed on 10 well-oriented villi and crypts from each intestinal section of each animal, the number of intraepithelial lymphocytes (IEL) and Goblet cells (GC) were expressed per 100 µm of villus or crypt. On the basis of the cellular morphology, differences between the nuclei of enterocytes, goblet cells,

and lymphocytes will be clearly distinguishable at 400× magnification. All morphometric analysis was done by the same person, who was blind to treatment.

Fermentation Analysis

Samples of ileal and cecal digesta (2 mL) were collected in tubes containing 2 mL of a preservative solution (5 % (w/w) H_3PO_4, 1% mercuric chloride and 50 mM 4-methyl valerate and stored at -20ºC until short-chain fatty acids (SCFA) analysis. SCFA and lactic acid were analyzed by gas liquid chromatography using the method (Richardson et al., 1989) modified by (Jensen et al., 1995) using 4-methylvaleric acid as internal standard.

Ileal and cecal digesta (1 mL) of were also sampled in tubes containing 1 mL of HCl solution and stored at -20ºC until ammonia N analysis. Samples were centrifuged at 15000 x g for 15 min at 4ºC, and the supernatant was used to determine ammonia N by spectrophotometry (Libra S21, Biochrom Technology, Cambridge, UK) as described by (Chaney and Marbach, 1962).

Gut Microbiota Analysis

Sampling and DNA extraction

The terminal ileum (20 cm cranial to the ileocecal valve) and cecum were opened lengthwise along the mesenteric attachment, rinsed carefully with ice-cold 0.9% NaCl. Then the mucosa layer was scraped with a sterile cell scraper, and 2-3 g were frozen at -80ºC until the DNA extraction of the bacteria associated to mucosa. Additionally, samples of ileal and cecal digesta (1 mL) were collected in sterile tubes containing 3.0 mL of ethanol as a preservative for DNA analysis and kept at 4ºC until

DNA extraction. The DNA of the digesta samples was extracted and purified using the QIAmp DNA Stool Mini Kit (Qiagen, West Sussex, UK) and the DNA of the mucosa samples was extracted and purified using the QIAmp DNA Tissues and Blood Mini Kit (Qiagen, West Sussex, UK). The temperature of lysis was increased to 95ºC and an additional incubation step with lysozyme was included (10 mg/mL, 37ºC, 30 min) to improve the bacterial cell rupture.

Microbiota profiles by PCR-RFLP

The digesta and mucosa microbiota was then analyzed by PCR-RFLP. Briefly, a 580-bp fragment of 16S rRNA gene were amplified from DNA extracts by PCR using primers specific to conserved sequences flanking variable regions V3, V4 and V5: 5'-CTACGGGAGGCAGCAGT-3' (forward) and 5'-CCGTCWATTCMTTTGAGTTT-3' (reverse). Primers and PCR reaction conditions were those described by Lane (1991). The reaction was performed using a GeneAmp PCR System 9700 thermocycler (PE, Biosystems, Warrington, UK). The DNA amplification conditions were 94ºC (4 min); 35 cycles of denaturation at 94ºC (1 min), annealing at 45ºC (1 min) with an increment of 0.1ºC per cycle, extension at 72ºC (1 min 15 s); and a final extension at 72ºC (15 min). Following visual confirmation of PCR products with agarose gel electrophoresis, five independent enzymatic restrictions were carried out (AluI, RsaI, HpaII, Sau 3AI, CfoI; F. Hoffmann-LaRoche Ltd. Group, Basel, Switzerland). The digestions were performed as recommended by the manufacturer; with the appropriate restriction buffer at 37ºC for 3 h. Fragments were separated using a 2% high-resolution agarose gel. The size and the intensity of the bands within each lane of a gel were analyzed using the Gene Tools software (Syngene, Cambridge, UK), and the degree of microbial richness was measured as the total number of different bands obtained from the five independent

restriction digestions. For pairwise comparisons of the banding patterns and the construction of dendrograms, similarity matrices were generated based on the Manhattan distance (Kaufman and Rousseeuw, 1990) which takes into account the size and the intensity of the bands generated.

Microbiota profiles by deep sequencing analysis

The microbiota from ileum and cecal digesta was analyzed by massive sequencing using Ion Torrent methodology. Firstly, 400-bp amplicons were obtained from DNA extracts using PCR and specific primers to conserved sequences flanking V4-V5 variable regions of 16S rRNA gene (Table 4). Amplifications were performed in a final volume of 50 μL using 32μL PCR-Master Mix (Applied Biosystems, Foster City, CA) containing 1.25 IU of Taq polymerase, 50 ng of DNA template, 0.4 μM of each primer, and the next cycling conditions: 94°C for 4 min, followed by 35 cycles of 94°C during 1 min, 63°C during 1 min, and 72°C during 1 min and 15 s. The last extension cycle was continued for 15 min.

For each amplicon, both concentration and quality were determined using Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, Thermo Fisher Scientific, USA) under manufacturer's conditions. Aliquots of 80 ng per amplicon were mixed and sequenced on an Ion Torrent Personal Genome Machine (PGM) with the Ion 318C Chip Kit v2 (Ion Torrent, Thermo Fisher Scientific, USA) under manufacturer's conditions in the Genomics and Next Generation Sequencing Services of Centre for Research in Agricultural Genomics (CRAG, Bellaterra, Spain).

Amplicon sequences obtained with Ion Torrent system were de-multiplexed, quality-filtered and analysed using QIIME 1.9.1 software (Caporaso et al., 2010b).

Analyses were performed at depth between 174.877 and 385.836 sequences per sample. Reads were clustered into operational taxonomic units (OTUs) at 97% similarity, using UCLUST option into the QIIME's assign_taxonomy.py script. Taxonomic assignment of representative OTUs was performed using the RDP Classifier(Wang et al., 2007) against Greengenes v13.8 database. Alignment of sequences was performed using PyNast option (Caporaso et al., 2010a) into the QIIME's align_seqs.py script. Summarize OTU by Category (barcode, age, sample type) and Plot Taxonomy Summary were conducted using the QIIME's summarize_taxa_through_plots.py script. Alpha diversity was used to evaluate the Shannon index using the QIIME's alpha_diversity.py scrip.

Statistical Analysis

Data were analyzed using the SAS MIXED procedure (SAS Institute Inc, 2011) using the animal as the experimental unit. The model included cereal type (C or WBR), enzyme (0 or 0.01%) and their interaction as fixed effects, and litter and mother as random effect. Results are presented as least squares means. Relative data was transformed to all by one and to arc sinus in order to proceed with statistical analysis. The level of significance was set at $P < 0.05$ and trends were discussed at $P < 0.1$.

RESULTS

Humoral and gut immune response

The humoral and gut immune responses are presented in Table 5. The sera IgG and IgM concentration was not affected by carbohydrase addition or cereal composition. Nevertheless, in absence of carbohydrase, the pigs fed the C and WBR diet presented a higher concentration of IgA in serum ($P = 0.04$) compared to animals

feed with supplemented diets. At bile level, animals fed with WBR diets presented an increased IgA concentration compared to C diets (P = 0.04).

GC and neutral GC were not influenced by cereal source or enzyme supplementation at villus and crypt level. The non-supplementation of C diet increased the IEL in the villous compared to C+, WBR- and WBR+ diets (P = 0.0002, P < 0.0001, P = 0.0002; respectively). However, in the crypt, the IEL were affected by diet where animals fed with corn diets presented an increased number of IEL compared to WBR diets (P = 0.05)

Microbial Environments

Fermentation

The ileal and caecal digesta fermentation are shown in Table 6. The ammonia concentration was not influenced by enzyme supplementation of C and WBR diets in ileum digesta. Nevertheless, at caecal level, a cereal effect was observed; animals fed with C diets presented an increased ammonia concentration compared to WBR diets (P = 0.05). A tendency was observed for enzyme supplementation, diets supplemented with enzyme trends to decrease the ammonia concentration in the caecum digesta (P = 0.08).

Pigs fed with C diet presented an increased formate and decreased lactate concentration at ileal level compared to the WBR diet (P = 0.02 and P = 0.0005, respectively). In addition, an interaction trend for lactate concentration was observed; the non-carbohydrase supplementation tends to reduce the acid latic concentration in animals fed with C diet and increase in animals fed with WBR diet (P = 0.06).

However, no differences for short-chain fatty acids were found with carbohydrase supplementation of C and WBR diets.

At caecal level, pigs fed with C diet presented an increased ammonia concentration compared to WBR diet (P = 0.05) and in absence of enzymes the ammonia concentration trend to increase (P = 0.09), mainly in C diets. The SCFA were affected considerably by fiber composition and enzyme supplementation. In absence of enzymes, the animals fed with C diet, presented a decreased acetate, propionate and total SCFA concentration compared to C+ diet (P = 0.001, P = 0.02 and P < 0.0001, respectively). In the case of butyrate, a cereal effect is observed (P = 0.02). Besides, the carbohydrase supplementation of C diets shrank butyrate concentration in the caecum compared to WBR+ diet (P = 0.05). Animals fed with WBR diet presented an increased valerate concentration and trend to present a decreased BCFA compared to C diets (P=0.01 and P=0.06, respectively).

Microbiota biodiversity

The ileal and caecal biodiversity of digesta by OTUs and ileal and caecal biodiversity of digesta and mucosa by RFLP analysis are shown in Table 7. No differences were found for biodiversity by RFLP of ileal digesta and ileal and caecal mucosa. Nevertheless, an enzyme effect was observed for biodiversity of caecal digesta by RFLP. In absence of enzymes, the caecal digesta biodiversity of pigs fed both cereals type was significantly lower than the obtained with supplementation (P = 0.05). No differences between treatments were observed in the biodiversity estimated based on richness of OTUS.

Microbiota profile

When using RFLP to analyze variations in the bacterial community, changes in band patterns related to type diet and enzyme supplementation were observed (Figure 1). At ileal level, the microbial profile of pigs fed the WBR+ clustered separately with 70% of microbial similarity compared to others treatment. (Figure 1A). Whereas, in the pigs fed the corn diet, the enzyme not modulated the microbiota. When the digesta arrived at the cecum, the microbiota presented a higher modulation. The clusters were grouped firstly by type diet and secondly by carbohydrase addition (Figure 1B).

The enzyme supplementation presented different effect on microbial profile of ileal mucosa depending on feed composition (C or WBR). When the enzyme was added to WBR promoted an ileal modulation more similar to C-. Whereas, in absence of supplementation, the WBR presented 89% of the microbial similarity in the ileal mucosa compared to C+ diet- (Figure 1C). At caecal level, the microbial associated to mucosa of pigs fed WBR presented an enzyme effect with 83% of microbial profile similarity, the supplementation of C diet promoted a cluster more similar to WBR based diets than C-. The non-supplementation of corn promoted a higher distance to the rest of treatments, resulting with 75% of bacterial profile similarity compared to others treatments.

Based on Shanonn-Wieneer Index, a NJ phylogenetic trees show the difference between the treatments on digesta microbiota (Figure 2). One hand, the groups were divided by gut segments; the ileal digesta (ID) is showed on the top whereas cecal digesta (CD) is in the bottom of tree. In other hand, the treatments presented a diet effect followed by an enzyme supplementation effect. The carbohydrase supplementation of corn promoted microbiota differentiation compared to WBR diets. Besides, the WBR+ was highly differentiated compared to other diets. The cecal digesta

presented higher differentiation between treatments. In the cecum, the corn diets presented a higher enzyme effect compared to the same diets in the ileum. A similar distribution was observed in the RFLP results. The WBR diets presented a higher carbohydrase supplementation effect, showing a higher distance between WBR- and WBR + diets.

In the ileal digesta, with deep sequencing analysis of microbiota, 85% to 94% and 5% to 13% of sequences were identified respectively as Firmicutes and Proteobacterias Phylum. Nevertheless, none difference at phylum level of microbial population were found between treatments in the ileal digesta. However, considering class level, animals fed with C diet presented a significant increment of Clostridia compared to WBR diets ($P = 0.02$). When order, family or genus level was considered, several effects of diet, enzyme or interaction were detected at ileal microbiota (Figure 3). Animals fed with WBR presented a significant decreased number of OTUs for *Staphylococcus* spp. compared to C ($P < 0.001$). Besides, the enzyme supplementation of C diet presented an increased number of OTUs of *Staphylococcus* spp. compared to C- (7% vs 0.02%, $P < 0.0001$). Animals fed with non-supplemented corn diet presented a decreased number of *Leuconostoc* spp. compared to the supplemented corn diet ($P = 0.0004$), WBR- ($P = 0.001$) and WBR+ ($P < 0.0001$). A cereal effect was found in some Clostridiales populations, animals fed with WBR diets presented a decreased number of Peptostreptococcaceae family, Clostridiaceae family and *Clostridium* spp. compared to C diets ($P = 0.005$, $P = 0.02$ and $P = 0.005$, respectively). Animals supplemented with carbohydrase presented a significant increment for an OUT identified as Gammaproteobacteria class compared to non-supplemented diets ($P = 0.04$).

In the caecum, the microbiota is mainly composed by Bacteroidetes (from 68% to 59%) and Firmicutes phylum (from 35% to 27%) depending on treatment. The enzyme addition present a different effects in Bacteroidia class depending on cereal diet composition (P = 0.02), where the carbohydrase supplementation of WBR diet was 9 percentage points more higher than C+ diets. The Cyanobacteria phylum also presented an interaction effect (P = 0.009), when enzyme is added to WBR diets, this population trends to increase when compared to non-suplemented WBR diets (P = 0.07). The corn diets presented an increment of Firmicutes population compared to WBR diets (P = 0.05), and the enzyme supplementation trend to present a different effect depending on cereal based diet (P = 0.06), where animals fed C + diets presented an increased Firmicutes population compared to WBR+. For Spirochaetes class, the enzyme presented a significant effect (P = 0.03), animals fed with WBR+ present an increased Spirochaetes population compared to WBR- diets (P = 0.02). Animals fed with WBR diets trend to present an increased population of Betaproteobacterias compared to corn diets (P = 0.07). The carbohydrase supplementation increased the population of YRC22 bacteria from Paraprevotellaceae decreased the polulation of Clostridiales compared to diets without supplementation (P = 0.04 and P = 0.004, respectively). Animals fed with C+ diets increased Lactobacillales compared to C-, WBR – and WBR+ diets (P = 0.006, P = 0.006 and P = 0.006, respectively). Corn diets increased the population of Clostridiaceae and Veillonellaceae family (P = 0.02 and P = 0.002, respectively) and decreased Ruminococcaceae (P = 0.02) compared to WBR diets. The population of *Megasphaera* spp., *Coprococcus* spp., *Lachnospira* spp. and *Veillonella* spp presented an interaction effect (P = 0.05, P = 0.03, P = 0.007 and P = 0.003, respectively), where the enzyme supplementation of corn diets increased these population compared to WBR+ diets. Animals fed with WBR diet presented an increment of *Roseburia* spp.

compared to C diets (P = 0.001), the non-supplementation presented different effects depending on cereal diet: decreasing population in C diets and increasing in WBR diets (P = 0.003).

DISCUSSION

The diet composition appears to play significant roles defining the gut microbiota (Isaacson and Kim, 2012). Several studies have established the gut microbiota as a significant contributor to the digestion of feed substrates that influences the overall physiological growth, immunologic responses, and pathogenesis in the host (Richards et al., 2005). Within this context of interaction between feed enzyme and gut microbiota, enzymes act in specific components of feed ingredients in most cases explains the role of enzymes in modulating gut microbiota.

Even corn and soyabean meal are not so tan rich in NSP than WBR based diet (Irish and Balnave, 1993; Dierick and Decuypere, 1996), these cereals contain NSP that can be retaining protein and antinutritional factors. The presence of high levels of dietary trypsin inhibitors from soybeans can promote substantial reductions in protein and amino acid digestibilities in rats and pigs (Gilani et al.). This could be promoting an upper stimulation of mucosa and, as consequence, increasing the number of IEL in villus of animals fed corn based diets without the degrading action of carbohydrases. Acording (Morrison and Preston, 2016), in a human study, suggested that the increase $CD3^+$ intra-epithelial lymphocytes and $CD68^+$ lamina propria macrophages observed on high fat/low fiber diets suggesting increased inflammation in the absence of saccharolytic breakdown of fiber. An undigested protein at ileal level, also can explain the higher concentration of ammonia at caecal level in these animals. Considering that corn diets are rich in glucose as energy source, this fact can be promoting an increased

population of bacterias suggars-fermenting like Clostridiales bacterias from Clostridiaceae family and Peptostreptococcaceae familiy. This supposed extra sugar also could be an explanation for a higher formate concentration in the ileum of animals fed with corn diets, due to the glucose fermentation by Embden-Meyrhof via resulting in pyruvate, a metabolite predecessor of formic acid. Feed enzymes can make an impact on GIT microbial ecology by reducing undigested substrates and producing oligosaccharides that can be fermented by microbiota (Kiarie et al., 2013). In our study, the absence of enzyme in corn diets decreased the fermentation carrying out a decreased concentration of acetate, butyrate and propionate at caecal level. In the same way, these animals presented an increased population of Firmicutes, Lactobacillales, *Coprococcus* spp., *Lachnospira* spp compared with the animals fed with C+ diet.

The NSP contents in wheat, rye, and are greater than corn, with around 11% in wheat and rye and 16% in barley (Chesson, 1993; Slominski et al., 2004). The diets rich in fiber promoted a decreased of ammonia and increased the SCFA concentration in the caecum. This high concentration of NSP can be explain an increased lactate concentration and also bacterias Lactobacillales such as *Leuconostoc* spp., which are producers of lactate (Marchandin and Jumas-Bilak, 2014), at the same way decrease Clostridiales population. This microbiota modulation at ileal level also could be an explanation for an increased IgA concentration in the bile. It can suggest the development of a microbiota that stimulates positively the immune system response.

In vitro utilization of acetate by *Roseburia spp*. has been evidenced (Duncan et al., 2004), in our study the diets that presented a higher concentration of acetic acid also presented an increased population of *Roseburia* spp. in the caecum. As is well-know, *Roseburia* spp. is a butyrate producing bacteria (Walker et al., 2005), the animals fed

with C- diets presented current study was observed decreased butyrate production and also a decrease of *Roseburia* spp. in the caecum digesta.

The Bacteroidetes phylum mainly produces acetate and propionate, whereas the Firmicutes phylum has butyrate as its primary metabolic end product (Macfarlane and Macfarlane, 2003). However this direct relation was not observed in this study. Animals fed with corn without carbohydrase supplementation presented decreased acetate, propionate, butyrate and total SCFA concentration compared to others diets, nevertheless the bacterial population presented a different composition.

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Table 1. Ingredient composition of the common pre-experimental diet, as-fed basis

Ingredient, %	Content
Barley	28.94
Whey, acid, dehydrated	16.67
Corn	15.46
Soybean, whole extruded	14.64
Soybean meal, 48% CP	11.42
Fish meal	8.00
Soybean oil	4.03
Vitamin/mineral premix[1]	0.40
Calcium carbonate	0.17
L-Lys·HCl	0.13
DL-Met	0.11
L-Thr	0.04

[1] Providing per kg of diet: vitamin A, 1.5 mg; vitamin D_3, 0.025 mg; vitamin E, 15 mg; thiamin, 1.3 mg; riboflavin, 3.5 mg; vitamin B_{12}, 0.025 mg; vitamin B_6, 1.5 mg; calcium pantothenate, 10 mg; nicotinic acid, 15 mg; biotin, 0.1 mg; folic acid, 0.6 mg; vitamin K_3, 2 mg; Fe, 80 mg as iron sulfate; Cu, 6 mg as copper sulfate; Co, 0.75 mg as cobalt sulfate; Zn, 60 mg as zinc oxide; Mn, 30 mg as manganese sulfate; I, 0.75 mg as potassium iodate; Se, 0.10 mg as sodium selenite; and ethoxiquin, 150 mg.

Table 2. Ingredient composition and chemical analysis of the experimental diets, as-fed basis

Cereal:	Corn		Wheat-barley-rye	
Ingredient, % Enzyme,[1] %:	0	0.01	0	0.01
Corn	71.11	71.11	0	0
Wheat	0	0	24.58	24.58
Barley	0	0	24.58	24.58
Rye	0	0	22.72	22.72
Soybean meal, 44% CP	17.83	17.83	13.55	13.55
Sunflower meal	6.88	6.88	6.88	6.88
Lard	0.03	0.03	3.47	3.47
Calcium carbonate	1.15	1.15	1.19	1.19
Dicalcium phosphate	1.28	1.28	1.17	1.17
Sodium chloride	0.37	0.37	0.50	0.50
Vitamin/mineral premix[2]	0.40	0.40	0.40	0.40
L-Lys·HCl	0.34	0.34	0.36	0.36
L-Thr	0.04	0.04	0.07	0.07
DL-Met	0.03	0.03	0.04	0.04
L-Trp	0.04	0.04	0.01	0.01
Titanium dioxide	0.50	0.50	0.50	0.50
Analyzed composition				
DM, %	86.82	86.89	88.78	88.96
Ash, %	5.32	5.46	5.62	5.55
GE, kcal/kg	3,888	3,858	4,031	4,045
Ether extract, %	3.23	3.13	4.56	4.7
CP, %	15.17	15.23	16.33	15.88
Crude fiber, %	4.21	3.72	4.76	4.63
NSP,[3]%	9.25	9.27	13.68	14.05
Arabinose, %	3.22	3.29	5.37	5.18
Xylose, %	1.38	1.31	1.86	1.29
Mannose, %	0.32	0.34	0.51	0.26
Glucose, %	3.08	3.04	4.86	6.29
Galactose, %	1.25	1.29	1.08	1.04
Enzyme recovery, %				
β-glucanase	-	92	-	92
Xylanase	-	106	-	103

[1] Rovabio Excel AP (Adisseo France SAS, Antony, France).

[2] Providing per kg of diet: vitamin A, 1.5 mg; vitamin D_3, 0.025 mg; vitamin E, 15 mg; thiamin, 1.3 mg; riboflavin, 3.5 mg; vitamin B_{12}, 0.025 mg; vitamin B_6, 1.5 mg; calcium pantothenate, 10 mg; nicotinic acid, 15 mg; biotin, 0.1 mg; folic acid, 0.6 mg; vitamin K_3, 2 mg; Fe, 80 mg as iron sulfate; Cu, 6 mg as copper sulfate; Co, 0.75 mg as cobalt sulfate; Zn, 60 mg as zinc oxide; Mn, 30 mg as manganese sulfate; I, 0.75 mg as potassium iodate; Se, 0.10 mg as sodium selenite; and ethoxiquin, 150 mg.

[3] Non-starch polysaccharides; total neutral sugars.

Table 3. Monoclonal antibodies and concentrations used for ELISA.

	ELISA Abs		
	Coat	Conjugate	Sample
IgG (serum)	1:100	1:75000	1:90
IgM (serum)	1:100	1:75000	1:90
IgA (serum)	1:100	1:60000	1:60
IgA (bile)	1:200	1:60000	1:80

Table 4. Primers used for massive sequencing analysis of gut microbiota of ileum and caecum.

Primer Identification	Primer sequence
R0512BC01-F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGCTAAGGTAACAGCAGCCGCGGTAATA-3’
R0512BC02-F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGTAAGGAGAACAGCAGCCGCGGTAATA-3’
R0512BC03-F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGAAGAGGATTCAGCAGCCGCGGTAATA-3’
R0512BC04-F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGTACCAAGATCAGCAGCCGCGGTAATA-3’
R0512BC05-F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGCAGAAGGAACAGCAGCCGCGGTAATA-3’
R0512BC06-F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGCTGCAAGTTCAGCAGCCGCGGTAATA-3’
R0512BC07-F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCGTGATTCAGCAGCCGCGGTAATA-3’
R0512BC08-F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCCGATAACAGCAGCCGCGGTAATA-3’
R0512BC09-F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGTGAGCGGAACAGCAGCCGCGGTAATA-3’
R0512BC10-F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGCTGACCGAACAGCAGCCGCGGTAATA-3’
R0512BC11-F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGTCCTCGAATCAGCAGCCGCGGTAATA-3’
R0512BC12-F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGTAGGTGGTTCAGCAGCCGCGGTAATA-3’
R0512BC13-F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTAACGGACAGCAGCCGCGGTAATA-3’
R0512BC14-F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGTTGGAGTGTCAGCAGCCGCGGTAATA-3’
R0512BC15-F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTAGAGGTCAGCAGCCGCGGTAATA-3’
R0512BC16-F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTGGATGACAGCAGCCGCGGTAATA-3’
R0512BC17-F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTATTCGTCAGCAGCCGCGGTAATA-3’
R0512BC18-F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGAGGCAATTGCAGCAGCCGCGGTAATA-3’
R0512BC19-F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGTTAGTCGGACAGCAGCCGCGGTAATA-3’
R0512BC20-F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGCAGATCCATCAGCAGCCGCGGTAATA-3’
R0512BC21-F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGCAATTACAGCAGCCGCGGTAATA-3’
R0512BC22-F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCGAGACGCAGCAGCCGCGGTAATA-3’
R0512BC23-F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGTGCCACGAACAGCAGCCGCGGTAATA-3’
R0512BC24-F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGAACCTCATTCAGCAGCCGCGGTAATA-3’
R0512BC25-F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGCCTGAGATACAGCAGCCGCGGTAATA-3’
R0512BC26-F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGTTACAACCTCAGCAGCCGCGGTAATA-3’
R0512BC27-F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGAACCATCCGCAGCAGCCGCGGTAATA-3’
R0512BC28-F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGATCCGGAATCAGCAGCCGCGGTAATA-3’
R0512BC29-F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGACCACTCAGCAGCCGCGGTAATA-3’
R0512BC30-F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGCGAGGTTATCAGCAGCCGCGGTAATA-3’
R0512BC31-F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGTCCAAGCTGCAGCAGCCGCGGTAATA-3’
R0512BC32-F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTTACACACAGCAGCCGCGGTAATA-3’
R0512BC33-F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCTCATTGAACAGCAGCCGCGGTAATA-3’
R0512BC34-F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGCATCGTTCAGCAGCCGCGGTAATA-3’
R0512BC35-F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGTAAGCCATTGTCAGCAGCCGCGGTAATA-3’
R0512BC36-F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGAAGGAATCGTCAGCAGCCGCGGTAATA-3’
R0512BC37-F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGCTTGAGAATGTCAGCAGCCGCGGTAATA-3’
R0512BC38-F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGTGGAGGACGGACAGCAGCCGCGGTAATA-3’
R0512BC39-F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGTAACAATCGGCAGCAGCCGCGGTAATA-3’
R0512BC40-F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGCTGACATAATCAGCAGCCGCGGTAATA-3’
R0907trP1-R	5’-CCTCTCTATGGGCAGTCGGTGATCCGTCWATTCMTTTGAGTTT-3’

Table 5. Effect of carbohydrase supplementation of corn or wheat-barley-rye based diets on the ileal mucosa and humoral immune response in growing pigs.

Item	Cereal	Corn		Wheat-barley-rye		SED	*P*-value		
	Enzyme	0	0.01	0	0.01		Cereal	Enzyme	Cereal x Enzyme
Gut immune response									
Villus									
Goblet cells (number of cells/100µm)		3.8	3.6	2.8	3.6	0.31	0.1411	0.3824	0.1408
Neutral Goblet cells (number of cells/100µm)		1.7	2.6	2.3	2.8		0.3141	0.0802	0.6641
Intraepithelial lymphocytes (number of cells/100µm)		4.8[a]	3.2[b]	2.7[b]	3.2[b]	0.25	0.0002	0.0144	0.0002
Crypt									
Goblet cells (number of cells/100µm)		9.5	9.3	7.9	9.4	0.54	0.1974	0.2134	0.1291
Neutral Goblet cells (number of cells/100µm)		7.7	8.7	8.7	9.8		0.2483	0.2725	0.9889
Intraepithelial lymphocytes (number of cells/100µm)		0.29[a]	0.28[a]	0.22[b]	0.19[b]	0.036	0.0470	0.5586	0.6832
IgA bile (µg/µL)		31.5[b]	27.6[b]	37.9[a]	48.6[a]	6.29	0.0371	0.5914	0.2523
Hummoral immune response									
IgA (µg/µL)		59.3[b]	62.7[a]	59.1[b]	67.7[a]	2.81	0.3937	0.0401	0.3543
IgG (µg/µL)		49.1	55.4	50.7	52.2	2.55	0.7402	0.1226	0.3440
IgM (µg/µL)		33.4	35.7	36.1	34.7	1.06	0.4087	0.6912	0.0800

[ab] means within a row without a common superscript differ ($P < 0.05$).

Table 6. Effect of carbohydrase supplementation of corn or wheat-barley-rye based diets on ileal and cecal digesta fermentation in growing pigs.

Cereal	Corn		Wheat-barley-rye		SED	*P*-value		
Enzyme	0	0.01	0	0.01		Cereal	Enzyme	Cereal x Enzyme
Ileal fermentation								
Ammonia (mg/100ml FM)	34.7	30.4	24.5	25.6	4.93	0.1352	0.7476	0.5861
Formate (µmol/g FM)	11. 7^{a}	15.1^{a}	3.7^{b}	8.9^{b}	2.62	0.0115	0.1149	0.9088
Lactate (µmol/g FM)	8.4^{b}	29.1^{b}	60.5 ^{a}	46.1^{a}	8.80	0.0005	0.7218	0.0529
SCFA (µmol/g FM)								
Acetate	15.5	20.5	9.7	15.7	3.29	0.1177	0.1075	0.8780
Propionate	0.14	0.19	0.13	0.26	0.148	0.8333	0.5501	0.7660
Butyrate	0.10	0.42	0.14	0.47	0.186	0.8263	0.0936	0.9763
Valerate	nd	nd	nd	nd				
BCFA	nd	nd	nd	nd				
Total	16.4	21.6	10.7	16.8	3.48	0.1382	0.1124	0.9098
Cecal fermentation								
Ammonia (mg/100ml FM)	84.5^{a}	23.5 ^{a}	17.7^{b}	11.9^{b}	18.98	0.0470	0.0878	0.1561
Formate (µmol/g FM)	0.42	0.00	0.02	0.00	0.296	0.3457	0.3053	0.3457
Lactate (µmol/g FM)	1.14	0.00	3.52	1.42	1.518	0.2195	0.2926	0.7525
SCFA (µmol/g FM)								
Acetate	58.6^{b}	73.8^{a}	72.5^{a}	74.2^{a}	3.05	0.0263	0.0093	0.0335
Propionate	24.6^{b}	37.6 ^{a}	45.3^{a}	40.2^{a}	2.51	<0.0001	0.1251	0.0011
Butyrate	7.4^{b}	12.2^{ab}	12.6^{ab}	13.7^{a}	1.39	0.0223	0.0398	0.1806
Valerate	1.20^{b}	1.67^{b}	2.97^{a}	2.65^{a}	0.546	0.0143	0.8824	0.4596
BCFA	0.82	0.31	0.26	0.28	0.209	0.0579	0.1176	0.0826
Total	92.6^{b}	125.7^{a}	133.9^{a}	131.0^{a}	5.27	0.0001	0.0073	0.0017

nd = non detected

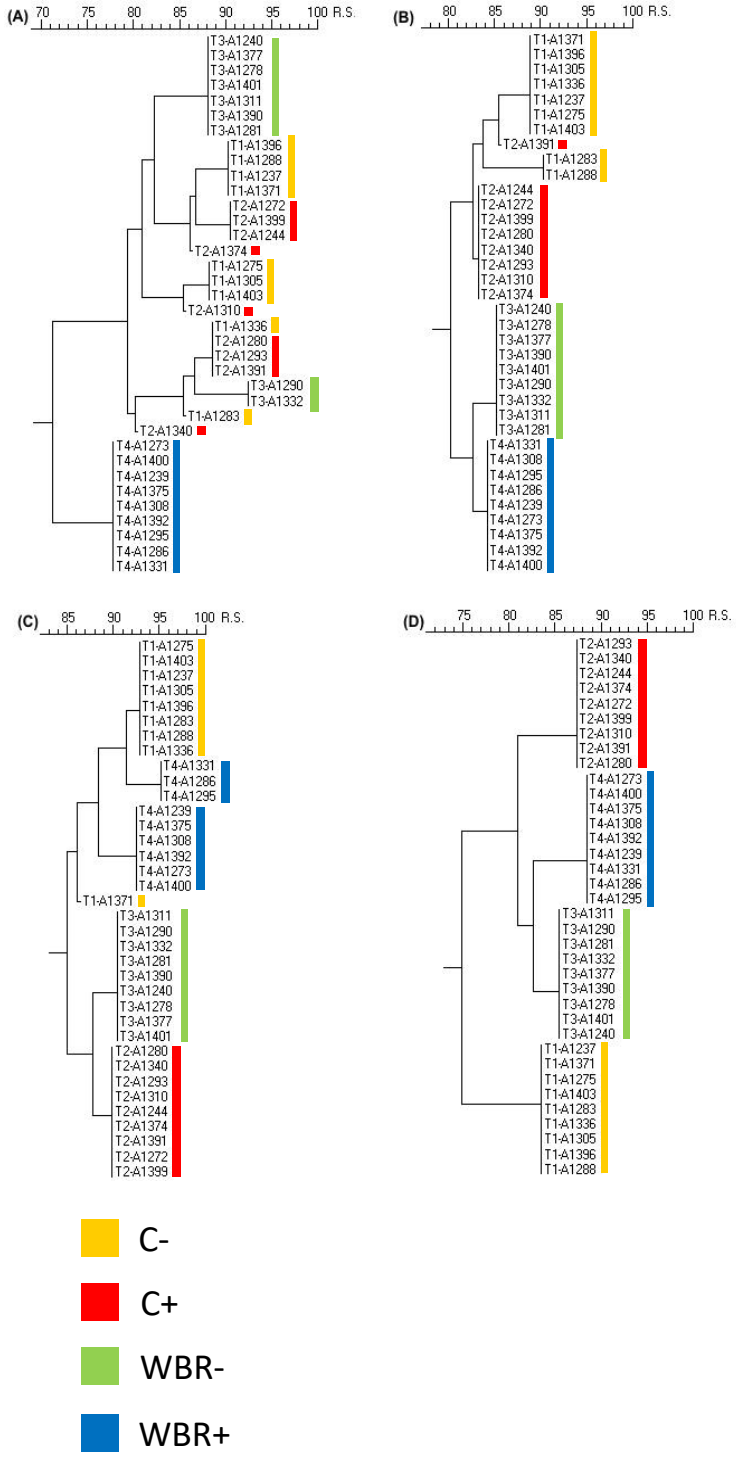
^{a,b,c} means within a row without a common superscript differ ($P < 0.05$).

Table 7. Effect of carbohydrase supplementation of corn or wheat-barley-rye based diets on the ileal and cecal biodiversity of digesta and mucosa in growing pigs.

Cereal	Corn		Wheat-barley-rye		SED	*P*-value		
Enzyme	0	0.01	0	0.01		Cereal	Enzyme	Cereal x Enzyme
Biodiversity by RFLP (number of band patterns)								
Ileal digesta	41.0	38.9	35.9	37.1	2.34	0.1510	0.8498	0.4798
Cecal digesta	50.1[a]	48.4[b]	52.1[a]	45.6[b]	1.97	0.8227	0.0446	0.2230
Ileal mucosa	47.0	47.0	45.1	48.1	2.03	0.8492	0.4651	0.4651
Cecal mucosa	43.4	43.7	42.8	44.7	1.90	0.9307	0.5828	0.6642
Biodiversity based on OTUs (number of OTUs)								
Ileal digesta	22.8	26.4	24.4	25.7	2.63	0.8155	0.2067	0.5226
Cecal digesta	44.0	46.8	43.8	46.2	1.70	0.8203	0.1340	0.9224

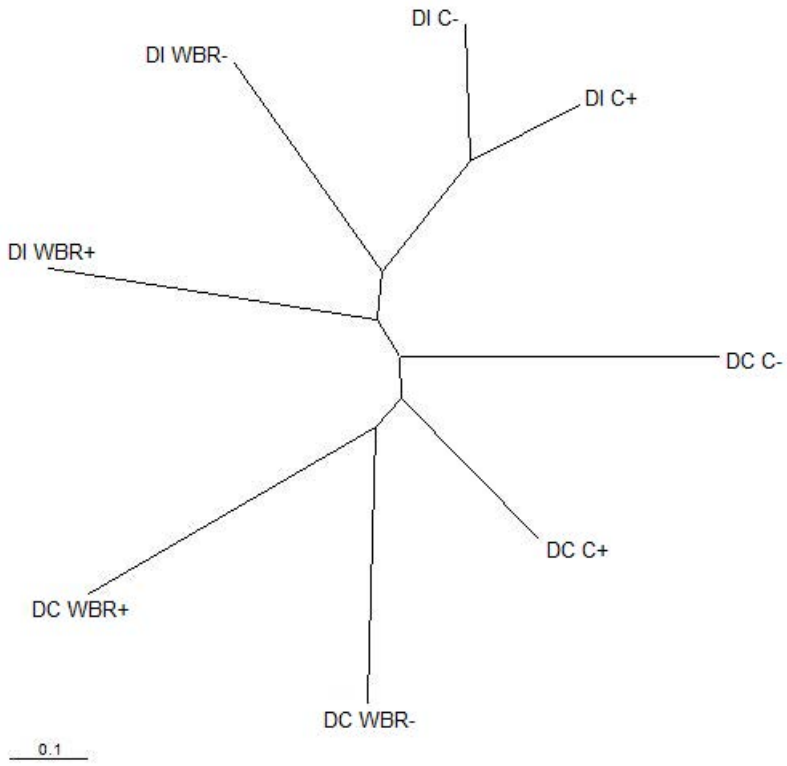
[ab] means within a row without a common superscript differ ($P < 0.05$).

Figure 1. Effect of carbohydrase supplementation of corn or wheat-barley-rye based diets on microbial profile in the ileal (A) and cecal (B) digesta and ileal (C) and cecal (D) mucosa.



C-: corn based diet without enzyme complex; C+: corn based diet with enzyme complex; WBR-: wheat-barley-rye based diet without enzyme complex; WBR+: wheat-barley-rye based diet with enzyme complex.

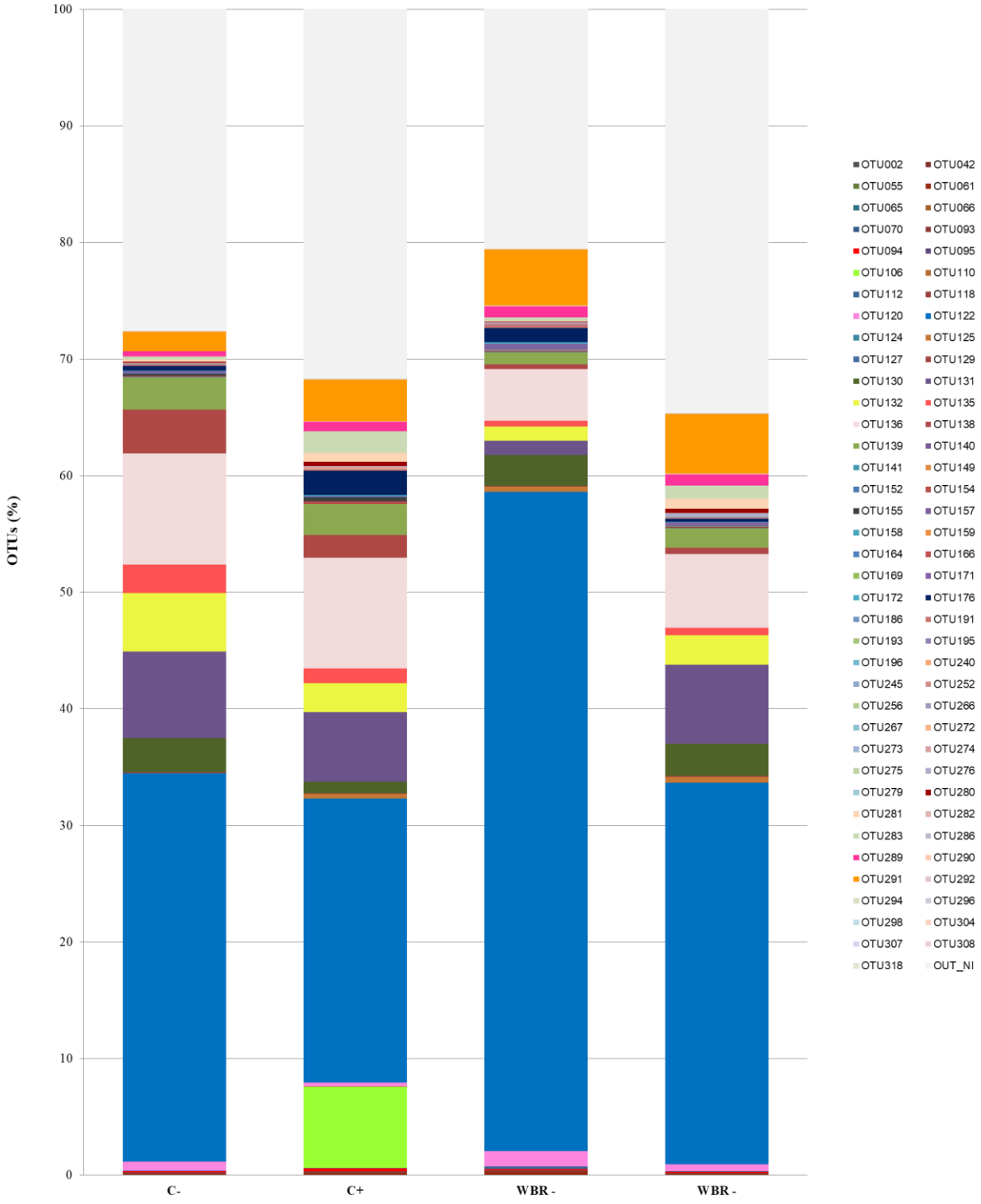
Figure 2. Neighbor-joining (NJ) phylogenetic trees for 16S rDNA, of gut microbiota based on Shannon-Wiener Index.



C-: corn based diet without enzyme complex; C+: corn based diet with enzyme complex; WBR-: wheat-barley-rye based diet without enzyme complex; WBR+: wheat-barley-rye based diet with enzyme complex.

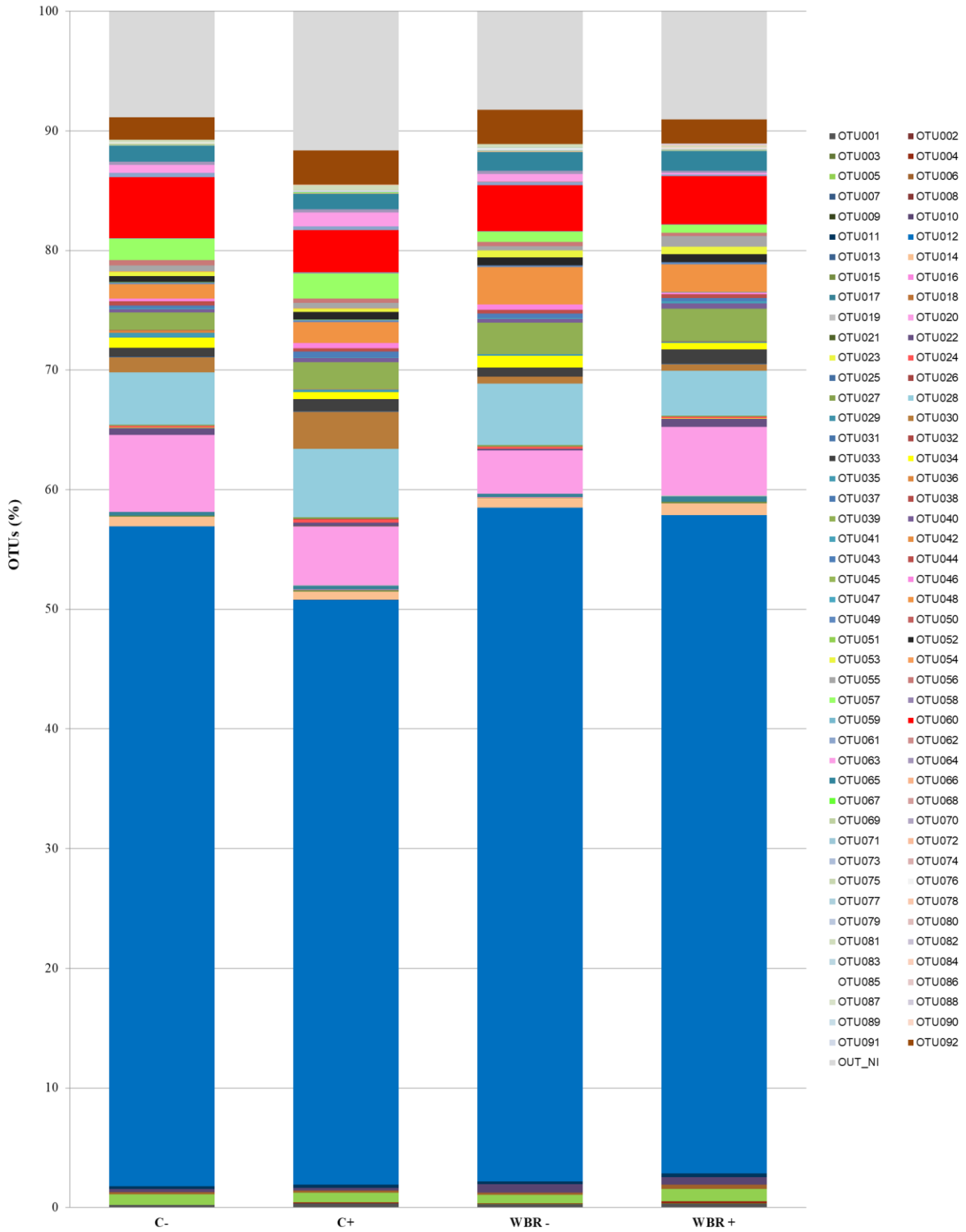
ID, ileum digesta DC, cecum digesta

Figure 3. Effect of carbohydrase supplementation of corn or wheat-barley-rye based diets on microbial profile in the ileum by 16S gene sequencing analysis.



C-: corn based diet without enzyme complex; C+: corn based diet with enzyme complex; WBR-: wheat-barley-rye based diet without enzyme complex; WBR+: wheat-barley-rye based diet with enzyme complex.

Figure 4. Effect of carbohydrase supplementation of corn or wheat-barley-rye based diets on microbial profile in the caecum by 16S gene sequencing analysis.



C-: corn based diet without enzyme complex; C+: corn based diet with enzyme complex; WBR-: wheat-barley-rye based diet without enzyme complex; WBR+: wheat-barley-rye based diet with enzyme complex.

Chapter 5.

General discussion

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The feed industry is continually looking for new feed additives and developing towards in the area of functional feed to modulate the intestinal microbiota in absence of the antibiotics growth promote (AGP). The aim of these products is to affect beneficially the gut microbial composition and its activities. Regarding to this, enzymes have been proposed as tool to enhance gut health based on the fact that carbohydrases broken NSP getting free small particles that can be fermented by microbiota

The intestine developed the “gut barrier function”, a defensive system involving various elements, both intra- and extracellular, those work in a coordinated way to hamper the passage of antigens, toxins, and microbial byproducts, and simultaneously preserve a correct development of the epithelial barrier, the immune system, and the acquisition of tolerance against dietary antigens and the intestinal microbiota. The layer of the mucus make by goblet cells can serve as a barrier between the luminal contents and the absorptive system of the gut, changes in this barrier may affect the absorption in the small intestine. Animals fed with corn diets without enzyme supplementation trend to present a lower mucosa protection (across neutral mucin production) and also an increased number of IEL in the villus compared with corn with enzyme supplementation. This suggested that carbohydrases help to diminish the mucosa aggression and stimulates the neutral mucus production which protects the mucosa against pathogens adherence, entailing an enhancement of the performance data.

Other data also related directly with animal performance is the architecture of mucosa. In both diets, the non-supplementation of booth cereals decreased the VH together with a numeric increase of crypt depth (mainly in animals fed with NSP rich diets), carrying out an important dropped of VH/CD ratio. This reduction is reverted for both diets with carbohydrase supplementation which higher villous height promotes absorption of the nutrients that leads to a better body weight gain. As shown by (Vente-Spreeuwenberg et al., 2003), VH is directly correlated to productive performance. In a diarrhea pathophysiology study, the VH are directly related to the absorptive capacity of the mucous membrane (Buddle and Bolton, 1992). Based in the villous height/crypt depth ratio is a useful criterion for estimating the digestive capacity in the small intestine (Montagne et al., 2003) and that some studies show that the gut mucosa is related with the substrate on intestinal lumen (Hedemann et al., 2006b; Nofrarias et al., 2006), the changes on small intestine substrate promoted by enzyme addition and diet type can modify the mucosa morphometry.

The most abundant microbial genes in the pig microbiome were related to carbohydrate metabolism. The observed changes in substrate composition of small intestine may also reflect differences in the degree of fermentation and microbial growth along the digestive tract. Differences in the RFLP patterns were mainly due to a change in the species composition (type of bands) of the microbial ecosystem with the use of enzymes and different fiber source. From our results, it could be suggested that a more complex microbial community would have a greater robustness in response to changes in the intestinal

environment promoted by different dietary ingredients and that the beneficial effects of enzymes could be related to an improvement of the adaptive capacity of commensal microbiota as a natural barrier defense against the overgrowth of pathogens more than to a reduction in bacteria numbers.

Several studies have shown that different fiber sources are fermented at different rates and release different amounts of SCFA. This is confirmed with this study by the differences in ileal concentration of lactate and formate was influenced by the source of carbohydrate. Taking into account that starch of corn can be considered as an easily fermentable, this fact can be increasing the population of suggars-fermenting bacterias like Clostridiales bacterias from Clostridiaceae and Peptostreptococcaceae families. This supposed extra sugar also could be an explanation for a higher formate concentration in the ileum of animals fed with corn diets in our study, due to the glucose fermentation by Embden-Meyrhof via resulting in pyruvate, a metabolite predecessor of formic acid. This indicates that the changes in the microbiota and SCFA concentration, promoted changes in the gut immunity and consequently in the performance data.

The NSP contents in wheat, rye, and are greater than corn, with around 11% in wheat and rye and 16% in barley (Chesson, 1993; Slominski et al., 2004). This high concentration of NSP can explain an increased lactate concentration and also bacterias Lactobacillales such as *Leuconostoc* spp., which are producers of lactate (Marchandin and Jumas-Bilak, 2014), at the same way decrease Clostridiales population. This microbiota modulation at ileal level also could be an explanation for an increased IgA concentration in the bile. It can suggest the

development of a microbiota that stimulates positively the immune system response.In our study, the most relevant changes on fermentation were observed at caecum level. Animals feed with corn without enzyme supplementation presented a lower acetate, propionate, butyrate and total SCFA concentration in the caecum. These data confirm the hypothesis that the diet enzyme supplementation has a “prebiotic effect” breaking the complex NSP chains, this new oligosaccharides production change the microbiota/fermentation profile and improve the gut health entailing an enhancement of the performance data.

In conclusion, the pigs undergo a dramatic physical development of the gastrointestinal tract (GIT) throughout their productive lives. The intestinal colonization and gut immune response may be affected by some factors, as diet formulations. Diet components, such as NSP, are potential modulators of the intestinal environment. Also, enzymes have been shown to influence the intestinal microbial ecosystem towards a healthier state. Therefore, it should be possible to manipulate the gut microbiota by dietary enzyme supplementation and dietary ingredients.

Chapter 6.

Conclusions

Chapter 6. Conclusions

1. The change in the substrate promoted by non-starch degrading enzymes leads to change in the microbiota and the fermentation metabolism. These changes promoted enhancement of the perfomance data.

2. The enzyme supplementation on the corn-based diet promoted a clear decrease in the gut aggression.

3. The changes found in the microbiota and the immnunology data suggest that the supplementation with non-starch degrading enzymes enhance the performance data by improving gut health.

Chapter 7.

List of references

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