EFFECTS OF DIETARY FIBER ON SWINE INTESTINAL EPITHELIAL AND IMMUNE RESPONSE

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Dedication

This document is dedicated to my family. To my parents, for being my role model, for their undying support and constant worrying, and for always encouraging me to persevere. This accomplishment would not have been possible without them. To my sister, for always believing in me. And to André, for being a loving partner, an immense source of support and for always inspiring me to be optimistic

It always seems impossible until it is done

– Nelson Mandela

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List of abbreviations

AACC – American Association of Cereal Chemists

ADF – Acid Detergent Fiber

CD – Cluster of Differentiation

CF – Crude Fiber

CFU – Colony-Forming Unit

CP – Crude Protein

DDGS – Dried Distiller's Grains with Solubles

DF – Dietary Fiber

DM – Dry Matter

EE – Ether Extract

FA – Fatty Acid

GALT – Gut Associated Lymphoid Tissue

GI – Gastrointestinal

IDF – Insoluble Dietary Fiber

IEC – Intestinal Epithelial Cell

IFN – Interferon

Ig – Immunoglobulin

IL – Interleukin

IPEC – Intestinal Porcine Epithelial Cell

NDF – Neutral Detergent Fiber

NE – Net Energy

NSP – Non-Starch Polysaccharides

PAMP – Pathogen-Associated Molecular Pattern

PRR – Pattern Recognition Receptor

PSC – Pluripotent Stem Cells

SBH – Soybean Hulls

SCFA – Short Chain Fatty Acid

SDF – Soluble Dietary Fiber

TDF – Total Dietary Fiber

TGF – Transforming Growth Factor

Th – T helper

TLR – Toll-Like Receptor

TNF – Tumor Necrosis Factor

Treg – T regulatory

WM –Wheat Middlings

WS –Wheat Straw

CHAPTER 1:

LITERATURE REVIEW

Introduction

The increasing demand of cereals for human consumption and availability of high fiber by-products of different industrial processes, i.e. the ethanol industry, have caused a rise in the use of high fiber ingredients in the diets of agricultural animals to reduce production costs (Noblet & Goff 2001). Dietary fiber (DF) is a major component of a healthy and balanced diet, and comprises polysaccharides and lignin resistant to enzymatic digestion in the upper part of the gastrointestinal (GI) tract (Dhingra et al. 2012).

For swine, there is not a recommended level of DF. However, it is a general consensus that minimal DF inclusion is needed in order to maintain normal intestinal function (Wenk 2001) and for optimization of GI health and animal welfare (Knudsen et al. 2012; Brownawell et al. 2012). Although diets with high content in fiber have lower energy content than low fiber diets, resulting in decreased animal performance (Noblet & Goff 2001), these effects may vary based on fiber properties and sources (Wenk 2001). Different strategies have been developed in order to increase the nutrient utilization of diets rich in fiber. Techniques such as supplementation with non-starch polysaccharides (NSP)-degrading enzymes have been shown to increase energy, amino acid, and protein digestibility in swine diets (Zijlstra et al. 2010) which might result in a more cost-effective production.

Different varieties and amounts of DF will differentially affect the host's microbiome (Kuo 2013). There is increasing evidence showing that fiber can have prebiotic effects in pigs due to interactions with the gut microenvironment and the gut associated immune system (Lindberg 2014). Although there have been considerable efforts to understand

how DF affects the gut and whether its effects are mediated by activating the immune system to modify the intestinal microenvironment, the mechanisms involved remain unclear.

The focus of this thesis is to: 1) identify the cytokine profile of the swine intestine caused by DF sources and 2) to define whether DF actions on the intestinal epithelium are mediated through the activation of a localized immune response.

1. The gastrointestinal tract

1.1. Basic aspects of the intestinal anatomy, physiology and nutrient digestion

The GI tract has a main function of digesting the food and absorbing nutrients. However, it also has a very important barrier role, since the GI tract's mucosal surface (eg. 200-300 m2 in an adult human) constitutes the largest interface of the body with the outside world. Anatomically speaking, the GI tract of monogastric animals has eight parts: mouth, pharynge, esophagus, stomach, small intestine, cecum, large intestine (colon) and anus. Each part has different structures and secretions that allow them to carry out their specific functions.

Histologically, a common architectural plane for the entire GI tract is evident from the esophagus to the anus, composed of four concentric layers that constitute the wall of the tract: serosa, muscularis externa, submucosa, muscularis mucosae and mucosa (Gartner & Hiatt 2012). The four layers are described from the outermost to the innermost (Figure 1.1): The serosa layer (adventitia in the esophagus) is the most distant layer from the lumen, formed by a continuous lamina of flat epithelial cells, called the mesothelium, and by a layer of elastic connective tissue that separates it from the underlying muscular layer. The external muscular layer is formed by smooth muscle cells distributed structurally in two layers: the circular, more internal, and the longitudinal, more external. Between the two muscular layers lies the Akerbach's or labyrosthetic plexus, formed by nerve structures organized in ganglia and interconnected nerve bundles, responsible for controlling the motor functions of the GI tract. The submucosa is composed of a thick layer of connective tissue that physically supports the mucosa. Within the submucosa, several structures can be found including a large number of mucus secretory glands,

blood vessels, cells of the immune system and the submucosal plexus, or Meissner's plexus, with neurons that control secretion. The submucosa also hosts the Peyer patches and the immune follicles. The intestine has about 250 Peyer patches (e.g. aggregates of 5 or more follicles) and thousands of single follicles. Each follicle is in charge of transporting the antigens through the epithelium, so that the immune cells can recognize them (Parkin & Cohen 2001). The muscularis mucosa is a thin layer of muscle outside the mucosa lamina propria that separates it from the submucosa. It is composed of several thin layers of smooth muscle fibers oriented in different ways to keep the mucosal surface and underlying glands in a constant state of gentle agitation. These muscles fibers also work to expel the contents of glandular crypts and enhance contact between epithelium and the contents of the lumen. The mucosa is the innermost layer and is in direct contact with the exterior. It is composed of three concentric layers: 1) an epithelial lining with secretory and absorptive functions; 2) the lamina propria, formed by connective tissue containing glands, blood vessels and a large variety of cells with immune and intercellular communication functions; and 3) the muscularis mucosa.

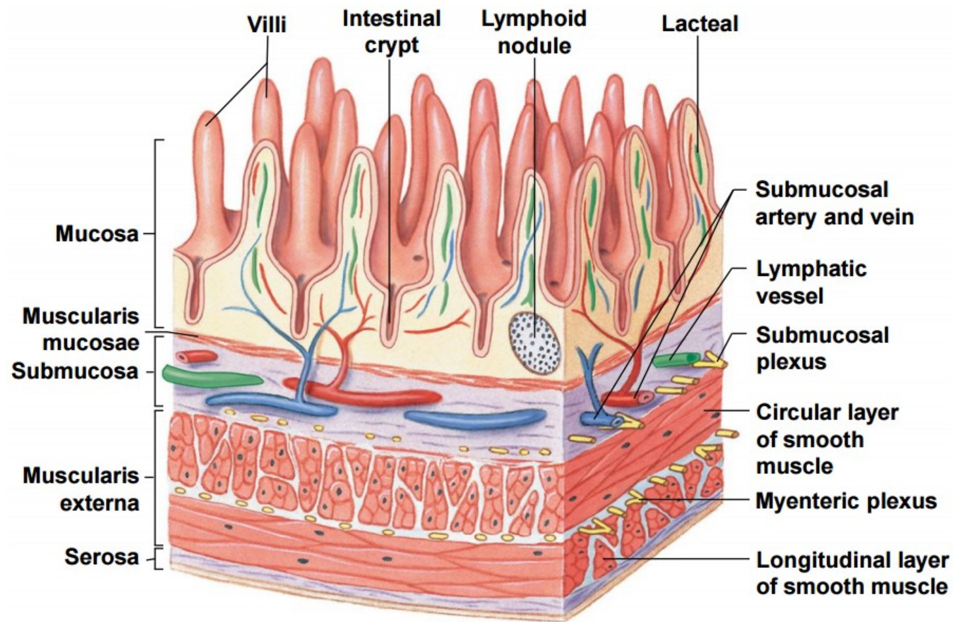

Figure 1.1. The organization of the intestinal layers (Martini, Frederic H; Bartholomew, Edwin F., Essentials of Anatomy & Physiology, 6th, ©2013. Reprinted by permission of Pearson Education, Inc., New York, New York)

Cell types

The intestinal epithelial cells can be divided into two main groups depending on their function and differentiation origin (Figure 1.2). These groups comprise cells belonging to the absorptive lineage and cells belonging to the secretory lineage.

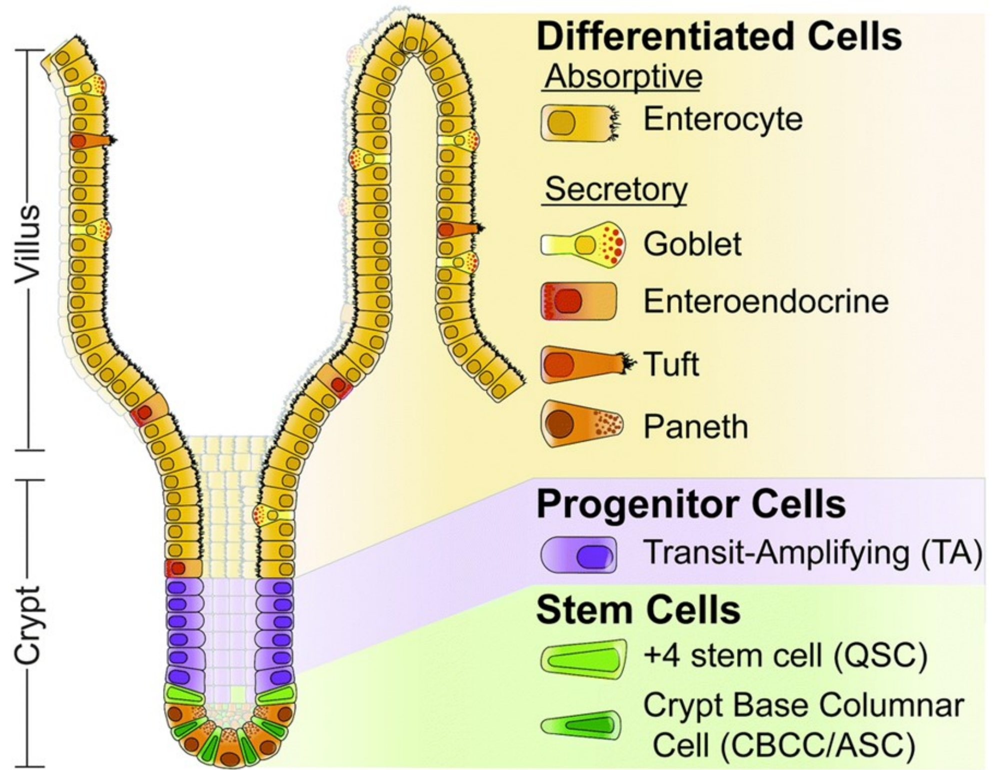

Figure 1.2. Intestinal epithelial organization (Carulli et al. 2014) - Published by The Royal Society of Chemistry

Enterocytes belong to the absorptive lineage of intestinal epithelial cells and play a crucial role in the acquisition of nutrients from the lumen. Enterocytes are defined as simple columnar epithelial cells and are characterized by an apical end that displays a prominent striated border called brush border. This brush border is a layer of densely packed microvilli covered by glycocalyx through which nutrients are absorbed. Other than nutrient uptake, enterocytes also have a role in the reabsorption of unconjugated bile salts and the secretion of immunoglobulins (Ig).

Several cell types can be classified as secretory cells (Figure 1.2). Goblet cells are epithelial cells specialized in mucus secretion in order to protect mucous membranes. Mucus is mainly formed by mucins, large glycoproteins that are able to form gels by attracting large quantities of water. Mucins are stored in granules inside the goblet cells before being released to the lumen, action that occurs after stimulation by irritants, presence of certain virus and bacteria, and potentially, immune system signaling molecules such as cytokines. Enteroendocrine cells are present in variable numbers throughout the length of the small intestine and they secrete numerous peptide hormones which are used to classify the enteroendocrine cells in subtypes. Paneth cells, located in the basal portion of the intestinal crypts close to the stem cells, are exocrine cells with large secretory granules in their apical cytoplasm that contain antimicrobial substances such as lysozyme. Paneth cells also have an important role in innate immunity and in regulating the microenvironment of the stem cells in the intestinal crypts.

Digestion and absorption

One of the main functions of the GI tract is the digestion of nutrients in the diet. Different enzymes are secreted along the GI tract, with the purpose of hydrolyzing complex molecules into much simpler ones to facilitate absorption across the mucosa and into the circulation. This process starts within the mouth. The salivary glands secrete amylase, that partially degrades starch, and lipase that acts on lipids breaking them down into fatty acids (FA). Once within the stomach, pepsin and acid will degrade proteins. In the duodenum, bile acids and several enzymes will be released from the liver and pancreas, respectively. Bile acids act to allow enzymes, like pancreatic lipase, to digest fats in an aqueous medium. Pancreatic amylase will continue degrading starch, while trypsin and

chymotrypsin will act on the peptides resulted from the gastric pepsin protein degradation. The mucosa of the jejunum and ileum produce lactase, maltase and different peptidases that will complete the digestion of the remaining nutrients from the diet. The non-absorbed fractions and undigested material will move on to the large intestine where intestinal microbiota will ferment and further degrade them, and remaining materials then will be excreted.

1.2. Microbiota

The GI tract is colonized by an extremely complex microbial community. Microbial communities increase in amount and complexity distally through the GI tract. The stomach, with its low pH, allows for the growth of few bacterial species. In the small intestine, pancreatic and biliary juices; and peristaltic movements are the main limiting factors for microbiota growth. At this point, microorganism levels increase progressively, from 10^3 – 10^4 bacteria/ml of content in the duodenum, to more than 10^8 bacteria/ml content in distal ileum (Sender et al. 2016). Both small and large intestines have a pH closer to neutrality and a very reducing environment. By contrast, in the large intestine, transit is slower and pancreatic and biliary secretions are diluted, allowing for larger amounts of bacteria to populate this section of the GI tract (10^{11} bacteria/ml content) (Sender et al. 2016). The most abundant genera found under normal conditions in mammalian large intestine are *Bacteriodes, Eubacterium, Bifidobacterium, Peptoestreptococcus, Ruminococcus* and *Clostridium* (Tannock 2002). Facultative anaerobic populations of bacteria such as *Lactobacillus, Enterobacterium, Enterococcum* and *Streptococcum* can be found in concentrations between 10^5 and 10^8 CFU/g

(Holzapfel et al. 1998). These species, despite their lower number, can be essential for microbial homeostasis in the large intestine.

Intestinal bacteria play four fundamental functions in the GI tract:

a. Nutritional function: Commensal bacteria are an important source of vitamins (like Vitamin K). However, their main metabolic function is to ferment the remains of the diet that were not digested. In humans, it is estimated that 20 – 60 g of carbohydrates and 5 – 20 g of protein reach the colon undigested (Xaus et al. 2001). Microbial diversity results in activation of different enzymes and biochemical pathways, allowing the recovery of some energy of the diet that, without the microbiota, would have been lost.

b. Trophic function: Microbial fermentation of undigested carbohydrates results in short chain fatty acid (SCFA) production, mainly acetate, propionate and butyrate. Those SCFA exert an important trophic action over the intestinal epithelium, regulating not just mechanical processes but also proliferation and differentiation processes (Frankel et al. 1994).

c. Protective function: Another important function of the commensal bacteria is the regulation and stabilization of the ecosystem, limiting colonization by pathogenic microorganisms. There is a constant competition for space and for available nutrients between the commensal bacteria and the pathogenic bacteria. Also, the microbiota help to stablish pH and sometimes even produce bacteriocines and other substances that block foreign bacterial colonization (Kuo 2013).

Immunological function: The proper development of the immune system in the mucosa depends on the intercommunication between commensal bacteria in the mucosa and the

immunocompetent cells. The presence of the commensal bacteria is, indeed, needed for the normal development, balance and regulation of the immune system (Kuo 2013).

2. An overview of the immune system

2.1. The immune system

The host's defensive response against pathogenic microorganisms is based on two well-differentiated but synergistic components: innate immunity and adaptive or acquired immunity. The adaptive response is characterized by a clonal selection of antigen-specific lymphocytes that in the long run, provide a lasting and specific protection. In contrast, the innate immune response is not specific to any pathogen and does not generate immune memory (Parkin & Cohen 2001).

2.1.1. Innate immunity

The essential function of the innate immunity is to cause a rapid response against the pathogenic microorganisms without the need for induction or maturation of lymphocytes, which is the reason it is considered the first line of defense against infectious diseases. The innate immunity is mainly mediated by phagocytic cells: neutrophils, eosinophils, macrophages and dendritic cells. The main function of these cells is to phagocytize and destroy pathogens, coordinating other cell responses by the release of inflammatory mediators and cytokines such as TNFα and IL1 which activate complementarily acquired immunity (Parkin & Cohen 2001).

The main function of the innate immune response is to detect the presence of invading microorganisms via the recognition of specific microbial products (Ulevitch 2004). A

functional immune system must be able to have a pathogen recognition mechanism, while maintaining tolerance against the "self" antigens. The sensitivity and specificity of this important function is caused, at least in part, by the ability to recognize specific microbial patterns. For this reason, the first challenge faced is the capacity of recognition of a large number of pathogens by a limited number of receptors. The problem has been solved by the creation of receptors that recognize fragments of pathogenic microorganisms that are not found in eukaryotic cells and that are subject to little to no mutations over evolution, since they are part of structures essential for the life of the microorganisms. These fragments are called pathogen-associated molecular patterns (PAMPs) (Parkin & Cohen 2001). Receptors that recognize PAMPs have therefore been referred to as pattern recognition receptors (PRRs) (Parkin & Cohen 2001). Pathogen-associated molecular patterns include cell wall components of bacteria such as lipopolysaccharide (LPS), peptidoglycans or teichoic acids, and other components of fungi, yeasts and protozoa. There are two fundamental types of PRRs, those that mediate the mechanisms of phagocytosis and those that induce pathways of activation of pro-inflammatory mediators. The first investigations on these receptors were performed on *Drosophila melanogaster*, which has no acquired immunity, and resulted in the discovery of a whole family of these membrane receptors called Toll-like receptors (TLR) (Lien & Ingalls 2002). To date, ten different TLRs have been discovered in mammals and some of their mechanisms of action have been described. Activation of the membrane receptor initiates an intracellular cascade of kinases that results in the translocation of transcription factors from the cytoplasm to the nucleus. The most important transcription factor to be activated is NF-κB which stimulates the production of a large number of immune

mediators and cytokines such as TNFα, IL6, IL1, IL8 or IL12. It also stimulates molecules necessary for the activation of T lymphocytes by antigen-presenting cells, creating a link between the detection of the pathogenic microorganism, innate immunity and acquired immunity (Parkin & Cohen 2001).

2.1.2. Adaptive immunity

In addition to innate immunity, an acquired or adaptive immune system has been developed in the body to protect mucosal surfaces (Parkin & Cohen 2001). In contrast to innate immunity, acquired immunity is specifically mounted against particular antigens and generates immunological memory, thus inducing a lasting defense. This type of immunity is characterized by B-cell and T-cell lymphocytes. Effector B-cells, also known as plasma cells, are responsible for producing immunoglobulins (Ig) that act by exclusion and elimination of antigens. Antibodies of IgG and IgM families act at the systemic level while IgA is produced locally at the intestinal level and secreted to the GI lumen and intracellular space. IgA-producing intestinal plasma cells are derived from the B2 cells located in the Peyer's patches or in the solitary follicles (mainly located on the lower ileum), as well as by the B1 cells residing in the peritoneal cavity (Fagarasan & Honjo 2003). The immune reaction is initiated by the contact of the antigen with the antigen-presenting cells that process and present antigens to the lymphocytes located in the Peyer's patches and the follicles, generating the proliferation of an antigen-specific clone that is released to the bloodstream, distributed as memory cells, and then returns to the lamina propria where it is permanently housed. In response to new contact with the antigen, the antigen-specific T lymphocytes proliferate as a cellular immune response is

triggered and, at the same time, stimulating the transformation of B lymphocytes into plasma cells (Fagarasan et al. 2001).

Cellular or cell-mediated immunity is one of the main components of the adaptive immune system. Cell mediated immunity is an immune response that does not involve antibody production and release, but rather involves phagocyte and antigen-specific cytotoxic T-cell activation and the release of several cytokines. Cellular immunity, according to the co-stimulatory molecules expressed by the antigen-presenting cells, can be polarized in a Th1 profile if TNFα and IFNγ predominate (cytokines that activate macrophages and induce cytotoxic mechanisms); or in a Th2 profile, where IL4, IL5 and IL13 cytokines induce the production of IgE and the activation of eosinophils in allergic type reactions. The balance between these two profiles depends in part on the secretion of anti-inflammatory cytokines by Treg lymphocytes also activated by antigen presenting cells, mainly dendritic cells (Stagg et al. 2004).

2.2. Immunity in the gut and oral tolerance

The gut associated lymphoid tissue (GALT) represents the largest mass of lymphoid tissue in the body (Parkin & Cohen 2001). The regulatory functions of the intestinal immune response occur in different physiological compartments, either in different locations throughout the mucosa and the epithelium (intraepithelial and lamina propria lymphocytes), or in aggregates in the lymphoid follicles and the Peyer patches.

In the intestine, the immune system is constantly processing antigens collected from the lumen; a reason why the lymphoid follicles located in the mucosa present are very active (MacDonald 2003). All evidence available to date suggests that the intestinal immune

system actively responds to food antigens and normal flora without inducing disease, mechanism also known as "physiological inflammation", characterized by a Th1 profile of cytokines, with high local levels of IL12 (Monteleone et al. 2003).

Oral Tolerance

The term can be defined as the physiological response to food antigens and commensal flora through the induction of a specific suppression of immune response to antigens (MacDonald 2001). In contrast to food and commensals, pathogenic microorganisms induce potent immune responses in the gut, indicating that the intestinal immune system is able to process and distinguish between harmless and potentially harmful antigens. The balance between tolerance (suppression) and active inflammatory response depends on several factors including genetics, nature of the antigen, dose and frequency of administration, age at first exposure, immunological status of the host and the route of exposure (Strobel 2001). The mechanisms that induce tolerance are not very clear and probably act at different levels of the immunological cascade, complementing and giving feedback to each other. Some studies suggest that the induction of oral tolerance depends mainly on the type of antigen exposure (Strobel & Mowat 1998; Strober et al. 1998). Thus, administration of high doses of the antigen would induce tolerance by a mechanism of lymphocyte deletion or anergy, while repeated administration of the antigen at low doses would induce it through cellular or biochemical regulatory mechanisms (Weiner 2000; Weiner & Wu 2011). Lymphocyte deletion consists of the elimination of clones of T lymphocytes specific for a particular antigen by apoptosis while anergy is the lack of response to an antigen that will pass into the blood in the absence of an inflammatory response. The alternative to deletion or anergy for the elimination of specific T

lymphocytes is the induction of Treg cells with inhibitory activity. These inhibitory cells are primarily Th3 lymphocytes that exert their function through the release of suppressor cytokines such as IL10 or TGFβ (Garside & Mowat 2001).

3. Dietary Fiber

3.1. Definition

Fiber comprises a wide variety of complex materials which makes it very difficult to define. Even though during the last decade several authors have tried to give a clear definition for DF, so far no definition has been internationally accepted. The term "dietary fiber" was coined by Hipsley in 1953. This initial definition included "non-digestible constituents of the vegetal cell wall" such as lignin, cellulose and hemicellulose (Hipsley 1953). In 1975, Burkitt and Trowell adopted a more wide term and defined DF as "the remaining components of the plant wall, which are resistant to hydrolysis by human intestinal enzymes" (Trowell & Burkitt 1976). This concept includes all compounds of the vegetal cell wall like cellulose, hemicellulose and lignin; plus other polysaccharides present in plants such as gum, mucilage, modified cellulose, olygosaccharides and pectin; which are all edible and resistant to the digestive processes. In 2001, the American Association of Cereal Chemist (AACC) expanded the definition of DF: "Dietary fiber is the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine. Dietary fiber includes polysaccharides, oligosaccharides, lignin, and associated plant substances. Dietary fibers promote beneficial physiological effects including laxation, and/or blood cholesterol attenuation, and/or blood glucose attenuation" (AACC 2001). For the purpose of this thesis, DF will

be defined as “edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the small intestine with complete or partial fermentation in the large intestine”.

3.2. Chemical components of fiber

Fiber components are commonly classified by their chemical structure (Table 1.1). Polysaccharides and oligosaccharides are classified together. Lignin needs to be classified independently due its high structural complexity, forming an independent group. Analogous carbohydrates, such as resistant starches, are the third and last group of the DF components (DeVries et al. 2001).

Table 1.1 Dietary fiber components defined by the AACC (DeVries et al. 2001)

1. Non starch Polysaccharides and Oligosaccharides

- 1.1 Cellulose
- 1.2 Hemicellulose
  - 1.2.1 Arabinoxylan
  - 1.2.2 Arabinogalactan
- 1.3 Polyfructoses
  - 1.3.1 Inulin
  - 1.3.2 Oligofructan
- 1.4 Galacto-oligosaccharide
- 1.5 Gum
- 1.6 Mucilage
- 1.7 Pectin

2. Analogous carbohydrates

- 2.1 Indigestible dextrins
  - 2.1.1 Resistant maltodextrin
  - 2.1.2 Resistant potato dextrin
- 2.2 Synthesized carbohydrates compounds
  - 2.2.1 Polydextrose
  - 2.2.2 Methyl cellulose
  - 2.2.3 Hydroxypropylmethyl cellulose
- 2.3 Resistant starch

3. **Lignin substances associated with the NSP and lignin complex**

3.1 Wax
3.2 Phytate
3.3 Cutin
3.4 Saponin
3.5 Suberin
3.6 Tannin

Non-starch polysaccharides (NSP) are all those polysaccharides resistant to digestion in the small intestine that act as microbial substrates in the large intestine (DeVries et al. 2001). Non-starch polysaccharides include cellulose, hemicellulose, pectin, modified cellulose, fructans, gum, and mucilage (DeVries et al. 2001). Cellulose is a lineal polysaccharide composed by thousands of glucose units linked by β-1, 4 glycosidic bonds (Dhingra et al. 2012). In their structure, cellulose polysaccharides contain several hydrogen bonds within the glucose units, yielding a final organization of crystalline microfibrils (Cosgrove 2005). Hemicellulose is shorter in size than cellulose, but is also formed by glucose units linked by β-1, 4 glycosidic bonds. Another difference is spatial structure, lineal for cellulose and branched for hemicellulose (Cosgrove 2005).
Resistant starch is defined as starch and products of starch degradation that are not absorbed in the small intestine of healthy individuals (Topping & Clifton 2001). Pectins are a group of polymers that contain 1,4-linked α-D-galacturonic acid residues (Ridley et al. 2001).

Oligosaccharides are saccharide polymers that contain 3 to 10 monosaccharides. One of the most studied types in the last years are fructans, formed of lineal chains of fructose bonded together by a β-(1-2) link (Watzl et al. 2005). Inulin is a special case of oligosaccharide with a polymerization degree of 2 to 60, and is not considered a strict

oligosaccharide but am oligo- and polysaccharide mixture. Both, inulin and fructans, only get digested in the large intestine since the upper GI tract does not contain any enzymes able to break the β-(1-2) linkage (Watzl et al. 2005).

Lignin is a molecule of high weight, resulting from the union of approximately 40 oxygenated phenylpropane units (Dhingra et al. 2012). Lignin can be found in whole grain cereals and is not digested or absorbed in the small intestine, or used by the colonic microbiota (Dhingra et al. 2012), this being the reason why the lignification process notably affects fiber digestibility.

3.3. Analytical methods for fiber classification

Over the years, several analytical methods to determine fiber concentration in the diet have been defined (Figure 1.3). However, many of those chemical determinations exclude fractions of the total carbohydrate load, while others overlap (Mertens 2003), leading to the final conclusion that there is no single method of analysis for DF that precisely measures all carbohydrates that characterize its nutritional definition (NRC 2007).

Crude Fiber (CF) is the residue of plant food left after sequential extraction with solvent, diluted aqueous acid, and dilute aqueous alkali (Trowell 1976). Crude fiber is determined by using a chemical-gravimetric technique. This method separates carbohydrates into two portions: CF and nitrogen-free extract. However, soluble fiber and part of lignin, cellulose, and hemicellulose is found in the nitrogen-free extract, so the analyzed concentration of CF does not adequately describe the actual fiber composition of a feed ingredient (Otten et al. 2006; NRC 2012). Detergent fibers such as neutral detergent fiber

(NDF, measures most of the structural components in plant cells including lignin, hemicellulose and cellulose but not pectin) and acid detergent fiber (ADF, includes lignin, cellulose and silica but not hemicellulose) also are determined using a chemical-gravimetric method. This method separates non-starch polysaccharides into NDF, ADF, and lignin (Robertson & Horvath 2001). The difference between the concentration of lignin and ADF gives as a result the concentration of cellulose, and the concentration of hemicellulose is calculated as the difference between ADF and NDF. Total Dietary Fiber (TDF) consists of the remnants of edible plant cells, polysaccharides, lignin, and associated substances resistant to digestion by the alimentary mammalian digestive system (Trowell 1976). This concept englobes oligosaccharides, polysaccharides, β-glucans, pectins and gums, hemicellulose, cellulose, lignin (NRC 2007). The TDF determination method is a more comprehensive enzymatic-gravimetric procedure to quantify all of the fiber fractions in a feed ingredient, and also separates the various carbohydrates into soluble and insoluble fiber fractions (NRC 2012). Non-starch polysaccharides, as mentioned before, are all those polysaccharides resistant to digestion in the small intestine that act as microbial substrates in the large intestine (DeVries et al. 2001). The NSP can be determined using two different methods: the Uppsala method that calculates dietary fiber as the sum of amylase-resistant polysaccharides, uronic acids, and Klason lignin (Mertens 2003); and the Englyst method, similar to the Uppsala method but excluding ligning and resistant starch (Englyst & Hudson 1987).

For the current project, NDF was the method selected to balance the animal diets. Neutral detergent fiber accounts for hemicellulose, cellulose and lignin content, excluding pectin, gums and β-glucans (Figure 1.3).

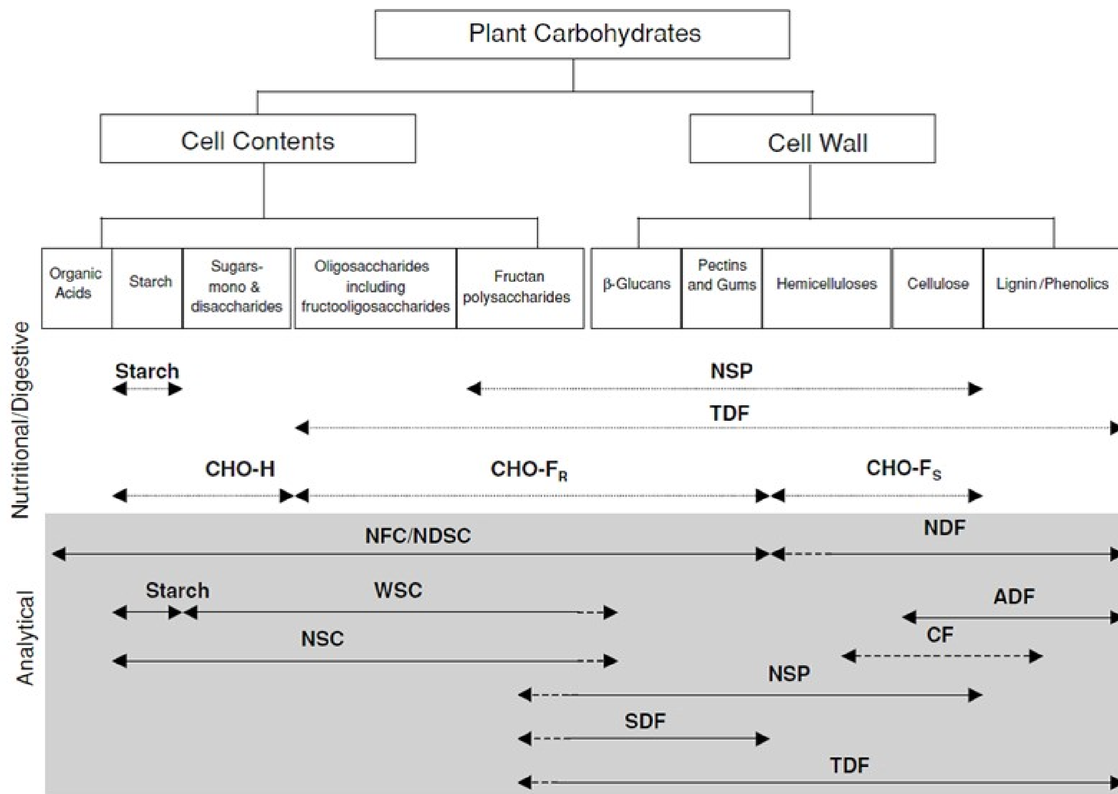

Figure 1.3. Plant carbohydrate analytical and nutritional composition (NRC 2007).

3.4. Characteristics of dietary fiber

Fiber types can be classified by their physical properties. Those properties are: fermentability, solubility, and viscosity (Table 1.2). Fermentability is the "fiber's capacity to be broken down into smaller, simpler carbohydrates by intestinal bacteria." Fermentability depends on the fiber chemical composition, structure, and transit time (El Oufir et al. 2000) . Fiber solubility is defined as fiber's behavior in water (Marlett 1992). Soluble fiber gets easily dispersed in water while insoluble fiber does not (Southgate et al. 1978). Viscosity is fiber's capacity to form gels when mixed with water. This capacity is affected by concentration, pH, and temperature (Dhingra et al. 2012). While

fermentability and solubility correlate (Eswaran et al. 2013), not much data about viscosity has been published.

Table 1.2. Classification of fiber components based on solubility, fermentability and viscosity (Slavin 2013)

Soluble fibers	β-glucans	Insoluble fibers	Cellulose
	Gums		Lignin
	Wheat dextrin		Some pectins
	Psyllium		Some hemicellulose
	Pectin		
	Inulin		
Fermentable fibers	Wheat dextrin	Non-fermentable fibers	Cellulose
	Pectins		Lignin
	β-glucans		
	Guar gum		
	Inulin		
Viscous fibers	Pectins	Non-viscous fibers	Polydextrose
	β-glucans		Inulin
	Some gums		
	Psyllium		

3.5. Fiber sources

Most of the DF is of plant origin, such as fruits, vegetables, cereals, legumes, and nuts (DeVries et al. 2001). For animal feeds, there are several high fiber ingredients available and many of them are derived from industrial processes rendering them to be unsuitable or undesirable for human consumption (Noblet & Goff 2001).

In the research presented in this thesis, four fiber sources were used that contained different fiber concentrations and composition, but are considered mainly sources of insoluble fiber (Table 1.3): corn distillers dried grains with solubles, soybean hulls, wheat straw, and wheat middlings.

Corn distillers dried grains with solubles (DDGS) is the primary co-product of fuel ethanol production from dry-grind processes (Rosentrater 2012). As an animal feed ingredient it serves as a source of energy, digestible amino acids, and phosphorus that partially replaces some of the corn, soybean meal, and inorganic phosphorus supplement in swine diets (Stein & Shurson 2009). However, optimizing the use of DDGS can be challenging due to the highly variable energy and nutrient content among sources (Stein & Shurson 2009), as well as its relatively variable concentration of fiber (18 to 47% NDF; Table 1.3), and variable fiber digestibility (Urriola et al. 2010). Apparent total tract digestibility of DDGS is less than 50%, which translates into reduced digestibility values for dry matter (DM) and net energy (NE). Despite those challenges, DDGS is a widely used feed ingredient in swine diets because of its abundant supply (about 42 million metric tons), high energy and nutrient content, and economical price ($137-172/metric ton, as on April 27, 2017 (US Grains Council 2017).

Soybean hulls (SBH) are a by-product of the processing of soybeans (Kornegay 1978), which are separated from the soybean seed during the solvent oil extraction process, and are high in fiber content (NDF 53 to 72%; Table 1.3). The amount of SBH used in swine diets is much less than DDGS and are used primarily in sow diets to reduce constipation (Whitney, Shurson, Johnston, et al. 2006). Approximately 17.3 million of metric of tons

of soybean hulls are produced annually (Schirmer-Michel et al. 2008) and sold at relatively inexpensive prices.

Wheat straw (WS) is a by-product of the plant after harvesting wheat grain, and is primarily used as bedding material in livestock housing systems. The nutritional value of WS is low compared with most other ingredients, and has very high fiber (65 to 86% NDF; Table 1.3), and low energy, crude protein, and ether extract content, which translates into decreased digestibility of protein, NDF and gross energy (Falkowska et al. 2006). Wholesale market commands about $30-40 per metric ton of WS (Lee & Grove 2015). While wheat straw is relatively inexpensive, it is not used in commercial swine diets because of its low metabolizable energy and digestible amino acid content.

Wheat middlings (WM) are one of the by-products of the wheat milling process for obtaining flour. In animal feed, WM can be a good source of protein, fiber (30 to 43% NDF; Table 1.3), phosphorous, and amino acids. Their inclusion in the animal diets improves quality of pellets by increasing firmness and particle adhesion (Cromwell et al. 2000). In addition to that, WM are a good source of nutrients at a low price ($65-100/metric ton as on April 27, 2017 (University of Missouri, Extension report, 2017), and abundant quantities are available (7 million of metric tons of flour milling by-products produced per year), making it a highly desirable ingredient for animal feed.

Table 1.3. Fiber composition (% dry matter basis) in corn distillers dried with solubles (DDGS), soybean hulls (SBH), wheat straw (WS) and wheat middlings (WM).

	CF	NDF	ADF	TDF	SDF	IDF	CP	EE
Corn DDGS	9.9	44.0	18.0	42.9	0.7	42.2	28.9	11.1
SBH	40.1	67.0	50.0	83.9	8.4	75.5	13.2	2.2
WS	41.6	85.0	54.0	71.5	0.5	71.0	4.0	1.5
WM	9.5	40.0	11.1	37.2	2.7	34.5	18.4	4.9

CF = crude fiber, NDF = neutral detergent fiber, ADF = acid detergent fiber, TDF = total dietary fiber, SDF = soluble dietary fiber, IDF = insoluble dietary fiber, CP = crude protein, EE = ether extract (Lackey 2010; Jaworski & Stein 2017).

3.6. Physiological functions of fiber

3.6.1. Prebiotic

Prebiotics are defined as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon" (Schrezenmeir & de Vrese 2001; Gibson & Roberfroid 1995). Prebiotics can have different properties such as anti-inflammatory, anti-carcinogenic, anti-osteoporotic, anti-microbial, hypolipidemic, and glucose modulators (Swinnen et al. 2006; Ewaschuk & Dieleman 2006).

Evidence shows that bacteria or probiotics prefer to metabolize small carbohydrates -oligosaccharides- than large ones -polysaccharides- (Cummings & Branch 1986). In one of our studies (Chapter 3), carbohydrases were added to the diet in order to further break down DF, making smaller carbohydrates more available to intestinal bacteria with the aim to increase fermentation and, as a result, increase digestibility of the high fiber diets.

3.6.2. Colonic production of short-chain fatty acids

Short-chain fatty acids, specially acetate, propionate, and butyrate; have an important role in the maintenance of colonic homeostasis (Mortensen & Clausen 1996). These SCFA are produced in the large intestine by anaerobic bacterial fermentation of carbohydrates and polysaccharides from the diet.

Butyrate is considered the main source of energy for colonocytes (Mortensen & Clausen 1996). Different studies have shown that diets rich in fiber, in addition to increasing SCFA production, also promote the utilization of butyrate by the colonocytes (Jacobasch et al. 1999) potentially inducing a shift from a pro-inflammatory profile to an anti-inflammatory profile (Maa et al. 2010). However, the mechanisms by which the SCFA modulate inflammation as well as if different fiber types can influence differently SCFA distribution, remain unknown (Chuang et al. 2011)

3.6.3. Motility and satiety

Fiber intake can modulate gastrointestinal motility (Eswaran et al. 2013). The secondary bile salts and SCFA produced by bacterial fermentation, which stimulate cholinergic-mediated intestinal circular smooth muscle contractions, can accelerate intestinal transit time (Eswaran et al. 2013; Soret et al. 2010). In one of their studies, Soret et al. (2010) proved that butyrate induces histone acetylation and general expression of the choline acetyltransferase (ChAT) gene in ChAT-immunoreactive myenteric neurons which results in a prokinetic effect on motility (Soret et al. 2010).

Increased dietary fiber intake promotes satiety (Slavin 2013). However, results differ according to the type of fiber, and whether it is added as an isolated fiber supplement,

rather than naturally occurring in food sources (Slavin 2013). Fiber physical properties also have an impact on fiber's capacity to induce satiety; increased fiber viscosity and decreased solubility has been linked with higher capacity to promote satiety; while non-viscous and soluble fibers have minimal effects on satiety, even if consumed in very large doses (Slavin & Green 2007).

3.6.4. Immunomodulatory effects

Studies to determine the effects of fiber on the GI immune system, including the GALT, are limited but such published research has revealed interesting results. Lim et al (1997) showed that feeding fibers of different solubility can alter cytokine concentrations in mesenteric lymph nodes in rats. In that experiment, rats were fed four different fiber sources: cellulose (water-insoluble), pectin, konjak mannan (water-soluble), and chitosan (acid-soluble); all at 5% w/w and levels of IFNγ and TNFα were measured in mesenteric lymph node cells cultured with and without concavalin A (a carbohydrate-binding protein) for 24 and 72 hours (Lim et al. 1997). Their results revealed that, after 24 hours, cells cultured without concavalin A showed undetectable concentrations of IFNγ and TNFα. However, after 72 hours pectin treated cells displayed high concentrations of IFNγ. They interpreted this, together with the fact that pectin caused a misbalance in $CD4^{+}$:$CD8^{+}$ ratio favoring $CD4^{+}$, suggesting that pectin may have the capacity to influence the differentiation of T-cells to become Th1 cells (Lim et al. 1997). In that same experiment, levels of Ig were also measured in order to assess fiber's capacity to induce hypersensitivity and/or immunosuppression. Their results showed that Ig levels in plasma were modified by the fiber type: pectin was able to induce greater levels of IgA

when compared to cellulose and chitosan, and greater levels of IgG when compared to the cellulose. However, cellulose had the greatest levels of circulating IgE when compared to any of the other types of fiber. Such results may indicate that pectin may have an anti-allergic effect, since IgA plays a role in preventing allergic reaction through inhibition of allergen absorption, while cellulose may have a more allergenic effect (Lim et al. 1997).

Other connections between fiber intake and the immune system have been reported in dogs where it was shown that a fermentable fiber mixture (8.7 g/kg body weight) composed by beet pulp, oligofructose and gum Arabic, increased the proportion of $CD8^+$ cells in the lamina propria and Peyer's patches, as well as a $CD4^+$ in the mesenteric lymph nodes and peripheral blood (Field et al. 1999). These findings are consistent with other studies performed in rats, where feeding a diet supplemented with sugar beet fiber (10% w/w) showed increased $CD8^+$ proportion in intraepithelial lymphocytes compared to rats fed a fiber-free diet. Other studies also showed alterations in number of lymphocytes and leukocytes in the spleen (Kudoh et al. 1998), peripheral blood (Kaufhold et al. 2000), and intestinal mucosa (Kudoh et al. 1998; Kudoh et al. 1999).

The mechanisms by which DF modulates the immune system remain unknown. When DF reaches the colon, depending on its composition and properties, it becomes a substrate for some particular bacterial species, altering the gut microbiome (McRorie et al. 1998; Kanauchi et al. 2008). However, most of the anti-inflammatory effects of fiber are attributed to changes on the intestinal microbiota rather than the effect of the fiber itself over the GI tract (De Vrese & Schrezenmeir 2008). Four major hypotheses have

been proposed so far on how DF induced microbiome shifts affect the immune system (Schley & Field 2002):

a. Direct contact of lactic acid bacteria with the GALT: The most direct mechanism of DF action is the immune stimulation of the GALT through contact with the microbiota. Bacteria, in small amounts, are able to cross the epithelial barrier into the Peyer's patches (Berg 1985). In an *in vitro* study performed by Marin et al. (1997), the co-culture of immune cells with *Bifidobacterium* increased the production of IL-6 and TNFα by macrophages, and IL-2 and IL-5 by $CD4^{+}$ cells. Similarly, Park et al. (1999), found that macrophages co-cultured with *Bifidobacterium* increased nitric oxide, hydrogen peroxide, IL-6 and TNFα production. Yasui and Ohwaki (1991) found that culturing mouse Peyer's patches with *Bifidobacterium breve* increased B-cell proliferation and antibody production.

b. Direct contact of bacterial products/metabolites with the GALT: Other authors hypothesized that is not the direct contact with bacteria that induces the intestinal immune changes, but the exposure to bacterial metabolites and products like cytoplasmic antigens or cell wall components that can pass the epithelial barrier and contact the GALT (De Simone et al. 1987; Perdigón et al. 1988; Takahashi et al. 1998). *In vitro* experiments showed that incubation of macrophages with cell-free extracts of *Bifidobacterium* and *Lactobacillus* caused a similar stimulation that co-culture with the whole bacteria (Hatcher & Lambrecht 1993). *In vivo* experiments performed in mice also demonstrated that administration of *Lactobacillus* culture supernatants stimulated phagocytic activity (Perdigón et al. 1988), and administration of cytoplasmic components of *Bifidobacterium* led to an increase in IgA production by the Peyer's patches

(Takahashi et al. 1998). The actual mechanism behind this immunomodulation is still unclear. It has been suggested that there may be receptor binding sites on the $CD4^+$ and $CD8^+$ lymphocytes for certain bacterial strains (De Simone et al. 1988). Also, it is known that peptidoglycans can bind to CD14, a co-receptor found in the membrane of macrophages and neutrophils, stimulating their activation (Matsuzaki 1998).

c. Production of SCFA: Fermentation of DF results in SCFA production, and some studies have reported immunomodulatory effects of SCFA on the GI tract (Bohmig et al. 1997; Pratt et al. 1996). *In vitro* studies showed that butyrate is able to suppress cytokine-induced expression of the transcription factor NFκB in colonic HT-29 cells (Inan et al. 2000). A hypothesis of what the mechanisms behind of the anti-inflammatory effects of SCFA are is that, in the colon, SCFA may reduce the colonocytes requirement for certain energy sources such as glutamine. Because glutamine is a preferred substrate for lymphatic tissue, it is possible that by sparing glutamine, immune cells function could be enhanced (Jenkins et al. 1999). However, is it not know if these effects occur at the concentrations of SCFA seen after a high fiber meal and if the type of fiber and physical characteristics will play a role on it (Schley & Field 2002).

d. Mucin production modulation: There is evidence that addition of fiber in high concentrations can increase mucin production in the intestine (Satchithanandam et al. 1990; Saqui-Salces et al. 2017), which also has an immunological role since mucin helps to avoid bacterial translocation across the intestinal epithelial barrier (Frankel et al. 1995; Xu et al. 1998; Desai et al. 2016). Previous studies have hypothesized that the increase in mucin production occurs because mucin gets secreted in response of the decreased pH caused by an increase of SCFA concentration in the lumen (Bustos-Fernandez et al.

1978). However our hypothesis for the present study is that is not the SCFA content what modulates mucin secretion but the cytokines present in the environment.

4. *In vitro* models of intestinal epithelial responses

4.1. Intestinal epithelial cell cultures

For the study of the intestinal epithelium, different cell lines have been developed, both normal and tumoral, derived from human tissue or other animals. The HT-29 and Caco-2 lines originated from moderately differentiated (stem cells evolved into specialized cells) grade II human colon tumors are capable of generating well differentiated enterocytic tumors when inoculated into mice (Zweibaum et al. 1984; Chantret et al. 1987). These lines cultured under the appropriate conditions express typical differentiation characteristics of normal enterocytes (Hauri et al. 1985; Rousset et al. 1985). However, they were not originated from normal cells, and display different pathways and molecules that may or not be present within normal entrocytes *in vivo*.

The IEC-6 line was established from rat jejunal epithelial cells. Since is derived from small intestinal crypts, it has undifferentiated phenotypic characteristics, capable of synthesizing fibronectin and collagen (Quaroni et al. 1979). This cell line has been used in cell proliferation (Sato et al. 1999; Jasleen et al. 2002; Tuhacek et al. 2004), differentiation (Carroll et al. 1988; Suh & Traber 1996; Soubeyran et al. 1999), repair of tissue damage (Tabel et al. 2003; Liu et al. 2005), evaluation of infections (Tabel et al. 2003; Upperman et al. 2003), and nutritional quality of foods studies (Thoreux et al. 1996).

Carroll et al. (1988) described a method for induction of morphological and functional differentiation of IEC-6 cells in culture. It consists of growing the cells on a basal membrane matrix obtained from rat Engelbreth-Hom-Swarm sarcoma tissue. Commercially, this material is known as Matrigel© and is composed of laminin, collagen IV, entactin and heparan sulfate proteoglycans (Kleinman et al. 1982). In addition, other authors have determined that IEC-6 cells are capable of differentiating themselves in the absence of Matrigel when the culture is in postconfluence (Ametani et al. 1996; Wood et al. 2003).

The behavior of IEC-6 cells in culture is similar to that experienced by epithelial cells *in vivo*. Initially the cells proliferate in an intense way as it happens in the base of the intestinal crypts. When confluence is reached, areas where the monolayer become thicker with more layers of cells. Afterwards, there is a proliferation blockage, followed by expression of differentiation markers such as intestinal alkaline phosphatase and development of microvilli on the cell surface. Finally, the cells lose adhesion to the substrate and are desquamated towards the culture medium, resulting in cell death by apoptosis. A balance established between differentiated cells that are disappearing and other proliferative undifferentiated cells maintain the number of cells constant in the postconfluent culture (Ametani et al. 1996).

Another important cell type for intestinal epithelial responses is the intestinal porcine enterocyte cell line (IPEC). There are two main IPEC cell liness: IPEC-1 and IPEC-J2, both established from normal intestinal epithelium cells isolated from the jejunum (for IPEC-1) and jejunum and ileum (IPEC-J2) of a neonatal (12 hours), unsuckled pig, and described as non-transformed (Nossol et al. 2015). These cell lines are considered a good

model for studying intestinal cellular mechanisms such as transport of nutrients, toxins, differentiation of intestinal epithelial cells and metabolism (Gonzalez-Vallina et al. 1996). Each line have slightly different morphology, while IPEC-1 are cobblestone shaped, IPEC-J2 have an elongated phenotype with a higher cell area. Unlike carcinoma-derived cell lines (such as Caco-2 cells), neither of the IPECs show anchorage independent growth or abnormaly high levels of Villin expression, making them a more "physiologically correct" model for epithelial response (Nossol et al. 2015). Other cell lines available from rat intestinal epithelium are IEC-17 and IEC-18 (Quaroni et al. 1999) although they are less widely used.

4.2. Intestinal organoids

Recent advances in stem cell research have resulted in the development of intestinal models that better reflect the normal physiology of the GI (Figure 1.4) and can contribute to the better understanding of the interaction of the GI with diet and microbiome, as well as the understanding of development of diseases such as colitis, inflammatory bowel disease, and infections (Gulati et al. 2008; Scoville et al. 2008).

As previously mentioned, the intestinal epithelium is organized in villi and crypts. Located at the bottom of the intestinal crypts, also known as crypts of Lieberkühn, leucine-rich repeat-containing G protein-coupled receptor 5-positive ($Lgr5^{+}$) stem cells are responsible for the continuous regeneration and maintenance of this tissue. Isolated intestinal crypts or $Lgr5^{+}$ cells under specific growth media conditions, are able to generate a new epithelium, forming 3D cell cultures. These cells *in vitro* are in constant division and they form invaginations similar to the intestinal folds found *in vivo*. After 1-

2 days in culture, the structures in culture result is a small "enteroid"; a structure consisting of numerous invaginations or crypts, surrounding a lumen and with a functionality that mimics the organ from which the stem cell was originally collected (Sato et al. 2009).

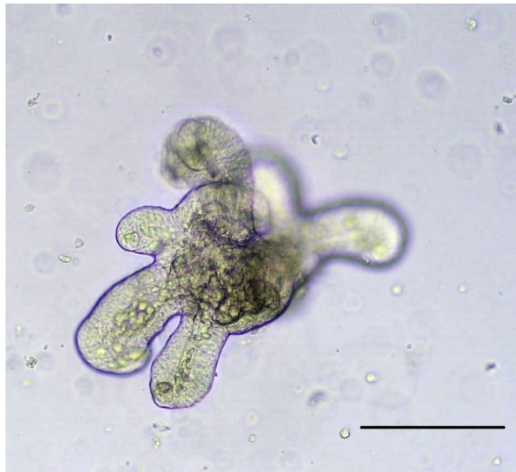

Figure 1.4. Mouse intestinal organoid. Scale bar represents 100μm.

Culture conditions for the organoids mimic those found in the intestine. Organoids are seeded in Matrigel© that, as explained before, provides extracellular matrix components (Kleinman et al. 1982) and support for tridimensional growth, but also laminin that prevents anoikis during the time of culture (Sato et al. 2009). Other factors required for culturing organoids are Wnt, in order to keep the functionality of the Wnt pathway which, upon activation, will cause an accumulation of β-catenin in the cytoplasm which will translocate into the nucleus to act as a transcriptional coactivator for cell proliferation. Other important factor is R-spondin1 (Rspo) which enhances the Wnt signal within cells but only in the presence of Wnt ligands, which may indicate that Rspo only acts over Wnt-activated cell and cells expressing Rspo receptors like Lgr5 (Kim et al. 2008). Epidermal growth factor (EGF), is supplemented in order to promote cell proliferation,

while the factor Noggin, is needed to inhibit the bone morphogenic protein (BMP) signaling pathway, which when activated, impedes intestinal self-renewal (Haramis et al. 2004; He et al. 2004).

Enteroids can be grown from isolated $Lgr5^+$ cells, a process that required additional growing factors added to the media in order to replace the signaling coming from the stem cell niche in the crypt. These additional factors include Jagged, a Notch ligand that activates Notch pathway or Y27632 that inhibits Rho kinase, causing an increase in levels of oncogenic factor phosphorylated Akt (pAkt) to promote cell survival (Li et al. 2005).

The enteroids (intestinal 3D organoids) grown *in vitro* will display all the differentiated epithelial cell types found in the intestine and will show a structure similar to the normal intestinal epithelium's structure (Sato et al. 2009).

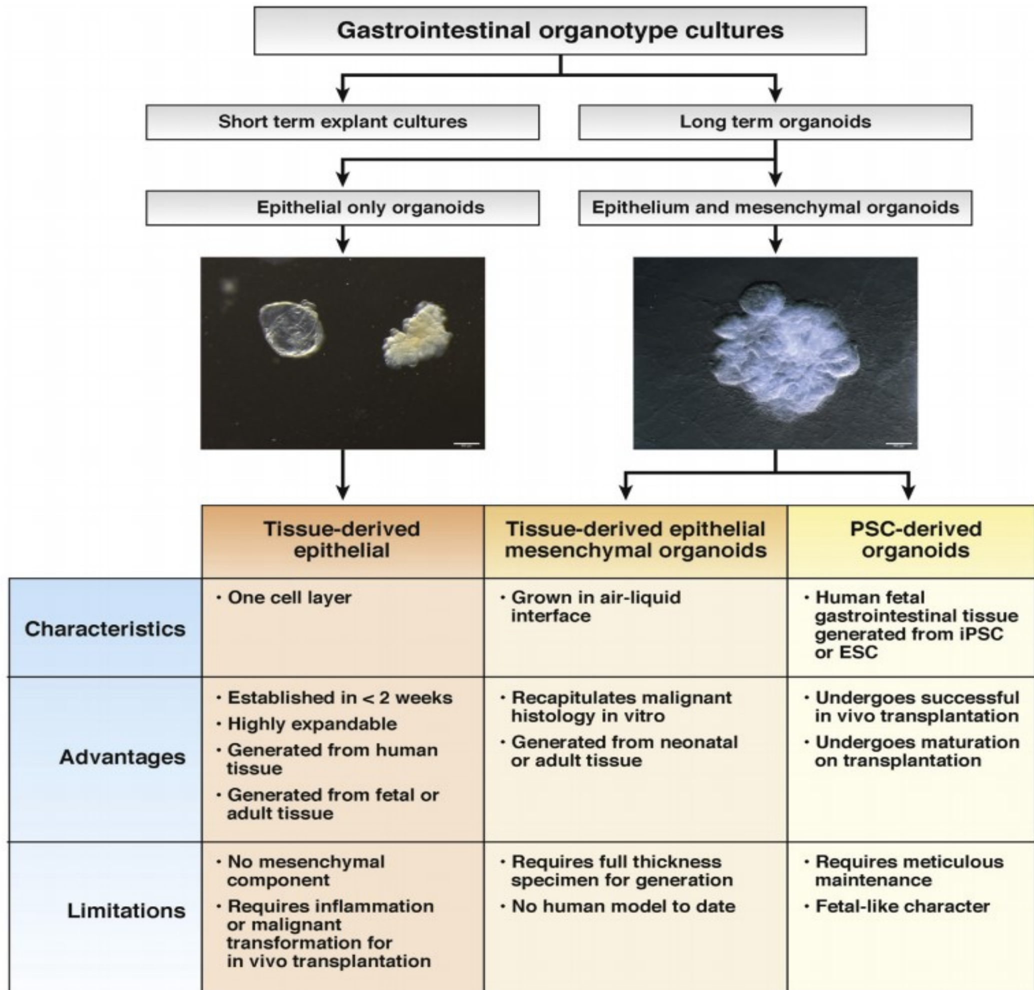

Figure 1.5. Composition, cellular sources, and nomenclature for GI organoids. GI organotypic cultures consist of short-term explant cultures and longterm organoid cultures. The 3 types of GI organoid cultures include (1) tissue-derived epithelial, (2) tissue-derived epithelial-mesenchymal, and (3) PSC-derived organoids. Tissue-derived epithelium and PSC-derived intestinal organoids are shown as examples of each type of tissue (Dedhia et al. 2016).

5. Synopsis

Dietary fiber is one of the most significant factors that affects gut physiology and health in humans (Slavin 2008) and livestock (Lindberg 2014) and its inclusion is considered to be important to promote and maintain gut motility and gut health (Viladomiu et al. 2013). For swine, there is not a recommended level of DF, however, it is a general consensus that minimal DF inclusion is needed in order to maintain normal intestinal function (Wenk 2001) and for optimization of gastrointestinal (GI) health and animal welfare (Knudsen et al. 2012; Brownawell et al. 2012). Although diets with high content in fiber have also been related with low energy content and decreased animal performance (Noblet & Goff 2001), these effects may vary based on fiber properties and sources (Wenk 2001).

Different varieties and amounts of fiber will differentially affect the host's microbiome (Kuo 2013). There is increasing evidence showing that fiber can have prebiotic effects in pigs due to interactions with the gut micro-environment and the gut associated immune system (GALT) (Lindberg 2014). Since fiber is not hydrolyzed or absorbed by the upper GI tract, it becomes a selective substrate for bacteria when it reaches the colon, altering the gut microbiome (McRorie et al. 1998; Kanauchi et al. 2008). Most of the anti-inflammatory effects of fiber are attributed to changes on the intestinal microbiota rather than the effect of the fiber itself over the GI tract (De Vrese & Schrezenmeir 2008); however other changes, like increased mucin production in the intestine, that are present when fiber is in high concentrations in the diet (Satchithanandam et al. 1990; Saqui-Salces et al. 2017) also play an immunological role since mucin helps to avoid bacterial translocation across the intestinal epithelial barrier (Frankel et al. 1995; Xu et al. 1998;

Desai et al. 2016). These changes seem to be more related to the specific properties of the fiber than because of the microbial composition modifications and yet have an important effect on the GI immune system capacity.

In summary, DF possesses the ability to modulate the immune response. Weather this immunomodulatory effect is caused directly by the fiber or through modifying other immune parameters remains unknown. The results of this project are important to understand the physiological significance of fiber sources on the GI inflammatory response, gut microbiota and mucin production. This knowledge will further help to define the best fiber concentrations and sources in the diet to improve animal performance and both human and animal health.

CHAPTER 2:

HIGH FIBER DIETS REDUCE PRO-INFLAMMATORY CYTOKINE LEVELS IN GROWING-FINISHING PIGS

SUMMARY

Dietary fiber plays an important role in the gastrointestinal health. We hypothesized that the swine intestinal immune response is modulated differentially by the type of fiber in the diet. Forty-six pigs (83.4 ± 6.7 kg) were divided into 4 groups and fed for 14 days diets formulated with 3 different fiber sources: wheat straw (WS; n=11), corn distiller's dried grains with solubles (DDGS; n=11), and soybean hulls (SBH; n=12). A corn-soybean meal diet was used as the control (CON, n=12). Test diets were formulated to contain approximately 17% NDF by adding 23% WS, 55% DDGS, or 30% SBH. Ileal tissue and blood samples were collected at euthanasia. Plasma concentrations of cytokines and chemokines were measured using a porcine immunoassay panel. Gene expression of cytokines in ileal tissue samples was analyzed. Statistical significance was calculated in GraphPad Prism 6.0 using ANOVA and corrected for multiple comparisons using Mann-Whitney U test. $P < 0.05$ was considered significant. In a systemic level (plasma), WS increased IFNγ, while DDGS and SBH decreased plasma levels of IL-2 and IL-6 respectively. There were no differences among fiber sources in plasma cytokine level for IL-1α, IL-1β, IL-1RA, IL-4, IL-8, IL-10, IL-12 and IL-18. In the ileum, the DDGS diet increased expression of *IL-12p40* and *IL-23A*, WS increased expression of *IFNγ*, *IL-12p40* and *IL-17A* and SBH increased *IL-1β*, *IL-11* and *IL-25*; and reduced *IL-8* expression when compared with the CON diet. Our results indicate that the intestinal cytokine profile is modulated differently by type of dietary fiber source, suggesting that fiber may be a good strategy to regulate GI inflammation.

INTRODUCTION

Dietary fiber (DF) is one of the most significant factors that affects gut physiology and health in humans (Slavin 2008) and livestock (Lindberg 2014), (Viladomiu et al. 2013). Fiber sources used for animal production are usually by-products derived from agro-industrial processes that are included in the feed in order to decrease cost and increase sustainability (Zijlstra & Beltranena 2013). However, high inclusion of fiber in pig diets can result in decreased dietary energy density, reduced nutrient digestibility and absorption, and increased manure output, which collectively impact the efficiency of commercial pork production systems (Urriola & Stein 2010; Gutierrez et al. 2014).

Epidemiological data suggest that high fiber diets reduce the incidence of GI inflammatory diseases in humans (Ewaschuk & Dieleman 2006; Ananthakrishnan et al. 2013), however, few studies have addressed this potential effect in swine. Previous studies have demonstrated that a 30% inclusion of corn DDGS reduced ileitis in pigs caused by *Lawsonia intracellularis* infection (Whitney, Shurson & Guedes 2006a; Whitney, Shurson & Guedes 2006b). In contrast, other studies have showed that inclusion of 30% DDGS in swine diets resulted in earlier onset of clinical signs of dysentery when compared with a control diet (Wilberts et al. 2014).

Mechanistically, DF may be altering the immune system by regulating the cytokine profile either locally within the GI tract and/or systemically. Weber et al. (2008) found an increase in *IL-1β*, *IL-6* and *IL-10* gene expression in ileum from piglets after feeding a diet containing 7.5% DDGS. Also, Pié et al. (2007) showed that supplementing pig diets with a mixture of fermentable fibers had an impact in colonic *IL-6* expression (Pié et al. 2007). However other studies have found no differences in cytokine profile after

supplementing swine diets with different fiber sources (Smith et al. 2011; Pieper et al. 2012), leading to inconclusive information about the effects of high fiber diets on the GI cytokine profile. The objective of this study was to determine the effects of 3 sources of DF (DDGS, soybean hulls and wheat straw) on intestinal morphology, intestinal cytokine expression and systemic cytokine expression in pigs.

MATERIALS AND METHODS

The animal use protocol was reviewed and approved by the University of Minnesota Institution Animal Care and Use Committee, protocol number 1312-31148A.

Animals and Diets

Forty-eight barrows (body weight, body weight 84 ± 7 kg, Large White × Danish Landrace) were individually housed in metabolism cages (198 cm × 84 cm × 71 cm) at the University of Minnesota Southern Research and Outreach Center (Waseca, MN). The pigs were allotted to 4 treatments, with 12 pigs per treatment, and the mean initial BW was similar among treatments. The 4 treatments included control diet, a diet with 23% inclusion of wheat straw (WS) diet, a diet with 55% inclusion of DDGS, and a 30% soybean hulls (SBH) diet (Table 2.1). Control diet was formulated to 8.5% NDF; WS, corn DDGS, and SBH diets were formulated to NDF concentration of about 20%. Corn and soybean meal were the sources of fiber in the control diet; WS, corn DDGS or SBH was the only source of fiber in WS, corn DDGS, or SBH diets, respectively. All the diets were formulated to meet the nutrient requirements in accordance to the National Swine Nutrition Guide (NSNG 2012).

Pigs were provided feed twice a day (0800 and 1600 h) at the calculated amount equivalent to 2.5% of their BW. Water was available *ad libitum* in nipple drinkers. The experiment lasted 14 days. Four pigs were excluded from harvesting because of sickness or failure to consume the experimental diets. At day 15, forty-four pigs were weighed after overnight fasting and then harvested at the Andrew Boss Meat Science Laboratory of the University of Minnesota (Saint Paul, MN). At the time of euthanasia, blood was withdrawn for plasma collection and 2 pieces of ileum of about 1 cm long each were collected about 10 cm proximal to the ileocecal valve. One piece was fixed in formalin and the second piece was snap frozen in liquid nitrogen and stored at -80^{0}C until further processing.

Cytokine plasma levels

Plasma concentrations of 13 different cytokines: interferon gamma (IFNγ), interleukin (IL) 1α (IL-1α), IL-1β, interleukin 1 receptor antagonist (IL-1RA), IL-2, IL-4, IL-6, IL-8, IL-10, IL-12 and IL-18; were measured using a Multiplex Map Kit (Porcine cytokine/chemokine Magnetic Bead Panel, Merck Millipore, Darmstadt, Germany). After pre-wetting the plates, 25 µl of matrix solution and 25 µl of standard or control reagents were added to the standard and control wells, respectively. Twenty-five µl of assay buffer and 25 µl of serum matrix were added to the background well, and 25 µl assay buffer and 25 µl sample (dilution 1:1) were added to all the sample wells. Twenty-five microliters of pre-combined beads were added to all the wells. After an overnight incubation at 4°C, the plates were washed twice, and 25 µl of detection antibody was added to each well. After antibody addition, the plates were incubated for 1 h on a plate shaker. Twenty-five

microliters of streptavidin-phycoerythrin were then added, and the plates were shaken for 30 min at room temperature. Finally, the plates were washed 3 times, and 150 µl of sheath fluid was added. Plates were read with a Luminex machine (Luminex 200, Austin Luminex, USA) and data were analyzed using Bio-plex manager 5.1 software (Bio-Rad), in accordance with the manufacturer's instructions.

Cytokine gene Expression

Total RNA from the ileal samples was isolated using the RNeasy Plus Universal Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Total RNA was quantified using a NanoDrop 2000 instrument (Thermo Scientific, Wilmington, DE), and 1000 ng of RNA were reverse transcribed using the High Capacity cDNA reverse Transcription Kit (Applied Biosystems, Foster City, CA). Gene expression (Table 2.2) of *interferon gamma* (*IFNγ*), *tumor necrosis factor alpha* (*TNFα*), *IL-1β*, *IL-4*, *IL-6*, *IL-8*, *IL-10*, *IL-11*, *interleukin 12 subunit 40* (*IL-12p40*), *IL-17A*, *IL-23A*, *IL-25*, *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) and *hypoxanthine-guanine phosphoribosyltransferase* (*HPRT*) was determined using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) in a StepOne-Plus system (Applied Biosystems, Foster City, CA). PCR conditions were: initial activation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec denaturation and annealing at 60°C for 60 secs.

Data Analysis

Relative gene expression was calculated using the primer efficiency values as described by Pfaffl (2001), Ct values > 38 were considered non-detectable. Geometric mean of the

housekeeping genes *GAPDH* and *HPRT* were used as reference genes. All data were tested for normality using D'Agostino and Pearson tests. Relative gene expression levels were analyzed using ANOVA followed by Tukey's multiple comparisons test using Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA) software.

RESULTS

Effects of different fiber sources on systemic immune response

There were no differences among fiber sources in plasma cytokine level for IL-1α, IL-1β, IL-1RA, IL-4, IL-8, IL-10, IL-12 and IL-18 (Table 2.3). IFNγ concentration was increased in the WS group when compared with the control group (P = 0.045). IL-6 concentration was decreased in pigs of the SBH group when compared with the controls (P = 0.039). Pigs fed DDGS as fiber source had lesser IL-2 concentrations when compared with the control diet (P = 0.045). The DDGS group also showed a significant decrease in the IL-10: IL-12, compared with the control group (P = 0.045).

Effects of fiber on cytokine mRNA expression in ileum

Dietary treatments affected the gene expression of cytokines in the ileum (Table 2.4). Only *IL-6* had no differences in mRNA expression among treatments. The *IFNγ* expression was not different for pigs fed WS and DDGS diets compared with those fed the control diet. However, pigs fed SBH had greater expression of *IFNγ* compared with those fed the control (P = 0.0015), WS (P < 0.0001) and DDGS (P < 0.0001) diets. Expression of *TNFα* did not differ between pigs fed the high-fiber diets compared with pigs fed the control diet, although it was induced by feeding the WS diet compared with

the SBH diet (P = 0.0097). Expression of *IL-1β* was not different for pigs fed WS and DDGS compared with those fed the control diet. However, pigs fed SBH had greater expression of *IL-1β* compared with those fed the control (P = 0.028), WS (P = 0.0001) and DDGS (P = 0.005) diets. Expression of *IL-4* was greater for pigs fed the control diet compared with those fed WS (P < 0.0001), DDGS (P < 0.0001) and SBH (P < 0.0001). Pigs fed SBH also had greater expression of *IL-4* compared with the pigs fed WS (P < 0.0001) and DDGS (P < 0.0001). Expression of *IL-8* in pigs fed the SBH diet was lesser than those fed the control (P = 0.0009), WS (P = 0.0158) and DDGS (P = 0.0003) diets. The expression of *IL-10* was lesser in pigs fed WS and DDGS compared with pigs fed the control and the SBH diets (P < 0.0001 and P < 0.0001 respectively). Expression of *IL-11* was greater for pigs fed SBH compared with pigs fed the control (P = 0.05), WS (P < 0.0001) and DDGS (P < 0.0001) diets. Pigs fed the control diet also had greater expression of *IL-11* compared with pigs fed WS (P = 0.0073) and DDGS (P = 0.0008). The expression of *IL-12p40* was increased by feeding WS and DDGS compared with the control (P < 0.0001) and SBH (P < 0.0001) diets. The *IL-17A* expression was increased by feeding the WS compared with the animals fed the control (P = 0.0004), DDGS (P = 0.0256), and SBH (P = 0.01) diets. Expression of *IL-23A* was greater in pigs fed the DDGS diet when compared with the control diet (P = 0.0012), and did not differ among the other dietary treatments. Expression of *IL-25* was greater in pigs fed SBH compared with those fed control (P < 0.0001), WS (P < 0.0001) and DDGS (P < 0.0001) diets. In addition, pigs fed the control diet had greater expression of *IL-25* compared with pigs fed WS (P < 0.0001) and DDGS (P < 0.0001). The IL-10:IL-12 ratio was greater for pigs fed the control diet compared with pigs fed DDGS (P < 0.0001), SBH (P < 0.0001) and WS

($P < 0.0001$). Feeding the SBH diet resulted in greater values for IL-10:IL-12 when compared with DDGS ($P = 0.0001$) and WS ($P < 0.0001$) diets.

DISCUSSION

In swine production, the available literature evaluating the amounts of fiber are best for an optimal GI function is limited, and although it is a general consensus that minimal DF inclusion is needed in order to maintain normal intestinal function (Wenk 2001), GI health and animal welfare (Knudsen et al. 2012; Brownawell et al. 2012), the effects of different fiber sources are not clear.

Plasma levels of 13 cytokines were determined in order to assess changes in systemic immune status potentially triggered by the fiber in the diets. Out of those 13 cytokines, only IFNγ, IL-6 and IL-2 showed changes. Not many studies in swine have been performed looking at IL-6 and IL-2 changes, but studies in humans have shown that DF can modify systemic levels of pro-inflammatory cytokines IL-1β, IL-5, IL-6 and TNF-α, as well as anti-inflammatory cytokines IL-4 and IL-13 (Ma et al. 2008; Chuang et al. 2011). Our results in pigs are consistent with the previously reported reductions in plasma concentration of IL-6 after insoluble fiber consumption in humans (Ma et al. 2008; Chuang et al. 2011). To our knowledge, there are no previous *in vivo* studies reporting changes in plasma levels of IL-2 or IFNγ associated to DF consumption in swine.

In contrast to what we found at the systemic level, IL-6 was the only cytokine which expression was not modified by the diet in the ileum. The scarce literature addressing the changes in intestinal cytokine expression after fiber addition to diet presents contradictory results. Weber et al. (2008) found an increase in *IL-1β*, *IL-6* and *IL-10* gene

expression in the ileum of piglets fed a diet containing 7.5% DDGS during 7 days post-weaning, but found no differences in the cytokine profile of piglets fed 7.5% SBH for the same amount of time when compared to the controls (Weber et al. 2008). Smith et al. (2011) found no differences in gene expression of *IFNγ*, *IL-1α*, *IL-6*, *IL-8*, *IL-10* or *TNFα* in ileal and colonic tissue of pigs fed 10 days a diet supplemented with laminarin, a soluble and fermentable fiber, derived from the brown algae *Laminaria digitate*. Pieper et al. (2012)compared high and low crude protein content diets combined with high and low content of insoluble fiber and found no changes in *IL-1β*, *IL-6*, *IL-10* and *TGFβ* expression related to the insoluble fiber content but some significances related to the crude protein content. Walsh et al. (2013) added laminarin to the diets of 24 day-old piglets for 8 days, and found that the diet supplemented with laminarin reduced expression of *IL-1β*, *IL-6*, *IL-10* and *IL-17A* in the proximal colon, but no data on ileum was reported.

The discrepancies in changes on cytokine levels in plasma and gene expression between our study and the previous literature may be attributed to the fiber sources used, the housing conditions, age of the animals, breed, and the length of dietary treatment.

Regarding the fiber sources used in this study, previous research performed in our group showed that, out of the 3 fiber sources, SBH had the greatest *in vitro* fermentability (Shurson et al. 2015). Higher fermentation rate by the microbiota in the intestine will yield higher short chain fatty acid (SCFA) concentrations in the intestinal content (Wong et al. 2006) and SCFA have been proven to have immunomodulatory capacity in the GI tract (Viladomiu et al. 2013; El Oufir et al. 2000; Wong et al. 2006; Watzl et al. 2005). This supports the idea that increased production of SCFA from fiber sources may be

driving the local ileal immunomodulation, since the SBH diet was the most fermentable one and the one inducing greater changes over the cytokine changes. An possible explanation for the increased expression of cytokines in the DDGS group is that remaining yeast cell wall components from the ethanol production process that could be interacting with the GALT (Weber et al. 2008), while the more pro-inflammatory profile triggered by the WS diet could be attributed to other physical characteristics of this ingredient, including low digestibility and high insolubility.

There is evidence that the capacity to utilize DF increases with age of the pig. Growing-finishing pigs have a well-developed GI tract, with a slower transit time, and higher cellulolytic activity than newly weaned piglets, which results in greater capacity of older pigs to digest fibrous components compared to young pigs (Lindberg 2014). Commercial pig genetic lines have been genetically selected for improved capacity to deposit protein and lipid at the expense of digestive capacity. Therefore, there has been no selection pressure to develop commercial genetic lines to have greater capacity to digest fiber (Lindberg 2014).

CONCLUSIONS

In conclusion, DF source has an effect on modulating intestinal cytokine expression and systemic cytokine profile in pigs.

TABLES AND FIGURES

Table 2.1. Experimental diets: ingredient composition and nutrient content.

Item	Control	WS	Corn DDGS	SBH
Ingredient (%)				
Corn, yellow dent	79.79	-	-	-
Soybean meal	17.86	-	-	-
Wheat straw (WS)	-	23.0	-	-
Corn distillers dried grains with solubles (DDGS)	-	-	55.0	-
Soybean hulls (SBH)	-	-	-	30.0
Plasma spray-dried	-	4.73	4.73	4.73
Corn starch	-	61.10	34.73	56.66
Casein	-	3.00	3.00	3.00
Fish meal, menhaden	-	6.74	-	3.77
Titanium dioxide	0.50	0.50	0.50	0.50
Dicalcium phosphate (18.5%)	0.65	-	-	0.30
Limestone	0.92	0.32	1.44	0.44
Sodium chloride	0.30	0.30	0.30	0.30
Grow-finish VTM premix[1]	0.25	0.30	0.30	0.30
Total	100.0	100.0	100.0	100.0
Analyzed nutrient composition, DM basis				
GE, kcal/kg	4340	4167	4475	4103
CP, %	17.60	13.00	23.07	13.33
EE[2], %	2.66	2.87	6.20	2.40
ADF, %	2.60	12.63	8.77	14.63
NDF, %	8.50	24.20	19.57	21.53
Titanium, %	0.40	0.32	0.37	0.29

[1]Grow-finish vitamin and trace mineral premix (ANS swine G-F premix) provided per Kg of diet: vitamin A, 3,527,392 I.U.; vitamin D3, 661,386 I.U.; vitamin E as dl-alpha tocopherol acetate, 13,228 I.U.; vitamin K (MPB), 1,323 mg; riboflavin, 2,205 mg; niacin, 13,228 mg; pantothenic acid, 8,818 mg; vitamin B12, 13 mg; iodine (EDDI), 119 mg; selenium (Na selenite), 119 mg; SQM organic zinc, 22,046 mg; SQM organic iron, 13,228 mg; SQM organic manganese, 454 mg; SQM organic copper, 1,543 mg.

[2] Ether extract with acid hydrolysis

Table 2.2. Sequences of primers used in this study.

Gene	Forward Sequence	Reverse Sequence	Reference
IFNγ	GCTTTTCAGCTTTGCGTGACT	TCACTCTCCTCTTTCCAATTCTTC	Self
TNFα	AGCACTGAGAGCATGATCCG	GACATTGGCTACAACGTGGG	Self
IL-1β	CCAATTCAGGGACCCTACC	CATGGCTGCTTCAGAAACCT	(Lapthorne et al. 2015)
IL-4	CCAACCCTGGTCTGCTTACTG	TTGTAAGGTGATGTCGCACTTGT	(Sweeney et al. 2012)
IL-6	TGAACTCCCTCTCCACAAGC	GGCAGTAGCCATCACCAGA	(Lapthorne et al. 2015)
IL-8	AAGCTTGTCAATGGAAAAGAG	CTGTTGTTGTTGCTTCTCAG	(Petrov et al. 2014)
IL-10	CACTGCTCTATTGCCTGATCTTCC	AAACTCTTCACTGGGCCGAAG	(Xun et al. 2015)
IL-11	CAAATTCCCAGCTGACGGAGA	GTAGGAAAACAGGTCTGCTCG	Self
IL-12p40	GAGGGTGAGTGAGTGCCTTG	ACTCCGCCTAGGTTCGACTT	(Lapthorne et al. 2015)
IL-17A	ATCCTCGTCCCTGTCACTGC	ACATGCTGAGGGAAGTTCTTGTC	(Stepanova et al. 2012)
IL-23A	CCAAGAGAAGAGGGAGATGATGA	TGCAAGCAGGACTGACTGTTGT	(Luo et al. 2015)
IL-25	GAACCCACACCTTCCATTTG	ATCTCCAGAGGAGGCATGAG	(Masure et al. 2013)
GAPDH	ATCCTGGGCTACACTGAGGAC	AAGTGGTCGTTGAGGGCAATG	(Nygard et al. 2007)
HPRT	GGACTTGAATCATGTTTGTG	CAGATGTTTCCAAACTCAAC	(Gonzalez et al. 2013)

Table 2.3. Plasma concentration of cytokines in pigs fed high-fiber diets[1].

Marker (pg/ml)	Fiber source				
	CON	WS	DDGS	SBH	*P*-value
IFNγ	1022.4±280[b]	2166.2±714[a]	1457.1±747[a,b]	1683.9±596[a,b]	0.05
IL-1α	33.1±34	15±16	5.9±2	8.2±4	0.05
IL-1β	816.8±951	130.8±190	31.2±23	63.8±54	0.11
IL-1RA	143.8±169	155±99	45.2±19	119.1±113	0.15
IL-2	448.6±590[a]	155.0±93[a,b]	71.5±45[b]	93.6±52[a,b]	0.04
IL-4	1360.0±2020	518.5±617	125.4±105	214.6±238	0.17
IL-6	339.6±391[a]	83.9±75[a,b]	84.6±143[a,b]	49.1±21[b]	0.04
IL-8	5.9±5	14.2±4	8.8±5	9.4±7	0.19
IL-10	424.8±662	165.8±219	49.4±27	80.1±43	0.12
IL-12	426.3±149	437.1±83	384.3±112	420.4±163	0.83
IL-18	1472.9±1711	769.9±674	388.3±195	608.4±211	0.09
IL-10:IL-12[2]	93.89±54	203±53	84.32±27	326.3±55	<0.001

[1]Values presented as mean ± S.D. (n=12 for control, n=10 for WS, n=11 for DDGS and n=11 for SBH). Different superscripts within each row differ ($P < 0.05$).

[2]IL-10:IL-12 ratio was calculated using IL-10 plasma concentration/IL-12 plasma concentration.

Table 2.4. Relative gene expression in ileum of pigs fed high-fiber diets[1, 2].

	Fiber source				
	CON	WS	DDGS	SBH	*P*-value
IFNγ	0.037±0.005[b]	0.034±0.002[b]	0.033±0.003[b]	0.046±0.007[a]	<0.0001
TNFα	29.30±6.3[a,b]	41.68±16.57[a]	33.33±6.1[a,b]	26.42±8.3[b]	0.0131
IL-1β	0.048±0.007[b]	0.042±0.008[b]	0.046±0.005[b]	0.057±0.006[a]	0.0002
IL-4	108.3±9.9[a]	33.5±5.8[c]	34.7±16.1[c]	77.14±16.4[b]	<0.0001
IL-6	0.011±0.002	0.010±0.003	0.013±0.002	0.011±0.002	0.119
IL-8	7.88±0.5[a]	7.54±0.7[a]	7.97±0.9[a]	6.47±0.7[b]	0.0001
IL-10	0.97±0.01[a]	0.90±0.03[b]	0.89±0.03[b]	0.97±0.02[a]	<0.0001
IL-11	221.3±2.6[b]	115.4±18.4[c]	94.09±16.38[c]	300±119[a]	<0.0001
IL-12p40	0.003±0.0005[b]	0.011±0.003[a]	0.011±0.003[a]	0.005±0.001[b]	<0.0001
IL-17A	192.8±34.4[b]	312.9±88.3[a]	235.6±35.6[b]	228±53[b]	0.0006
IL-23A	0.004±0.0007[b]	0.005±0.001[a,b]	0.006±0.0004[a]	0.005±0.001[a,b]	0.0026
IL-25	11.37±0.75[b]	7.26±0.6[c]	7.03±0.8[c]	13.46±1.38[a]	<0.0001
IL-10:IL-12[3]	0.126±0.06[a]	0.189±0.1[a,b]	0.396±0.56[b]	0.948±0.99[a,b]	0.0331

[1]Relative expression mean values ± S.D. (n=12 for control, n=10 for WS, n=11 for DDGS and n=11 for SBH). Different superscripts within each row differ ($P < 0.05$).
[2]Values are expressed as a relative ratio of the amount of target gene copies to the amount of *HPRT* and *GAPDH* (housekeeping genes) copies.
[3]*IL-10:IL-12* ratio was calculated using IL-10 relative expression/IL-12p40 relative expression

CHAPTER 3:

CYTOKINE PROFILE IN ILEAL AND COLONIC TISSUE OF PIGS IS MODULATED BY FIBER SOURCE AND MULTI-CARBOHYDRASE SUPPLEMENTATION

SUMMARY

Carbohydrases are currently used in the swine industry with the aim to increase nutrient digestibility in animal diets. It is unknown whether the addition of enzymes has an effect on the intestinal immune response. In this study, wee hypothesized that different types of fiber and presence of carbohydrases in the diet will modulate the swine immune response and the intestinal microbiota composition. Fifty-four pigs (25.33±0.41 kg) were divided into 6 groups and fed a standard diet or diets formulated with 2 different fiber sources, all with or without carbohydrase addition: control diet (CSB, n = 9), a corn distiller's grains with solubles diet (DDGS, n = 9), a wheat middling diet (WM, n = 9), CSB with enzymes (CSB+E, n = 8), DDGS with enzymes (DDGS+E, n = 10) and WM with enzymes (WM+E, n = 9). Carbohydrase enzyme cocktail (100 mg/kg) contained: 1,500 U/g xylanase, 1,100 U/g β-glucanase, 110 U/g mannanase and 35 U/g galactosidase. Test diets were formulated to contain the same metabolic energy and approximately 17% NDF by adding 30% DDGS or 20% WM. Pigs were housed individually and fed treatment diets for 28 days. Ileal and colonic tissues and contents were collected at euthanasia and analyzed for cytokine and MUC2 gene expression by qRT-PCR, and cytokine abundance by ELISA. The presence of specific bacterial phyla (Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria and Spirochaetes) was analyzed using qPCR in reference to total bacterial 16s content. Statistical significance was calculated in GraphPad Prism 6.0 using ANOVA and corrected for multiple comparisons using Tukey's test. $P < 0.05$ was considered significant. Out of 13 cytokines, IL-1β, IFNγ, TNFα, IL-4, IL-11, IL-17A and IL-25 were modified at gene expression level. At protein level, IL-1β, IFNγ, IL-4,

IL-11, IL-17A were the only cytokines that showed differences. Overall the diets with carbohydrase addition increased pro-inflammatory cytokines and decreased anti-inflammatory cytokines. All high-fiber diets, increased MUC2 expression in both tissues, ileum and colon. Abundance of *Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria* were affected by diets, with DDGS+E showing an overall decrease in all phyla. Our results showed that fiber source has an impact on the cytokine profile of pigs fed high fiber diets with and without enzymatic supplementation as well as is able to induce changes in mucin expression in both ileum and colonic tissues, without having a major impact in microbiota composition.

INTRODUCTION

Diets with high content in fiber have been related with low energy content and decreased animal performance (Noblet & Goff 2001), effects that may vary based on fiber properties and sources (Wenk 2001). Different strategies have been suggested in order to increase the nutrient utilization of diets rich in fiber. One of such strategies is the supplementation with NSP-degrading enzymes (Zijlstra et al. 2010) which might result in a more cost-effective production. However, it is unknown whether the addition of enzymes has an effect on other parameters of the intestinal physiology such as the immune response and the microbiome composition.

Different varieties and amounts of fiber will differentially affect the host's microbiome (Kuo 2013). There is increasing evidence showing that fiber can have prebiotic effects in pigs due to interactions with the gut micro-environment and the gut associated immune system (GALT) (Lindberg 2014). Since fiber is not hydrolyzed or absorbed by the upper

GI tract it becomes a selective substrate for bacteria when it reaches the colon, altering the gut microbiome (McRorie et al. 1998; Kanauchi et al. 2008). Most of the anti-inflammatory effects of fiber are attributed to changes on the intestinal microbiota rather than the effect of the fiber itself over the GI tract (De Vrese & Schrezenmeir 2008); however other changes, like increased mucin production in the intestine, that are present when fiber is in high concentrations in the diet (Satchithanandam et al. 1990; Saqui-Salces et al. 2017) also play an immunological role since mucin helps to avoid bacterial translocation across the intestinal epithelial barrier (Frankel et al. 1995; Xu et al. 1998; Desai et al. 2016). The objective of this study was to define the swine intestinal cytokine profile when fed different diets with fiber sources with and without enzymatic supplementation, as well as to identify major microbiota composition shifts caused by the diet, and changes in mucin expression.

MATERIALS AND METHODS

The animal use protocol was reviewed and approved by the University of Minnesota Institution Animal Care and Use Committee (Project #1604-33628A)

Animals and Diets

Fifty-four pigs (25.33 ± 0.41 kg) were housed in individual pens (1.5 x 15 m^2) at the University of Minnesota Southern Research and Outreach Center (Waseca, MN). The pigs were blocked by body weight and assigned randomly to 1 of 6 dietary treatments, with 9 animals per treatment. The treatment diets included a control diet (CSB), a corn DDGS diet (DDGS) and a wheat middling diet (WM) (Table 3.1). The remaining 3 diets

(CSB+E, DDGS+E, WM+E), consisted of each of the three diets supplemented with 100 mg/kg of carbohydrase cocktail composed of1,500 U/g xylanase, 1,100 U/g β-glucanase, 110 U/g mannanase, 35 U/g galactosidase. Titanium dioxide (0.5%) and phytate (1,000 FTU/kg) were added to all diets. All the diets were formulated to meet the nutrient requirements in accordance to the National Swine Nutrition Guide (NSNG 2012)
The experiment lasted 28 days. Pigs were provided *ad libitum* access to feed and water. At day 29, animals were euthanized after overnight fasting. During the harvesting, ileal and colonic samples were retrieved, snap frozen in liquid nitrogen and stored at -80°C until analysis.

Gene Expression

Total RNA from the ileal and colon samples was isolated using the RNeasy Plus Universal Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Total RNA was quantified using a NanoDrop 2000 instrument (Thermo Scientific, Wilmington, DE), and 500 ng of RNA were reverse transcribed using the High Capacity cDNA reverse Transcription Kit (Applied Biosystems, Foster City, CA). Gene expression of genes for *interferon gamma* (*IFNγ*), *tumor necrosis factor alpha* (*TNFα*), *interleukin* (*IL*) *1β* (*IL-1β*), *IL-4*, *IL-6*, *IL-8*, *IL-10*, *IL-11*, *IL-12p40*, *IL-17A*, *IL-23A*, *IL-25*, *mucin 2* (*MUC2*), *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*), and *hypoxanthine-guanine phosphoribosyltransferase* (*HPRT*) was determined using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) in a Quantum Studio 3 system (Applied Biosystems, Foster City, CA). PCR conditions were: initial activation at 95°C

for 10 min, followed by 40 cycles of 95ºC for 15 sec denaturation and annealing at 60ºC for 60 secs. The primer sequences used are listed in Table 3.2.

Cytokine tissue levels

Tissue protein was extracted by homogenization of ileal and colon samples in lysis buffer containing deoxycholic acid (5mM) , Igepal CA-630 (0.5%), 1M Tris-HCl pH 7.4 (50 mM), 5M NaCl (250 mM), 100 µl of protease inhibitor cocktail at 1x (Halt protease inhibitor Cocktail (100x), Thermo Fischer Scientific, Rockford, IL, US) at 7.4 pH and 30 ml of distilled water. Homogenized samples were centrifuged at 12,000 x g for 15 min at 4ºC. Total protein was then quantified using a NanoDrop 2000 instrument (Thermo Scientific, Wilmington, DE). Samples of each group were then adjusted to the same protein concentration and pooled in triads to an n = 3/group for cytokine analysis.

A Multiplex Map Kit (Porcine cytokine/chemokine Magnetic Bead Panel, Merck Millipore, Darmstadt, Germany) was used to quantify IFNγ, IL-1β and IL-4. Cytokine levels of IL-17A were measured using a RayBio Porcine IL-17 ELISA kit (Raybiotech Inc., Monterouge, France). Concentration of IL-11 was measured using the Nori Porcine IL-11 ELISA kit (Genorise Scientific Inc., Philadelphia, USA) and concentrations of IL-25 were determined using a Nori Porcine IL-25/IL-17E ELISA kit (Genorise Scientific Inc., Philadelphia, USA).

Bacterial DNA isolation from intestinal contents

Two hundred µl of 1x phosphate buffer saline (PBS) were added to 200 mg of ileal and colonic content samples. Samples were then vortexed and centrifuged at 300 x g during 3

minutes at room temperature. Two hundred μl of the supernatant were used for DNA extraction utilizing the QIAamp DNA Minikit (Qiagen Inc., Chatsworth, California) following a previously described protocol (Burbach et al. 2016). The DNA samples were then pooled to obtain an n = 3 per group and stored at -20°C until PCR amplification.

Quantitative PCR of intestinal bacterial DNA

To ascertain the composition of the main bacterial phyla present in the small and large intestine of the pigs, isolated bacterial DNA was submitted to quantitative PCR and amplified using previously described primers (Table 3.3). Bacterial gene expression was determined using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) in a Quantum Studio 3 system (Applied Biosystems, Foster City, CA). PCR conditions were: initial activation at 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 sec denaturation and annealing at 60ºC for 60 secs.

Data Analysis

Relative gene expression of tissue cytokines was calculated using the primer efficiency values as described by Pfaffl (2001), Ct values > 38 were considered non-detectable. Housekeeping genes *GAPDH* and *HPRT* were used as reference genes. For microbiota gene expression a total bacterial *16s* was used as reference gene. All gene expression data as well as cytokine levels in tissue were tested for normality using D'Agostino and Pearson test. Relative gene expression, cytokine tissue levels and microbiota gene expression levels were analyzed using ANOVA followed by Tukey's multiple comparisons test using Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA) software.

RESULTS

Effects of fiber sources and enzymatic supplementation on cytokine mRNA expression in ileum and colon

No differences were found in the expression of *IFNγ, TNFα, IL-2, IL-6, IL-8, IL-10, IL-12p40,* and *IL-23* in ileal tissues (Table 3.4). The DDGS+E (P = 0.0002) and WM+E (P < 0.0001) diets had greater levels of *IL-1β* when compared with the non-enzymatically supplemented diets. CSB+E did not differ from the CSB, DDGS and WM but showed lesser *IL-1β* expression than WM+E (P = 0.013). From the non-enzymatically supplemented diets, pigs fed WM showed greater levels of *IL-4* when compared with those fed DDGS (P = 0.0187). There was no difference in *IL-4* levels across the enzymatically supplemented diets, however DDGS+E (P = 0.0275) and WM+E (P = 0.0065) had lesser levels of *IL-4* when compared with WM. *IL-11* expression was greater in the CSB fed group (P = 0.0002) and the DDGS group (P = 0.0014) when compared with the WM group. DDGS+E (P < 0.0001) and WM+E (P < 0.0001) showed lesser levels of *IL-11* when compared with CSB+E. *IL-11* levels in all pigs fed non-enzyme added diets were greater than on .Pigs fed DDGS+E had lesser *IL-11* than pigs fed CSB (P < 0.0001), DDGS (P < 0.0001) and WM (P = 0.0197). Animals fed WM+E also had less *IL-11* than pigs fed CSB (P < 0.0001), DDGS (P < 0.0001) and WM (P = 0.0003). Compared to their respective diet without enzymes, pigs fed enzymatically supplemented diets showed no differences on *IL-17A* expression. However, pigs fed WM+E had lesser expression of *IL-17A* than pigs fed CSB (P = 0.0315) and DDGS (P = 0.0142). *IL-25*

levels were greater in the WM fed group compared with the CSB fed group (*P* = 0.0066). There were no differences across enzymatically treated diets but pigs fed DDGS+E showed greater expression of *IL-25* when compared with those fed CSB (*P* = 0.0424).

No differences were found for *IL-1β, IL-2, IL-4 IL-6, IL-8, IL-10, IL-12p40, IL-23* and *IL-25* expression in colonic tissue (Table 3.4). *IFNγ* levels were not different among non- or enzymatically treated diets; however, enzymatically treated high-fiber diets (DDGE+E and WM+E) had greater expression of *IFNγ* when compared with their respective non-enzymatically treated high-fiber diets. Pigs fed DDGS+E had greater *IFNγ* expression than pigs fed DDGS (*P* = 0.0306) and WM (*P* = 0.0443). Feeding WM+E had a similar effect than feeding DDGS+E (with *P* = 0.0214 and *P* = 0.013 respectively). Expression of *TNFα* was increased in pigs fed DDGS when compared with pigs fed DDGS+E (*P* = 0.005) and CSB (*P* = 0.029). CSB+E diet induced the expression of *TNFα* when compared to CSB (*P* = 0.0437) and DDGS+E (*P* = 0.0088) diets. Pigs fed WM+E showed decreased *TNFα* expression when compared with pigs fed DDGS (*P* = 0.0007), WM (*P* = 0.022) and CSB+E (*P* = 0.0014) diets. The expression of *IL-11* did not differ between CSB and high fiber diets, or when enzymatically treated diets were compared to each other. However, enzymatic treatment decreased *IL-11* expression in the high fiber diets.*IL-11* expression was greater in the pigs fed DDGS when compared with the animals fed DDGS+E (P < 0.0001) and WM+E (P < 0.0001). Pigs fed WM had greater expression of *IL-11* compared with the pigs fed DDGS+E (P < 0.0001) and WM+E (P < 0.0001). The pigs fed CSB also showed a greater level of *IL-11* than DDGS+E (P = 0.0017) and WM+E (P = 0.0006) while animals fed CSB+E had lesser levels of *IL-11* than DDGS (P = 0.0091) and WM (P = 0.015). Addition of enzymes reduced *IL-17A*

expression in all diets. Animals fed CSB had greater expression than CSB+E (P = 0.04), DDGS+E (P = 0.0237) and WM+E (P = 0.0002). Pigs fed DDGS had increased levels of IL-17A when compared with CSB+E (P = 0.0003), DDGS+E (P = 0.0001) and WM+E (P < 0.0001). And pigs fed WM had greater levels of IL-17A than CSB+E (P = 0.0268), DDGS+E (P = 0.0145) and WM+E (P = 0.0001).

Effects of fiber sources and enzymatic supplementation on cytokine protein levels in ileum and colon

No differences in levels of IFNγ, IL-17A and IL-25 were found in ileal tissue for any of the diets (Table 3.5). IL-1β levels were significantly greater in the pigs fed the enzymatically supplemented diets when compared with the non-supplemented diets. Pigs fed WM+E had the highest IL-1β levels of all groups. IL-1β concentrations in the pigs fed DDGS+E and CSB+E diets were greater than those of pigs on CSB (*P* = 0.0118 and *P* = 0.0229 respectively), DDGS (*P* = 0.0229 and *P* = 0.0444 respectively) and WM (*P* = 0.0229 and *P* = 0.0406 respectively) diets. IL-4 levels were greater in the WM fed group when compared with DDGS (*P* = 0.0243), CSB+E (*P* = 0.0397), DDGS+E (*P* = 0.0491) and WM+E (*P* = 0.0131). Feeding CSB increased levels of IL-11 when compared with feeding CSB+E (*P* = 0.0143), DDGS+E (*P* = 0.0058) and WM+E (*P* = 0.0059), and pigs fed DDGS followed the same pattern (*P* = 0.0299, *P* = 0.0122 and *P* = 0.0124 respectively.) WM diet increased ileal IL-11 when compared with the DDGS+E (*P* = 0.0285) and WM+E (*P* = 0.0288) diets but not CSB+E.

In colonic tissue, no differences in levels of IL-1β, IL-4 and IL-25 were found (Table 3.5). IFNγ concentrations in pigs fed DDGS+E were greater than on pigs fed CSB (*P* =

0.0328), DDGS (P = 0.0005), WM (P = 0.0004) and CSB+E (P = 0.0018). CSB and WM+E diets induced greater expressions of IFNγ when compared with DDGS (P = 0.0058 and P = 0.0037 respectively), WM (P = 0.0034 and P = 0.0023 respectively) and CSB+E (P = 0.0328 and P = 0.0186 respectively) diets. IL-11 levels were greater in the colon of pigs fed the non-supplemented diets when compared with animals fed enzymatically treated diets. Pigs fed CSB diet had greater IL-11 than pigs fed CSB+E (P = 0.0047), DDGS+E (P = 0.0085) and WM+E (P = 0.0057). DDGS and WM diets also had induced the levels of IL-11 when compared with CSB+E (P = 0.0144 and P = 0.0208 respectively), DDGS+E (P = 0.0279 and P = 0.0411 respectively) and WM+E (P = 0.0176 and P = 0.0256 respectively) diets. IL-17A levels were lesser in the pigs fed diets supplemented with enzymes when compared with the non-supplemented diets diets. Feeding DDGS resulted in greater IL-17A levels when compared with feeding CSB+E (P = 0.0343), DDGS+E (P = 0.0208) and WM+E (P = 0.0262). Feeding DDGS+E reduced levels of IL-17A compared with feeding WM (P = 0.0461).

Effects of fiber sources and enzymatic supplementation on MUC2 expression in ileum and colon

Expression of *MUC2* was increased by the high fiber diets (DDGS and WM) in both tissues when compared with CSB diet, however that increment was countered by the addition of enzymes (Figure 3.1). In the ileum, DDGS fed group showed greater levels of expression of *MUC2* when compared with the CSB group ($P < 0.0001$), CSB+E (P = 0.0068), DDGS+E (P = 0.0294) and WM+E (P = 0.0037). DDGS+E was the only

enzymatically supplemented diet that maintained elevated *MUC2* expression when compared with CSB (*P* = 0.007).

In colonic tissue, the DDGS fed animals showed greater expression of *MUC2* when compared with the CSB group (*P* < 0.0001), CSB+E (*P* = 0.0017), DDGS+E *(P* = 0.0114) and WM+E (*P* = 0.0234). The WM group also showed increased levels of *MUC2* when compared with CSB (*P* = 0.0005) fed group. From the enzymatically supplemented diets, DDGS+E (*P* = 0.0201) and WM+E (*P* = 0.0095) induced *MUC2* expression when compared with CSB diet.

Effects of fiber sources and enzymatic supplementation on microbiota composition on ileum and colon

From the five phyla analyzed in ileum, we were not able to detect *Spirochaetes*. The markers for presence of *Proteobacteria* and *Bacteroidetes* did not show differences among groups (Table 3.6). The *Firmicutes* marker expression was increased in ileal contents of pigs fed the WM+E when compared with DDGS+E (*P* = 0.0255) diet. *Actinobacteria* was increased in the pigs on the CSB group when compared with the ones fed DDGS (*P* = 0.0239) and the DDGS+E (*P* = 0.0011) diets. DDGS+E fed pigs expressed lesser levels of *Actinobacteria* when compared with pigs fed WM (*P* = 0.0052) and WM+E (*P* = 0.0091).

In the colonic samples, *Firmicutes* and *Spirochaetes* did not show any significant differences among groups (Table 3.6). *Bacteroidetes* expression was decreased in pigs fed DDGS+E when compared with CSB (*P* = 0.0256), WM (*P* = 0.0441) and CSB+E (*P* = 0.0372). Expression of *Actinobacteria* was lesser in samples from the DDGS+E group

when compared with CSB (*P* = 0.0324), CSB+E (*P* = 0.0191) and WM+E (*P* = 0.0498) groups. Levels of *Proteobacteria* were increased in pigs fed CSB+E diet when compared with CSB (*P* = 0.0237), DDGS (*P* = 0.0011), DDGS+E (*P* = 0.0003) and WM+E (*P* = 0.0264) diets. WM fed pigs showed greater expression of *Proteobacteria* when compared with pigs fed DDGS (*P* = 0.0298) and DDGS+E (*P* = 0.0078).

DISCUSSION

Supplementation of pig high-fiber diets with fiber-degrading enzymes is an approach to increase energy and amino acid digestibility (Zijlstra et al. 2010)that may result in a more cost-effective production method. Different components of fiber will modify the intestinal microbiome and microbial metabolites, likely leading to changes in the intestinal immune response that can in turn affect the whole body's immune response (Kuo 2013). However, it is not known whether the addition of fiber-degrading enzymes can alter the immune response triggered by fiber in the diet. Smith et al. (2011) found no differences in gene expression of *IFNγ*, *IL-1α*, *IL-6*, *IL-8*, *IL-10* or *TNFα* in ileal tissue of pigs fed a diet supplemented with laminarin, a soluble type of fiber, while Weber et al. (2008) found an increase in *IL-1β*, *IL-6* and *IL-10* gene expression in the ileum of piglets fed a diet containing 7.5% DDGS when compared to the corn-soybean meal fed control pigs. From these results we could infer that a soluble fiber had minimal effects on the ileal cytokine profile of pigs. We found that WM, which has higher solubility (2.7% soluble dietary fiber; SDF, 34.5% insoluble dietary fiber; IDF) of the fiber sources tested, had a greater impact on cytokine expression than DDGS, which is a more insoluble source of fiber (0.7% SDF and 42.2% IDF).

In the ileum, we observed that the high fiber diets supplemented with enzymes favored a pro-inflammatory cytokine profile by increasing levels of *IL-1β* and decreasing *IL-11* when compared with the basal diets and the CSB+E group. IL-1β is a cytokine produced by activated macrophages, and a potent pyrogen and pro-inflammatory molecule that has a central role in the intestinal inflammation amplification cascade (Al-Sadi & Ma 2007). IL-11 is an anti-inflammatory cytokine that, in the intestine, is secreted by the subepithelial myofibroblasts, and plays an important role in epithelial integrity after mucosal insult such as ulcerations, enterocyte apoptosis, and gut translocation of bacterial endotoxins and enteric pathogens (Opal & Keith 2000; Bamba et al. 2003). Diets without enzyme inclusion showed an anti-inflammatory effect on the ileum evidenced by the increased expressions of *IL-4* and *IL-25*. IL-4 is a pleiotropic cytokine that, among its multiple properties, has the ability to influence the Th1:Th2 balance by favoring Th2 responses (West et al. 1996). IL-25 functions as a negative regulator of inflammatory Th1 and Th17 responses in the gut (Yoshimoto & Yoshimoto 2014). In summary, in ileal tissue, diets supplemented with carbohydrases caused a pro-inflammatory Th1 response by increasing *IL-1β* and decreasing the Th2 cytokine *IL-11* while high fiber diets without enzymatic supplementation induced a more anti-inflammatory profile by increasing expression of Th2 cytokines *IL-4* and *IL-25*.

Expression of cytokines in the colon was differently modulated by the addition of the carbohydrase mixture, with major changes observed on *IFNγ*, *TNFα,* at the gene level, andIL-11 and IL-17A at the protein level. IFNγ and TNFα are both very well-studied pro-inflammatory cytokines that are released in a very wide variety of situations, and by a wide variety of cells (Yoshimoto & Yoshimoto 2014). Walsch et al. (2013) added

laminarin to the diets of piglets and found reduced expression of *IL-1β*, *IL-6*, *IL-10* and *IL-17A* in the proximal colon (Walsh et al. 2013) while Weber et al (2008) did not found differences in *IL-1β*, *IL-6* and *TNFα* expression in colons of animals fed 7.5%. Our data suggest that, even though *TNFα* expression was greater in non-enzymatically supplemented diets compared to the supplemented ones, the low levels of *IFNγ* and greater levels of *IL-11* in the non-supplemented diets indicate a potential anti-inflammatory effect.

As expected from our previous study (Chapter 2) (Saqui-Salces et al. 2017), *mucin* expression in both ileal and colonic tissues was increased by the high fiber diets. Previous studies performed on different animal models like rats (Hino et al. 2013; Hino et al. 2012; Ito et al. 2009; Satchithanandam et al. 1990; Tanabe et al. 2005; Barcelo et al. 2000) and swine (Smith et al. 2011) had similar findings. In our study carbohydrase addition increased mucin expression in all diets when compared to the control diet, however, the mucin levels were still lesser than the mucin levels on those animals fed DDGS and WM without enzymes. Not much information has been published about effects of carbohydrases on mucin secretion. In poultry, it has been suggested that part of the improvements in digestibility seen after carbohydrase supplementation is explained by a reduction of mucin secretion caused by the enhanced digestion of fiber (Cowieson & Bedford 2009). Our results support Cowieson and Bedford's suggestion about mucin reduction, since the diets supplemented with enzymes showed less expression of mucin when compared to the high fiber diets, however, our animals did not show any improvement on digestibility of fiber.

The mechanisms by which DF modulates the immune system remain unknown; however, a common theory is that fiber affects the immune system via modulating the gut microbiome (Schley & Field 2002; McRorie et al. 1998). In the present study, microbial composition changes of the five phyla analyzed after fiber supplementation where rather modest. In adult swine, approximately 96% of the gut bacteria are represented by *Firmicutes* (91.8%) and *Bacteroidetes* (5%) (Zhao et al. 2015). The ratio of *Firmicutes* to *Bacteroidetes* in the intestine is a good measure of overall gut microbiota balance and can be correlated with changes on intestinal immune profile (Carding et al. 2015). Antibiotic-associated diarrhea, and other inflammatory diseases such as Crohn's disease and ulcerative colitis, have been correlated with decreases in *Firmicutes* and increases in *Bacteroidetes* as well as reduced gut biodiversity (Carding et al. 2015; Ott 2004). In our current study, *Firmicutes* were modified in the ileum and *Bacteroidetes* in the colon contents; in both tissues, DDGS+E diet lowered bacterial numbers, and the *Firmicutes* to *Bacteroidetes* ratio was not significantly different between diets, with no clear relationship of changes between the phyla or intestinal location. *Actinobacteria* was the only phyla from the 5 screened that changed in both ileum and colon. Animals fed DDGS with and without carbohydrases showed lowered abundance of *Actinobacteria* while the pigs fed CSB and CSB+E had the highest concentrations. Increased numbers on *Actinobacteria* have been reported in obese mice when compared to lean mice (Clarke et al. 2012). Even though so far there is no data about how changes in *Actinobacteria* correlates to inflammatory changes in the gut, modifications in this bacterial phyla may be related to increases in visceral adipose tissue deposition, which is associated with a

pro-inflammatory effect. Overall, pigs fed the DDGS+E diet showed greater impact on the 5 phyla studied.

In our study, the DDGS diet increased IL-17A, a Th17 cytokine present in the intestine under steady-state conditions. The expression of IL-17A is known to be dependent on the commensal gut-residing filamentous bacteria (Yoshimoto & Yoshimoto 2014) suggesting that the DDGS diet may have affected the presence of these bacteria; however, a more accurate approach to identify total microbial composition is needed in order to make conclusions about overall microbial population changes. Different varieties and amounts of fiber will differentially affect the host's microbiome (Kuo 2013). Since fiber is not hydrolyzed or absorbed by the upper GI tract, it becomes a selective substrate for bacteria when it reaches the colon, altering the gut microbiome (McRorie et al. 1998; Kanauchi et al. 2008). In summary, DF possesses the ability to modulate the immune response. Weather this immunomodulatory effect is caused directly by the fiber or through modifying other immune parameters remains unknown.

CONCLUSION

Fiber sources have an impact on the cytokine profile of pigs when fed high fiber diets with and without enzymatic supplementation, with enzymatic treatment promoting a pro-inflammatory profile compared with standard and high fiber diets. Fiber sources also affect mucin expression in both ileum and colonic tissues, without having a major impact in microbiota composition (Figure 3.2).

TABLES AND FIGURES

Table 3.1. Experimental diets: ingredient composition and nutrient content.

Item	CSB	DDGS	WM
Ingredient (%)			
Corn	72.00	42.02	46.66
Soybean meal	25.00	15.00	18.00
Corn distillers dried grains with solubles (DDGS)	-	40.00	-
Weat Middling (WM)	-	-	30.00
Soybean oil	-	-	2.23
Others	3.00	2.98	3.11
Total	100	100	100
Calculated nutrient composition			
ME, kcal/kg	3,285	3,295	3,285
CP, %	18.18	22.76	17.56
EE, %	2.89	5.10	5.14
NDF, %	8.61	17.30	17.22
ADF, %	3.39	6.15	5.78

ME – Metabolic energy, CP – Crude protein, EE – Ether extract with acid hydrolysis, NDF – Neutral detergent fiber, ADF – Acid detergent fiber.

Table 3.2. Sequences of primers used in this study for cytokine profiling.

Gene	Forward Sequence	Reverse Sequence	Reference
IFNγ	GCTTTTCAGCTTTGCGTGACT	TCACTCTCCTCTTTCCAATTCTTC	Self
TNFα	AGCACTGAGAGCATGATCCG	GACATTGGCTACAACGTGGG	Self
IL-1β	CCAATTCAGGGACCCTACC	CATGGCTGCTTCAGAAACCT	(Lapthorne et al. 2015)
IL-4	CCAACCCTGGTCTGCTTACTG	TTGTAAGGTGATGTCGCACTTGT	(Sweeney et al. 2012)
IL-6	TGAACTCCCTCTCCACAAGC	GGCAGTAGCCATCACCAGA	(Lapthorne et al. 2015)
IL-8	AAGCTTGTCAATGGAAAAGAG	CTGTTGTTGTTGCTTCTCAG	(Petrov et al. 2014)
IL-10	CACTGCTCTATTGCCTGATCTTCC	AAACTCTTCACTGGGCCGAAG	(Xun et al. 2015)
IL-11	CAAATTCCCAGCTGACGGAGA	GTAGGAAAACAGGTCTGCTCG	Self
IL-12p40	GAGGGTGAGTGAGTGCCTTG	ACTCCGCCTAGGTTCGACTT	(Lapthorne et al. 2015)
IL-17A	ATCCTCGTCCCTGTCACTGC	ACATGCTGAGGGAAGTTCTTGTC	(Stepanova et al. 2012)
IL-23A	CCAAGAGAAGAGGGAGATGATGA	TGCAAGCAGGACTGACTGTTGT	(Luo et al. 2015)
IL-25	GAACCCACACCTTCCATTTG	ATCTCCAGAGGAGGCATGAG	(Masure et al. 2013)
GAPDH	ATCCTGGGCTACACTGAGGAC	AAGTGGTCGTTGAGGGCAATG	(Nygard et al. 2007)
HPRT	GGACTTGAATCATGTTTGTG	CAGATGTTTCCAAACTCAAC	(Gonzalez et al. 2013)

Table 3.3. Sequences of primers used in this study for microbiota profiling[1].

Phylum	Forward Sequence	Reverse Sequence	Reference
Firmicutes	CTGATGGAGCAACGCCGCGT	ACACYTAGYACTCATCGTTT	(Hermann-Bank et al. 2013)
Bacteroidetes	CCGGAWTYATTGGGTTTAAAGGG	GGTAAGGTTCCTCGCGTA	(Hermann-Bank et al. 2013)
Actinobacteria	GCGKCCTATCAGCTTGTT	CCGCCTACGAGCYCTTTACGC	(Hermann-Bank et al. 2013)
Proteobacteria	TGGTGTAGGGGTAAAATCCG	AGGTAAGGTTCTTCGYGTATC	(Hermann-Bank et al. 2013)
Spirochaetes	GGTGTAGGAGTGAARTCCGT	TACGTGTGTAGCCCTRGRC	(Hermann-Bank et al. 2013)

[1]Nucleotide explanation: Y = C/T; R = A/G; W = A/T; K = G/T; M = A/C.

Table 3.4. Relative gene expression of cytokines in ileum and colon of pigs fed high-fiber diets[1, 2].

	Fiber source						
	CSB	DDGS	WM	CSB+E	DDGS+E	WM+E	*P*-value
Ileum							
IFNγ	0.069±0.008	0.065±0.009	0.069±0.01	0.069±0.008	0.068±0.007	0.071±0.01	0.89
TNFα	97±29.7	107.8±47.9	95.6±15.7	86.2±12.9	82.8±27.3	101.6±52.1	0.64
IL-1β	0.059±0.006[c]	0.059±0.01[c]	0.061±0.007[c]	0.071±0.02[b,c]	0.086±0.01[a,b]	0.091±0.01[a]	<0.0001
IL-2	1.95±0.09	1.99±0.14	1.92±0.04	1.94±0.08	1.87±0.05	1.91±0.11	0.13
IL-4	0.035±0.006[a,b]	0.032±0.004[b]	0.043±0.01[a]	0.033±0.004[a,b]	0.032±0.007[b]	0.031±0.003[b]	0.006
IL-6	0.028±0.004	0.027±0.007	0.024±0.003	0.025±0.004	0.023±0.003	0.025±0.003	0.22
IL-8	12.3±0.9	12.5±1.2	11.7±1.1	11.7±1.3	11.6±1.1	11.4±1.5	0.34
IL-10	1.98±0.08	1.99±0.1	1.97±0.04	1.96±0.07	1.92±0.04	1.94±0.08	0.38
IL-11	459.5±107[a]	435.8±77[a]	297.9±81[b]	422.4±79[a]	196.7±40[c]	140.4±21[c]	<0.0001
IL-12p40	0.0051±0.0008	0.0052±0.0008	0.0054±0.0008	0.0052±0.0001	0.0062±0.0007	0.0059±0.0001	0.08
IL-17A	675±110[a]	692.4±159[a]	590.3±107[a,b]	548.3±82[a,b]	575.6±165[a,b]	486.8±82[b]	0.008
IL-23A	479.7±99	502.3±155	513.3±101	521.4±168	530.5±133	614.9±230	0.54
IL-25	0.13±0.02[b]	0.14±0.01[a,b]	0.17±0.02[a]	0.15±0.01[a,b]	0.16±0.02[a]	0.15±0.02[a,b]	0.007
Colon							
IFNγ	0.071±0.007[a,b]	0.063±0.007[b]	0.064±0.006[b]	0.065±0.009[a,b]	0.076±0.009[a]	0.077±0.009[a]	0.0015
TNFα	119.2±16[b]	143.6±14[a]	134.3±18[a,b]	142.9±19[a]	114.6±15[b]	108.9±14[b]	<0.0001
IL-1β	0.049±0.007	0.047±0.004	0.049±0.008	0.044±0.007	0.051±0.01	0.045±0.004	0.45
IL-2	1.93±0.04	2±0.06	1.95±0.08	1.97±0.05	1.99±0.08	1.95±0.05	0.18
IL-4	0.017±0.002	0.018±0.002	0.019±0.002	0.017±0.001	0.017±0.001	0.017±0.001	0.09
IL-6	0.033±0.004	0.032±0.006	0.031±0.005	0.029±0.004	0.032±0.006	0.031±0.003	0.64
IL-8	12.28±0.87	13.69±0.81	12.95±0.99	13.30±1.34	13.42±1.33	13.3±1.04	0.12
IL-10	1.95±0.05	2±0.06	1.96±0.08	2±0.06	2.05±0.08	2±0.05	0.06
IL-11	494.2±96[a,c]	537.5±98[a]	532.7±123[a]	396.9±46[b,c]	339.5±32[b]	321.9±60[b]	<0.0001
IL-12p40	0.0043±0.0003	0.0043±0.0004	0.0046±0.001	0.0042±0.0004	0.0051±0.0008	0.0048±0.0008	0.07
IL-17A	1295±219[a]	1499±323[a]	1318±301[a]	953±181[b]	940±248[b]	740±117[b]	<0.0001
IL-23A	785.6±129	963.1±150	822.9±100	1006±207	960.5±144	977±160	0.013

IL-25	0.117±0.01	0.117±0.009	0.115±0.01	0.116±0.01	0.12±0.008	0.13±0.01	0.017

[1]Relative expression data are means ± S.D. (n=12 for control, n=10 for WS, n=11 for DDGS and n=11 for SBH). Different superscripts within each row differ ($P < 0.05$).
[2]Values are expressed as a relative ratio of the amount of target gene copies to the amount of *HPRT* and *GAPDH* (housekeeping genes) copies.

Table 3.5. Concentration of cytokines in ileal and colonic tissue of pigs fed high fiber diets[1].

	Fiber source						
Marker (pg/mg total protein)	CSB	DDGS	WM	CSB+E	DDGS+E	WM+E	*P*-value
Ileum							
IFNγ	0.37±0.21	0.32±0.13	0.57±0.17	0.51±0.06	0.36±0.17	0.45±0.2	0.6907
IL-1β	1.37±0.15^{c}	1.56±0.21^{c}	1.5±0.07^{c}	2.14±0.15^{b}	2.23±0.31^{b}	2.88±0.23^{a}	0.0023
IL-4	0.0018±0.01^{a,b}	0.0011±0.01^{b}	0.0037±0.01^{a}	0.0015±0.01^{b}	0.0011±0.01^{b}	0.0006±0.01^{b}	0.0416
IL-11	0.018±0.01^{a}	0.017±0.01^{a}	0.014±0.02^{a,b}	0.008±0.02^{b,c}	0.005±0.01^{c}	0.005±0.01^{c}	0.0011
IL-17A	0.54±0.15	0.64±0.12	0.65±0.02	0.43±0.02	0.3±0.01	0.38±0.07	0.0356
IL-25	0.44±0.08	3.77±0.06	0.3±0.07	0.36±0.06	0.36±0.13	0.38±0.004	0.7109
Colon							
IFNγ	3.78±0.27^{b}	2.56±0.38^{c}	2.38±0.57^{c}	2.99±0.2^{c}	4.44±0.37^{a}	4.28±0.55^{a,b}	0.0091
IL-1β	1.55±0.26	1.59±0.58	1.44±0.31	1.92±0.67	1.89±0.008	2.53±0.22	0.2318
IL-4	0.0015±0.01	0.0008±0.01	0.0013±0.01	0.0011±0.02	0.0009±0.04	0.0012±0.02	0.8946
IL-11	0.025±0.01^{a}	0.021±0.01^{a}	0.019±0.01^{a}	0.004±0.01^{b}	0.007±0.01^{b}	0.006±0.01^{b}	0.0210
IL-17A	2.02±0.84^{a,b,c}	2.78±1.37^{a}	2.29±0.65^{a,b}	0.90±0.01^{b}	0.61±0.05^{b,c}	0.833±0.25^{b}	0.0437
IL-25	0.41±0.18	0.29±0.01	0.33±0.11	0.42±0.12	0.4±0.15	0.43±0.06	0.8266

[1]Values presented as mean ± S.D. (n = 3/group). Different superscripts within each row differ ($P < 0.05$).

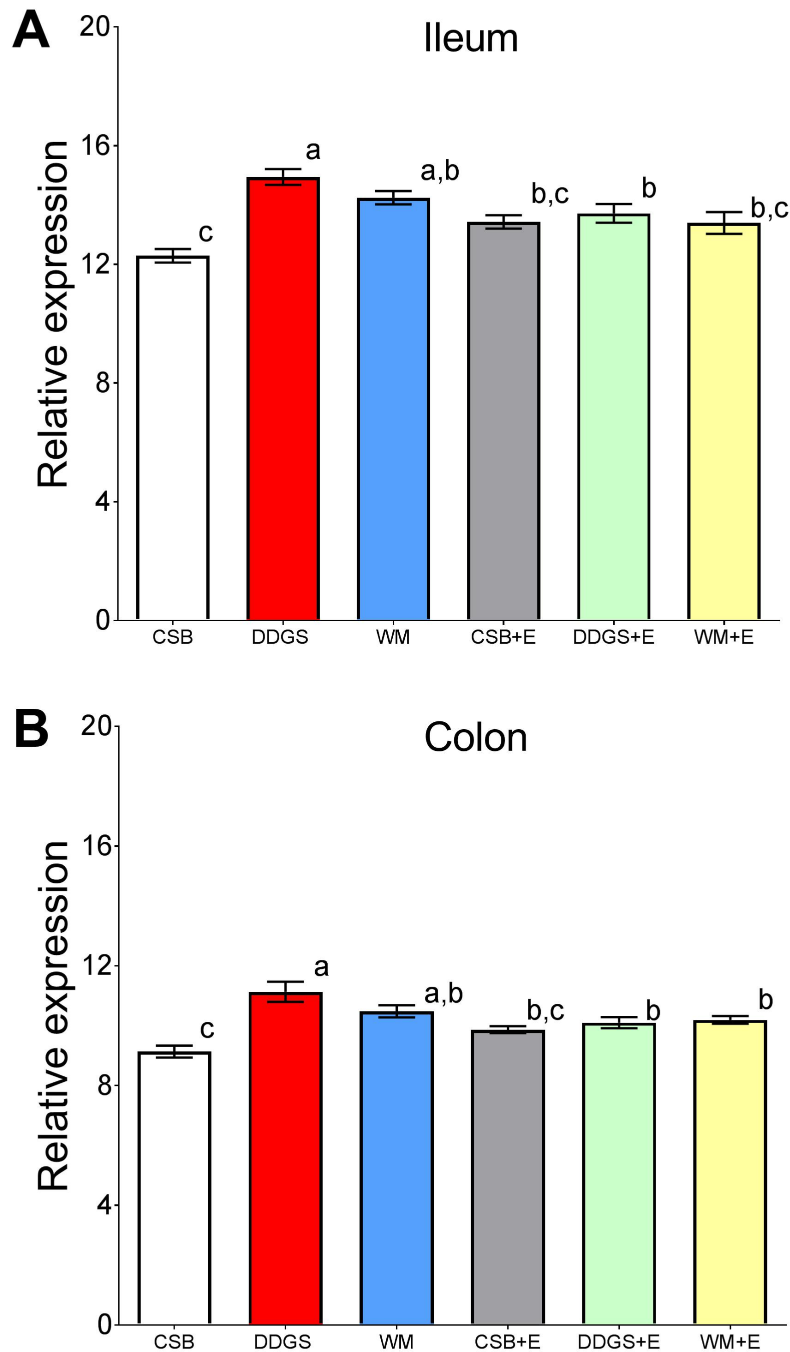

Figure 3.1. *MUC2* expression (relative to *GAPDH*, *HPRT* and *18s*) in ileum (A) and colon (B) of pigs fed high fiber diets with or without carbohydrase supplementation for 28 days. Bars represent S.D. Different superscripts between groups differ ($P > 0.05$).

Table 3.6. Relative gene expression of different phyla in ileum and colon of pigs fed high-fiber diets[1, 2].

	Fiber source						
	CSB	DDGS	WM	CSB+E	DDGS+E	WM+E	*P*-value
Ileum							
Firmicutes	0.023±0.003[a,b]	0.021±0.001[a,b]	0.024±0.001[a,b]	0.022±0.001[a,b]	0.017±0.001[b]	0.028±0.006[a]	0.0321
Bacteroidetes	0.01±0.002	0.013±0.006	0.012±0.001	0.009±0.001	0.008±0.001	0.0012±0.002	0.23
Actinobacteria	0.007±0.001[a]	0.004±0.008[b,c]	0.006±0.005[a,c]	0.005±0.001[a,b]	0.003±0.004[b]	0.006±0.008[a,c]	0.0011
Proteobacteria	0.006±0.001	0.004±0.009	0.006±0.005	0.005±0.008	0.003±0.001	0.005±0.001	0.0514
Spirochaetes	-	-	-	-	-	-	-
Colon							
Firmicutes	0.094±0.01	0.092±0.03	0.097±0.01	0.099±0.01	0.075±0.01	0.1±0.01	0.58
Bacteroidetes	0.068±0.004[a]	0.059±0.01[a,b]	0.066±0.005[a]	0.067±0.005[a]	0.054±0.005[b]	0.065±0.002[a,b]	0.15
Actinobacteria	0.016±0.001[a]	0.011±0.002[a,b]	0.014±0.002[a,b]	0.016±0.002[a]	0.01±0.001[b]	0.015±0.002[a]	0.007
Proteobacteria	0.004±0.001[b,c]	0.003±0.001[b]	0.006±0.001[a,c]	0.007±0.001[a]	0.003±0.001[b]	0.005±0.001[b,c]	0.003
Spirochaetes	0.004±0.002	0.004±0.003	0.004±0.001	0.004±0.001	0.004±0.001	0.003±0.001	0.96

[1]Relative expression data are means ± S.D. (n = 3/group). Different superscripts within each row differ ($P < 0.05$).
[2]Values are expressed as a relative ratio of the amount of target gene copies to the amount of *Total bacterial 16s* (housekeeping gene) copies.

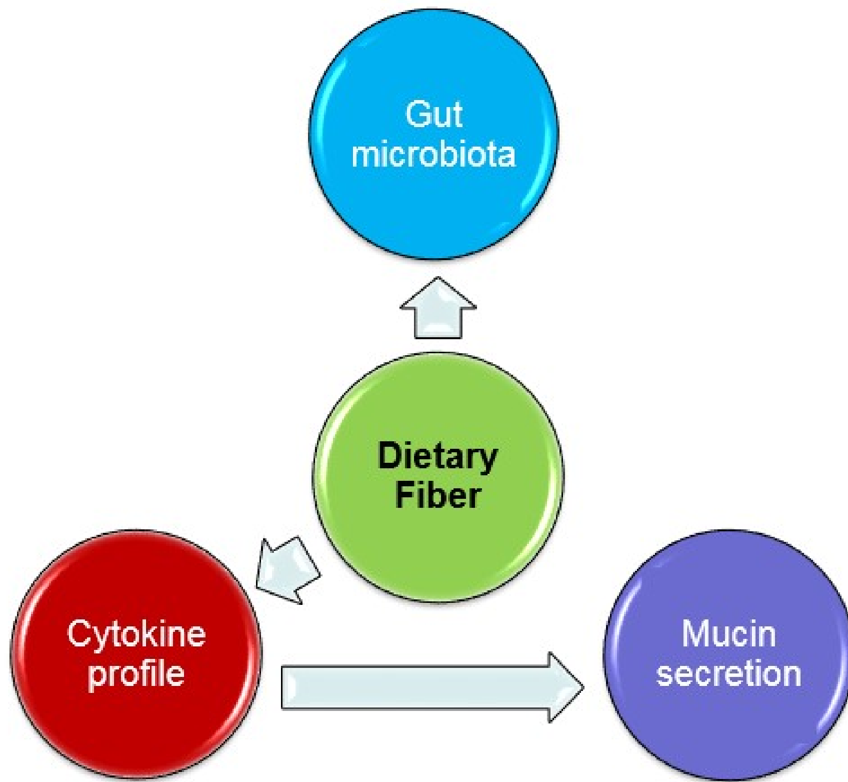

Figure 3.2. Schematic representation of the effects observed in this study.

CHAPTER 4:

CYTOKINE TREATMENT MODIFIES MUCIN EXPRESSION IN SMALL INTESTINAL ORGANOID (ENTEROID) MODEL

SUMMARY

High fiber diets can increase the expression of mucins, i.e. *Muc2*, and modify the immune profile in the gastrointestinal tract. In our previous studies, we have shown that different fiber sources uniquely modify the cytokine profile in the ileum and colon of pigs. We then hypothesized that cytokines in the intestine drive the increase in *Muc2* expression observed under high fiber diets. An *in vitro* enteroid model was used to test the effects of four interleukins we previously identified as elevated in the ileum of pigs fed high fiber diet, on *Muc2* expression. Murine enteroids (n = 10 – 12 / well) were stimulated with 1 ng/ml of IL-1β, IL-4, IL-11 or IL-17A for 48 hours. Total RNA from enteroids was isolated and 500 ng used for cDNA synthesis. Gene expression was determined by qPCR and the ΔCt of *Muc2* was calculated in reference to *Hprt* and *Gapdh*, considering primer efficiency. Differences were determined using ANOVA and followed by Tukey's test with correction for multiple comparisons and a $P < 0.05$ was considered significant. We found no differences in *Muc2* expression of enteroids treated with IL-1β, IL-17A compared with the controls. IL-4 and IL-11 treatments increased *Muc2* expression when compared with the controls. Our study provides evidence that can explain, at least partially, the increase in *Muc2* observed in the intestine of animals fed high fiber diets.

INTRODUCTION

The epithelium of the small intestine is covered by a mucus layer composed mainly of mucin glycoproteins that are synthesized and secreted by goblet cells (Frankel et al. 1995). This layer has a barrier function between the luminal contents and the absorptive system of the intestine, protecting the epithelium from luminal insults (Forstner & Forstner 1994). Changes in the properties of this barrier may affect the absorption of both dietary nutrients and endogenous macromolecules and ions in the small intestine (Dryden et al. 1985; Quarterman 1987), as well as can have an impact on the immune response in this location since the mucus layer also plays a protective role by maintaining a physical separation between luminal bacteria and epithelial cells (Frankel et al. 1995)

Our previous published studies (Saqui-Salces et al. 2017), as well as studies presented earlier in this document (Chapter 3), showed that ingredients with high insoluble fiber content commonly used for formulating swine feeds such as distiller's grains with solubles (DDGS), soybean hulls (SBH), wheat straw (WS) and wheat middlings (WM) increase *MUC2* gene expression in the small and large intestine of pigs after 14 or 28 days of diet exposure. However, when analyzing the expression of genes related to Notch and Wnt signaling, that regulate cell differentiation and proliferation in the intestine, we no able to identify a strong driver for goblet cell differentiation (Saqui-Salces et al. 2017), suggesting that other signals may participate in mucin induction. Other groups have showed that certain cytokines, like IL-4 and IL-13, have the ability to modulate mucin secretion (Blanchard et al. 2004; Iwashita et al. 2003), suggesting that cytokines in the intestine may participate in this process. .

Enteroids are 3D structures grown from isolated intestinal crypts that mimic the intestinal epithelial layer (Sato et al. 2009). Enteroids contain all epithelial cell types found in the normal intestinal epithelium including stem cells, enterocytes, and Paneth, goblet and enteroendocrine cells, and they demonstrate many of the biological and physiological properties of the small intestinal epithelium, such as mucin and hormone secretion as well as nutrient absorption and ion transport (Saxena et al. 2016). Although hold by extracellular matrix, the enteroid culture system lacks of other cell types normally present in intestine that crosstalk with the epithelium, such as myofibroblasts, enteric nerves, endothelial, and immune cells (Fatehullah et al. 2016; Saxena et al. 2016). The lack of stromal components has the advantage of allowing the study of epithelial responses mediated by specific factors, with the limitation of missing the regulatory cross-talk between the mesenchyme and epithelium occurring in the intestine.

MATERIALS AND METHODS

Animals

Male and female C57BL/6J were used to generate enteroids. Animals were maintained at the University of Minnesota animal facilities and housed under standard conditions. Mice were used at 5-10 weeks of age. The animal use protocol was reviewed and approved by the University of Minnesota Institution Animal Care and Use Committee. No. 1606-33871A

Crypt isolation and enteroid culture

After euthanasia, the mice abdomen was open and the small intestine gently pulled out of the abdominal cavity. A 1 cm piece of small intestine was then excised and kept in ice-cold advanced Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) with 1% antibiotic-antimycotic (final concentration: 100 units/mL of penicillin, 100 µg/mL of streptomycin, and 0.25 µg/mL of Amphotericin B). After collection, intestinal samples were opened longitudinally and washed in ice-cold phosphate-buffered saline (PBS) with 1% antibiotic-antimycotic. Tissues were then incubated in dissociating solution containing 30 mM ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT) and penicillin-streptomycin (100 units/mL of penicillin, 100 µg/mL of streptomycin) in Hank's Balanced Salt Solution (HBSS) for 5 - 10 minutes at 37 °C and vigorously shaken every 2 minutes. The tissue was transferred to a Petri dish with 1 ml of the dissociating solution and the epithelium pulled out by suction using a transfer pipette. The crypt suspension was collected in a 12 ml tube and DMEM supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic immediately added. The tube was then centrifuged at 150 x g for 4 minutes at room temperature. The supernatant was removed and the pellet resuspended in 1 ml of DMEM supplemented with 10% FBS and 1% Antibiotic-Antimycotic, transferred to a 1.7 ml Eppendorf tube and centrifuged at 150 x g for 4 minutes. After centrifugation, supernatant was removed and the pellet re-suspended in 1 ml of mouse enteroid media (Table 4.1). Fourty microliters of the suspension were mixed with 120 µl of Matrigel and approximately 40 µl of the mixture plated in the center of a well in a new 24 well plate. The plate was then placed in a 37°C,

5% CO_2 incubator for 10 minutes to allow for Matrigel polymerization. After 10 minutes, 500 µl of mouse enteroid media was added to each well, covering the Matrigel.

Approximately every 48 hours, the culture medium was replaced. Formulation of enteroids media can be found in Table 4.2. The enteroids were split and passaged every 7 days. For this process, the medium from all wells was removed together with the Matrigel and the organoids and pipetted into a 2 ml tube. Tubes were then centrifuged at 150 x g for 4 minutes. After centrifugation, the medium and the old Matrigel were pipetted out and discarded without disturbing the enteroid pellet. Enteroids were re-suspended in 1 ml of mouse enteroid media and passed 1 – 2 times through a 27½-G needle to dissociate. Fourty microliters of the suspension were mixed with 120 µl of Matrigel and approximately 40 µl of the mixture plated in the center of a well in a new 24 well plate. The plate was then placed in a 37°C, 5% CO_2 incubator for 10 minutes to allow for Matrigel polymerization. After 10 minutes, 500 µl of mouse enteroid media was added to each well, covering the Matrigel.

In order to obtain between 10 and 12 enteroids per well for treatment experiments, the amount of enteroids found in 100 µl of media was assessed with a microscope and the preparation was further diluted to obtain an approximate number of 50 organoids per each 50 µl of suspension before proceeding with plating.

Cytokine treatment

Recombinant mouse IL-1β, IL-4, IL-11 and IL-17A were purchased from PeproTech (Rocky Hill, NJ) and diluted in sterile water containing 0.1 % BSA as protein carrier

Establishment of treatment conditions

In order to determine optimal treatment time and concentration, a preliminary experiment was performed. An average of 10 enteroids was plated in each of 15 wells of a 24 well plate.Enteroids were stimulated with 500 µl of enteroid media containing either IL-1β, IL-4, IL-11 or IL-17A at 1 ng/ml, 10 ng/ml or 100 ng/ml. Three wells were stimulated with 500 µl of enteroid media containing the same amount of vehicle (PBS with 0.1% Bovine Serum Albumin) as control wells. Media was changed every 24 h and plates were kept in an incubator at 37°C, 5% CO_2. Three separated plates were used in total: the first plate was collected 24 h after cytokine treatment, the second at 48 h and the third at 72 h.

Cytokine treatment

An average of 10 enteroids were plated in each of 30 wells within a 48 well plate. Enteroids were stimulated with 300 µl of enteroid media containing each cytokine at 1 ng/ml, in triplicate.. Plates were kept in an incubator at 37°C, 5% CO_2. Media was changed every 24 h and after 48 h organoids were collected. The experiment was repeated independently three times and the means of each experimental triplicates used for the final data analisis.

Gene Expression

Total RNA from the enteroids was isolated and purified using the RNeasy Plus Universal Micro Kit (Qiagen, Valencia, CA). Enteroids were collected in the Matrigel patty together with the media and transferred to a 2 ml conical tube. After spinning down at 300 x g for 4 minutes at room temperature, the media and Matrigel were removed without disturbing the enteroid pellet. TRIzol reagent (350 µl) was added to the Eppendorf tube

and tissue disrupted mechanically by passing through a 27½-G needle. The tube was left to rest at room temperature for 5 minutes and 70 µl of chloroform were added to the tube. After vigorously shaking for 15 seconds, the tube was left to sit at room temperature for 3 minutes and then centrifuged at 12,000 x g for 15 minutes at 4°C. After centrifugation, the upper aqueous phase was transferred into a new tube without carrying the interphase, and 175 µl of isopropanol were added. The purification was performed following the kit manufacturer's instructions.

Total RNA was quantified using a NanoDrop 2000 instrument (Thermo Scientific, Wilmington, DE), and 500 ng of RNA were reverse transcribed using the High Capacity cDNA reverse Transcription Kit (Applied Biosystems, Foster City, CA). Gene expression for *Mucin 2* (*Muc2*), *glyceraldehyde 3-phosphate dehydrogenase* (*Gapdh*) and *hypoxanthine-guanine phosphoribosyltransferase* (*Hprt*) was determined using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) in a Quantum Studio 3 system (Applied Biosystems, Foster City, CA). PCR conditions were: initial activation at 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 sec denaturation and annealing at 60ºC for 60 secs. The primer sequences used are listed in Table 4.3.

Data Analysis

Relative gene expression was calculated using the primer efficiency values as described by Pfaffl (2001), Ct values > 38 were considered non-detectable. Housekeeping genes *Gapdh* and *Hprt* were used as reference genes. Data analyzed were the result of three independent experiments performed with three replicates each. All data were tested for normality using D'Agostino and Pearson tests. Relative gene expression levels were

analyzed using ANOVA followed by Tukey's multiple comparisons test using Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA) software.

RESULTS

Definition of treatment conditions

Our preliminary data showed no significant differences in *Muc2* expression when comparing cytokine dosages between 1 ng/ml, 10 ng/ml or 100 ng/ml (Figure 4.1). Relative expression of *Muc2* induced by IL-4 was the only one affected by time. The organoids treated with 1 ng/ml of IL-4 for 72 h showed decreased expression of *Muc2* when compared to the ones treated for 24 h and 48 h. Those organoids that were treated with 10 ng/ml of IL-4 for 72 h showed decreased *Muc2* compared to the 24 h organoids and the 48 h organoids. Finally, the organoids treated with 100 ng/ml of IL-4 for 72 h also showed lesser expression of *Muc2* when compared to the ones treated for 24 h and 48 h. No statistics were performed for this data since for this preliminary trial each of the treatments had an n = 1.

After this preliminary results, and since no differences among doses were found, we decided to proceed with 1 ng/ml cytokine treatment for all the different cytokines. Since the effect to be evaluated was a *Muc2* response, and not seeing differences in *Muc2* expression between 24 and 48 h cultures, a 48 h culture was chosen for the final experiment to allow enteroids to grow large enough as to perform histological analysis (not shown).

Effects of different cytokines over Muc2 expression

There were no differences in *Muc2* expression between treatments with IL-1β, IL-17A and the controls (Figure 4.2). IL-4 treated enteroids showed increased expression of *Muc2* when compared to the controls ($P < 0.0001$), and treated with IL-1β ($P = 0.0002$), IL-11 ($P = 0.0141$) and IL-17A ($P = 0.0005$). IL-11 also induced expression of *Muc2* when compared to the control organoids ($P = 0.0064$) and treated with IL-1β ($P = 0.0366$).

DISCUSSION

As shown in Chapters 2 and 3 of this document, high fiber diets had an effect on intestinal cytokine profile: in ileum, high fiber diets increased expression of Th2 cytokines *IL-4* and *IL-25* while in colon, high fiber diets lowered levels of *IFNγ* and increased levels of *IL-11* indicating a potential anti-inflammatory effect. These observations are in accordance with previously published data, where diets supplemented with different types of fiber had an impact on the cytokine profile in the intestine (Weber et al. 2008; Pié et al. 2007; Iwashita et al. 2003; Blanchard et al. 2004). It was our hypothesis that the cytokines induced by high fiber diets could increase mucin production. We used enteroids because as a three dimensional (3D) cell culture model that mimics the original tissue structure, enteroids display all the differentiated epithelial cell types that would be found within the intestine in physiological conditions and show a structure very similar to the normal intestinal epithelium (Sato et al. 2009), allowing for an analysis of the epithelial response independent of other signals present in the organ.

We exposed enteroids to IL-1β, IL-4, IL-11 and IL-17A, thatwere the cytokines which expressions were modified in ileal tissue in our second *in vivo* experiment (Chapter 3). Our observations in this study are in concordance to previously published data were IL-4 was found to increase *Muc2* expression *in vitro*, in cultured airway epithelial cells and *in vivo*, in mice airway epithelial cells (Dabbagh et. al. 1999; Iwashita et al. 2003). To our knowledge, no effects of IL-11 over mucin expression have been reported so far. Our results are of significance since this knowledge can be used in the future to modify (either enhance or diminish) mucin expression in the intestine by altering the cytokine profile. Mucin expression modifications can be useful in certain situations such as intestinal infections with intracellular bacteria or parasites, where enhancing mucin secretion could be protective.

The limitations of the mouse enteroid model should be considered. We used murine enteroids , and not swine, system to model the changes observed in the *in vivo* swine models (Chapter 2 and 3). There are several reasons to use the murine over the swine model. The main reason being that swine enteroids are not long-lived as mouse enteroids, this fact limits their utilization for since it is still technically difficult to define the swine enteroids health and whether changes observed are due to enteroids frailty or the treatment applied. Swine enteroids seem to be more sensitive than mouse enteroids to manipulation, and change their normal behavior even with minor environmental modifications, and regular manipulation, as passaging. Another important reason for using the mouse model is the availability of reagents and resources, which is somewhat limited when working with swine. There are known species differences on immune responses between mice and pigs, but not clear ones at the level of the intestinal

epithelium. Nevertheless, performing the experiment with a swine enteroid model would be ideal in order to verify the results obtained and validate the use of mouse enteroids as models for swine intestinal epithelium.

In our *in vivo* experiments, the expression of the anti-inflammatory cytokine IL-4 was increased in animals fed a high fiber DDGS diet. The major function of IL-4 is to induce differentiation of naïve T cells into Th2 cells. In addition to reduce inflammation, our *in vitro* experiment showed that IL-4 can also induce *Muc2* gene expression. Similar findings were also observed with IL-11. Further studies are required to identify the mechanism of how IL-4 and IL-11 modify mucin gene expression and their role in nutrient availability reduction seeing in high fiber diets.

CONCLUSION

In conclusion, changes in intestinal mucin secretion seen in animals fed high fiber diets can be driven by cytokines induced by diet.

TABLES AND FIGURES

Table 4.1. Reagents used for mouse intestinal crypt isolation.

Reagent name	Supplier	Cat. No.	Stock conc.	Final conc.
Advanced DMEM/F12	Gibco	12634-010	1x	-
Antibiotic-Antimycotic	Gibco	15240-062	10,000 μg/ml	10 μg/ml
HBSS	Gibco	14175-095	1x	-
EDTA	Promega	V4231	0.5 M	30 mM
DPBS	Gibco	14190-144	1x	-
DTT	-	-	1 M	1 mM
Pen/Strep	Gibco	15140-122	10,000 μg/ml	100 μg/ml
FBS	Invitrogen	16140063	1x	10%
Matrigel	Cornig	354234	-	-

DMEM – Dulbecco's Modified Eagle Medium, HBSS - Hank's Balanced Salt Solution, EDTA – ethylenediaminetetraacetic acid, DPBS – Dulbecco's phosphate buffered saline, DTT – dithiothreitol, Pen/Strep - Penicillin-Streptomycin, FBS – Fetal Bovine Serum.

Table 4.2. Mouse enteroid culture media formulation

Reagent name	Supplier	Cat. No.	Stock conc.	Final conc.
Stock Media				
Advanced DMEM/F12	Gibco	12634-010	1x	-
R-spondin conditioned media	-	-	-	-
HEPES, 1M	Gibco	15630-080	1 M	10 Mm
GlutaMAX	Gibco	35050-061	100x	1x
Pen/Strep	Gibco	15140-122	10,000 µg/ml	10 µg/ml
N2 Supplement	Gibco	17502-048	100x	1x
B27 Supplement	Gibco	17504-044	50 x	1x
Growth factors and hormones				
EGF	Sigma-Aldrich	E9644-2MG	2 mg	50 ng/ml
Noggin	R&D	6057-NG/CF	25 µg	100 ng/ml

DMEM – Dulbecco's Modified Eagle Medium, HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, Pen/Strep - Penicillin-Streptomycin, EGF - Epidermal growth factor

1

Table 4.3. Sequences of primers used for mouse enteroids.

Gene	Forward Sequence	Reverse Sequence	
Hprt	AGGACCTCTCGAAGTGTTGGATAC	AACTTGCGCTCATCTTAGGCTTTG	(Saqui-Salces et al. 2012)
Gapdh	TCAAGAAGGTGGTGAAGCAGG	TATTATGGGGGTCTGGGATGG	(Waghray et al. 2010)
Muc2	AGAACGATGCCTACACCAAG	CATTGAAGTCCCCGCAGAG	(Waghray et al. 2010)

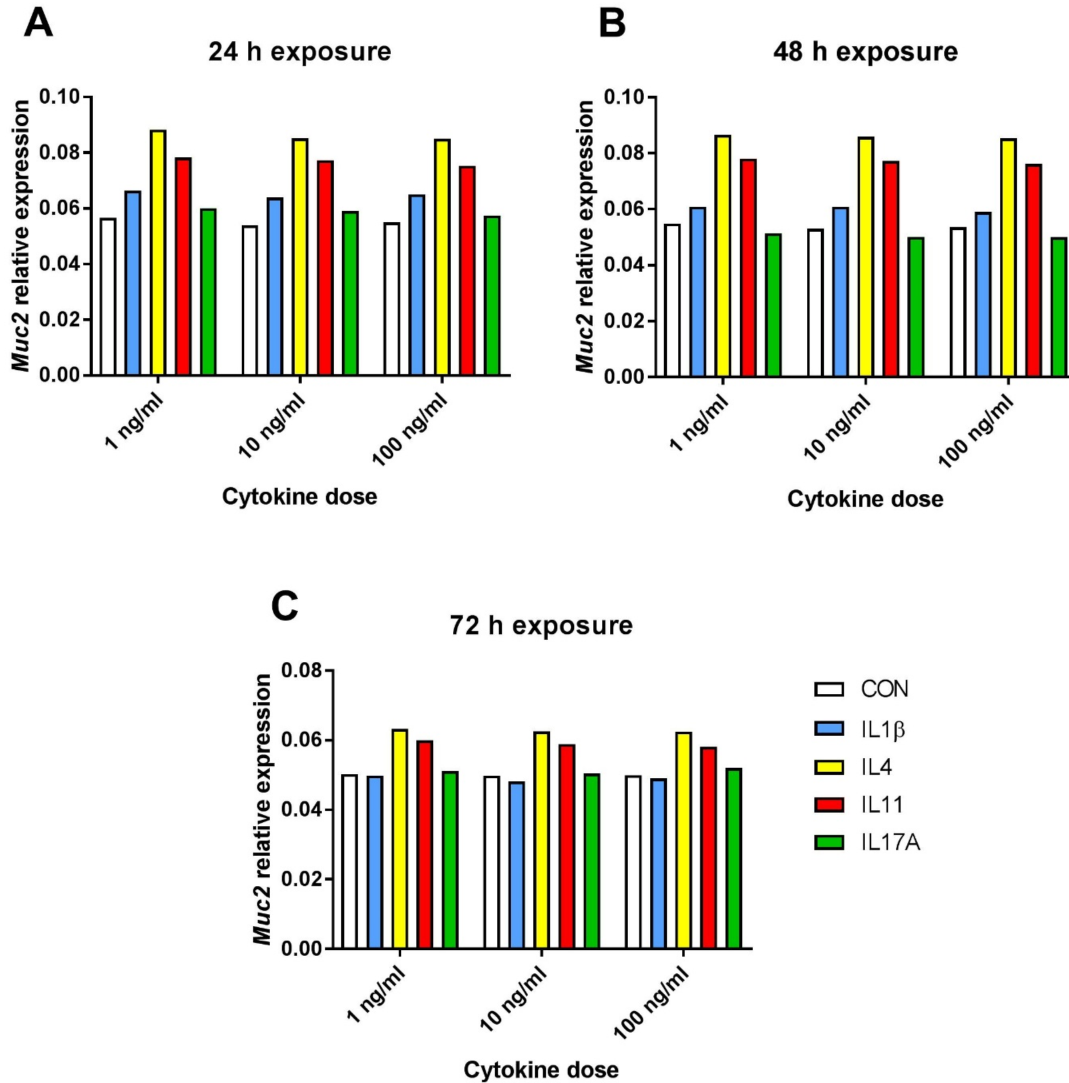

Figure 4.1. *Muc2* expression in mouse enteroids treated with3 different concentrations (1 ng/ml, 10 ng/ml and 100 ng/ml) of IL-1β, IL-4, IL-11 and IL-17A for 24 h (A), 48 h (B) or 72 h (C). Bars represent individual measurements. No statistic tests were performed for these samples.

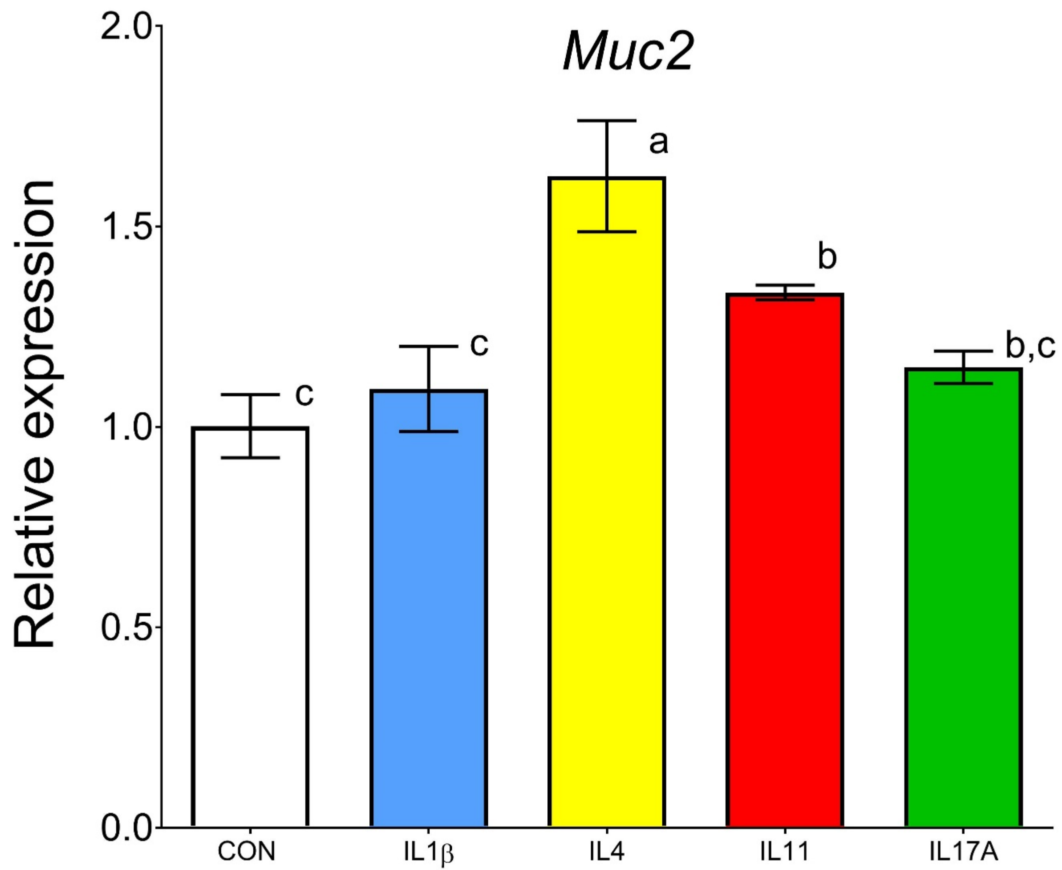

Figure 4.2. *Muc2* expression (relative to *Gapdh* and *Hprt*) in mouse enteroids after treatment with IL-1β, IL-4, IL-11 and IL-17A (1 ng/ml) during 48 h. Bars represent means ± S.D. Different superscripts between groups differ ($P > 0.05$).

CHAPTER 5:

DISCUSSION

Overall summary

Dietary fiber is a major component of a healthy and balanced diet with the capacity to modify the immune system at a GI level. The most accepted theory on how fiber modulates the immune system is that different sources and amounts of fiber will differentially affect the host's microbiome (Kuo 2013). These prebiotic effects are probably due to interactions with the gut micro-environment and GALT (Lindberg 2014), since non hydrolyzed or absorbed fiber by the upper GI is a good substrate for certain bacteria when it reaches the colon (McRorie et al. 1998; Kanauchi et al. 2008). Most studies in the literature attribute the anti-inflammatory effects of fiber to changes on the intestinal microbiota (De Vrese & Schrezenmeir 2008), however very little studies have considered that DF itself, because of its physical properties, may be modulating directly the immune response instead of doing it through modifying the microbiota. In our preliminary exploration of microbiota composition in the intestine of pigs high fiber diets, we did not observe changes that would support that DF is modulating the microbiome in a significant way. However, we approach used is limited and no conclusions can be made from our results in terms of microbiome composition.

Results in chapter 2 of this study suggest that dietary fiber can modify the swine cytokine profile at a systemic level as well as at a local level. In the experiment presented, DDGS decreased IL-10/Il-12 ratio at the systemic level when compared with the control animals, indicating an anti-inflammatory response potentially attributed to the fiber content in the diet. Also, we observed that SBH was the fiber that induced a more strong anti-inflammatory profile by lowering local gene expression of *TNFα* and *IL-8* and increasing

expression of *IL-10*, indicating that, in the ileum, fermentable fibers have the capacity to promote anti-inflammatory responses.

Chapter 3 results showed that in ileal tissue, high fiber diets favored an anti-inflammatory profile by increasing expression of IL-4 and IL-25, but adding enzymes to the diets shifted the response to pro-inflammatory by increasing IL-1β and decreasing IL-11 expressions. . In the colon, even though *TNFα* expression was greater in non-enzyme supplemented diets compared to the supplemented ones, the low levels of *IFNγ* and greater levels of *IL-11* in the non-enzyme supplemented diets also indicate a potential anti-inflammatory effect.

Results from Chapter 4 suggest that, changes in mucin secretion seen in animals fed high fiber diets in Chapters 2 and 3 may be driven, at least in part, by cytokines induced by diet the intestine. In our *in vivo* experiments, the expression of the anti-inflammatory cytokine IL-4 was increased in animals fed a high fiber DDGS diet. In our *in vitro* experiment IL-4 showed that it can induce *Muc2* gene expression. Further studies are required to identify the mechanism of how IL-4 modifies mucin gene expression..

The overall conclusion of this project is that DF manipulations can be used to modify the intestinal cytokine profile in order to enhance or inhibit certain immune responses as well as modify mucin secretion, all without triggering major microbial changes in ileum and colon of pigs.

Limitations of the study

Some limitations of this study should be highlighted. First of all, animals in Chapter 2 and Chapter 3 were not fed the same types of fiber or concentrations and treatment duration was also different, therefore, when testing the same cytokines that were

modified by the diet from Chapter 2 in the animals from Chapter 3, we may be biasing our results by overlooking other cytokines that may be affected by the diets used in Chapter 3 but not in Chapter 2. This could have been avoided by running an ELISA kit for profiling key genes mediating inflammatory response, to get an overview of which genes are being more affected.

In Chapter 3, for measuring bacterial composition, intestinal content was used. This may also be limiting our findings by missing those bacteria that reside in the inner mucus layer or attached to the epithelium of the intestine. By profiling just microbiota in the content, we may be overlooking changes in composition happening at a deeper level and that may be more significant for the host. A way to overcome this problem is using tissue samples to extract bacterial DNA instead of intestinal content, since those samples also include the inner mucus layer and all the bacteria on it. We, unfortunately, did not have those samples for microbiome analysis.

For testing if the local immune response induced by diet can affect mucin production in Chapter 4, mouse enteroids were used as a model. This also may be introducing a bias in our results since the model was derived from different species and the mechanisms driving mucin secretion in mice may not be the same that the ones that drive it in swine. A pig enteroid model is already being studied in our lab in order to prove our results with a same specie model.

Future directions

A pig enteroid model is currently being developed in our lab. This new model will be used in order to prove that these cytokines have the same effect in pigs as that

observed in mice enteroids (see Chapter 4). We will also be able to test if direct exposure to fibers can trigger changes in the cytokine expression profiles *in vitro*. For such studies, the enteroids can be microinjected with purified fibers (absent of microbial interactions) to test their capacity to modulate the profile of secreted cytokines within these enteroids. In addition, co-culturing with immune cells is also possible for a better simulation of the localized intestinal microenvironment. Another future direction in order to test the anti-inflammatory properties of DF would be to challenge pigs while feeding them with a high fiber diet in order to asses any protective property that the fiber could have at an immune level.

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