Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

SCIENZE ZOOTECNICHE

Ciclo XXIV

Settore Concorsuale di afferenza: AGR 18

TITOLO TESI

IMPORTANZA DELLA DIETA SULL'ATTIVAZIONE E SULLA MATURAZIONE DEL CONTROLLO ALIMENTARE E DELLA BARRIERA GASTROINTESTINALE NEL SUINETTO

DIET EFFECTS ON ACTIVATION AND MATURATION OF FEED CONTROL OVER THE GASTROINTESTINAL DEFENCE BARRIER IN PIGLETS

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Esame finale anno 2012

ABSTRACT

Weaning is an important and complex step involving many stresses that interfere deeply with feed intake, gastro-intestinal tract (GIT) development and adaptation to the weaning diet in young pigs. The health of the pig at weaning, its nutrition in the immediate post-weaning period, and the physical, microbiological and psychological environment are all factors that interact to determine food intake and subsequent growth. GIT disorders, infections and diarrhoea increase at the time of weaning, in fact pathogens such as enterotoxigenic *Escherichia coli* (ETEC) are major causes of mucosal damage in post-weaning disease contributing to diarrhoea in suckling and post-weaned pigs. The European ban in 2006 put on antibiotic growth promoters (AGP) has stimulated research on the mechanisms of GIT disorders and on nutritional approaches for preventing or reducing such disturbances avoiding AGPs. Concerning these aspects here are presented five studies based on the interplay among nutrition, genomic, immunity and physiology with the aim to clarify some of these problematic issues around weaning period in piglets. The first three evaluate the effects of diets threonine or tryptophan enriched on gut defence and health as possible alternatives to AGP in the gut. The fourth is focused on the possible immunological function related with the development of the stomach. The fifth is a pilot study on the gastric sensing and orexygenic signal given by fasting or re-feeding conditions. Although some results are controversial, it appears that both tryptophan and threonine supplementation in weaning diets have a preventive role in E.coli PWD and favorable effects in the gut especially in relation to ETEC susceptible genotype. While the stomach is believed as almost aseptic organ, it shows an immune activity related with the mucosal maturation. Moreover it shows an orexygenic role of both oxyntic mucosa and pyloric mucosa, and its possible relation with nutrient sensing stimuli.

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SUMMARY and BACKGROUND

Weaning is a complex step involving dietary, environmental, social and psychological stresses which interfere deeply with feed intake, GIT development and adaptation to the weaning diet (reviews by Pluske et al, 1997; Lallès et al. 2004).

In the perinatal period the maturational program of the intestinal epithelium is influenced by a complex interplay of local, systemic and luminal factors (Trahair and Sangild, 1997).

The health of the pig at weaning , its nutrition in the immediate post-weaning period, and the physical, microbiological and psychological environment are all factors that interact to determine food intake and subsequent growth.

The influences of dietary change on gastrointestinal mucosa response and development are especially marked at weaning. During the same period, the rapidly changing mucosal surface becomes colonized by successions of gut bacterial groups. The dynamic balance between host physiology, diet and the gastrointestinal microbiota leads to the establishment of a stable microbial ecology characterized by the presence of commensal organisms that exert a positive influence in maintaining and establishing a healthy gut immune system.

However, perturbation of the gut ecosystem can often occur around weaning period and still represents the time of greatest pig morbidity and mortality often associated with reduction of feed intake and post weaning diarrhea (PWD). An important predisposing factor to enteric infections is the prevalence of binding sites for pathogens on the intestinal surfaces of the suckling pig. Pathogens such as enterotoxigenic *Escherichia coli* K88 are major causes of PWD and mortality bind to specific intestinal mucins controlled by pig genome.

The reduction in feed intake, growth rate, PWD and mortality that occurs following weaning is of major economic consequence to the pig industry. In the past a range of antimicrobial growth promoters (AGPs) was utilized in common growth practice but a worldwide concern about development of antimicrobial resistance and about transference of antibiotic resistance genes from animal to human microbiota led to banning the use of antibiotics as growth promoters in the European Union since January 1, 2006.

Thus, there is the need to look for viable alternatives that could enhance the natural defence mechanisms of animals and reduce the massive use of antibiotics.

One way is to use specific feed additives or dietary raw materials to favorably affect animal performance and welfare, particularly through the direct stimulation of animal immune system or indirectly modulation of the gut microbiota which plays a critical role in maintaining host health. A balanced gut microbiota constitutes an efficient barrier against pathogen colonization, produces metabolic substrates (e.g. vitamins and short-chain fatty acids) and stimulates the immune system in a non-inflammatory manner.

Investigations of the interactions between pre- and post-weaning nutrition, gut physiology and immunology and their relevance to body functions and health are fundamental to solving the problems of dietary change and post-weaning performance. The influences of dietary change on intestinal epithelial differentiation and growth are especially marked at birth and weaning.

The most important sensor and effector role in all these responses is the GIT which acts as complex interface between the animal and its environment. It has a remarkable capacity to respond and rapidly adapt to diverse array of endogenous and exogenous stimuli. Vital to this adaptive capacity is a complex mucosa-epithelial surface, which is continually undergoing regeneration and differentiation. The GIT plays a major role in the defence against harmful antigen and pathogen entry into the body thanks to a complex barrier. It includes the secretion of fluid, minerals, mucin and immunoglobulin (IgA). Permeability of the

intestinal epithelial cell monolayer is tightly regulated. Intestinal permeability and absorptive and secretory properties of the intestine are largely modulated by cellular and molecular components of immunity, e.g. mast cells and cytokines.
The growth, development and intrinsic differentiation of the digestive tract in neonatal pigs are profoundly influenced through interaction with dietary constituents and the flora.

After an introduction on the main topics considered, here are presented five studies in different chapters with the aim to clarify some of these aspects.
Briefly, the first one proposes to investigate the effects of a threonine enriched diet towards E.coli K88 challenge and the resulting gut effects on host defense parameters like IgA, mucins, villuos changes.
The second study proposes to investigate the effects of a tryptophan enriched diet towards E.coli K88 susceptible genotype and the resulting gut effects on defense related gene expression.
The third is related to the second as experimental design, so it proposes to investigate the effects of a tryptophan enriched diet toward E.coli K88 susceptible genotype but focusing on the microbiota changes.
The fourth proposes to investigate a hypothetical immunological function of the stomach in relation to different functional parts of the organ during the piglet development.
The fifth proposes to investigate the effects of fasting and re-feeding treatments towards ghrelin orexygenic signal and specific nutrient receptors probably related in the stomach mucosa.

THE GASTROINTESTINAL TRACT

The gastrointestinal system consists of the gastrointestinal tract (GIT)(oral cavity, oesophagus, stomach, small intestine, large intestine and rectum) and the associated glands (salivary glands, pancreas and liver) (Figure 1). The main function of the gastrointestinal system is to assimilate nutrients from the external environment into the animal's internal environment, where they are used for tissue growth and repair and for energy production. This function is carried out through coordinated activities of the entire gastrointestinal system, which include digestion, secretion, motility and absorption. Nutrients in most animal feeds exist as macromolecules, such as proteins and polysaccharides, which are unable to be absorbed across the gastrointestinal wall. These macromolecules need to be broken down into much smaller molecules before being absorbed. The digestion is a breakdown process accomplished through the actions of hydrochloric acid, bile and various digestive enzymes that are secreted by the associated organ glands and glands within the gastrointestinal wall. The motility processes facilitates the digestion and absorption by the contraction of the smooth muscles of the gastrointestinal tract, which mixes the luminal contents with the digestive fluids and moves the resulting digesta along the gastrointestinal tract.

The structure of different parts of the gastrointestinal system is highly adapted to their functions. For example, the stomach has a large lumen for food storage and a strong muscular wall for the mixing of food digesta; the small intestine has a very large luminal surface area facilitated by villus and microvillus projections and is capable of efficient nutrient absorption.

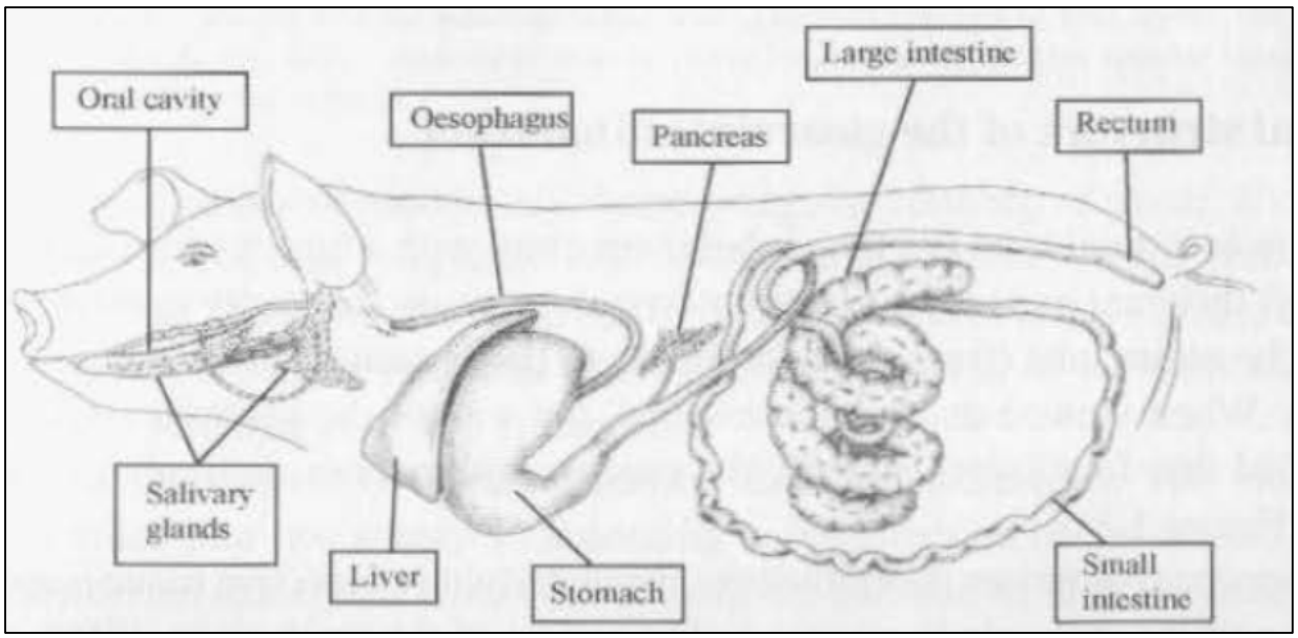

Figure 1. Overview of gastrointestinal system in the pig .

Although the gastrointestinal tract is a long tubular structure, different parts of the gastrointestinal tract vary markedly in morphology, the wall of the entire tube (from the oesophagus to the rectum) shares common structural features. When viewed under a microscope, the wall of the gastrointestinal tract can be divided into four layers, namely the mucosa, submucosa, muscularis extema and sierosa.

The mucosa comprises the epithelium, the underlying connective tissue named lamina propria, and the muscularis mucosae. The nature of the epithelium differs clearly along the gastrointestinal tract and is highly adapted to the function and content of the specific part of the tract. New epithelial cells that are derived from the basal region of the epithelium continuously replace the surface cells. The lamina propria consists of connective tissue and is rich in blood capillaries, lymph vessels, diffused leukocites and lymphatic nodules. The diffused leukocytes form an important defence mechanism against harmful luminal microorganisms and molecules that may penetrate through the epithelial lining. The muscularis mucosae consist of smooth muscle cells arranged in a circular and an outer longitudinal layer. Contractions of these smooth muscle cells stir the microenvironment close to the luminal epithelial surface and help local digestion and nutrient absorption.

The submucosa consists of moderately dense, irregular connective tissue. It contains numerous large blood and lymphatic vessels that send branches to the mucosa and muscularis externa The submucosa also contains nerve plexuses and lymphatic nodules. At certain parts of the gastrointestinal tract, the submucosal layer contains numerous exocrine glands with openings into the lumen. The muscularis externa consists of mainly smooth muscle cells. The muscularis externa is divided into two sub layers according to the direction of the smooth muscle cells. In the internal sub layer (close to the lumen), the orientation of the smooth muscle cells is generally circular, and in the external sub layer the orientation is mostly longitudinal.

The myoenteric nerve plexus lies between the two sublayers and controls the motility of the smooth muscles .The serosa is a serous membrane consisting of a single layer of simple squamous epithelial cells and underlying connective tissue. It is continuous with the mesentery, and supports the gastrointestinal tract in the abdominal cavity.

STOMACH

The stomach is a muscular and dilated organ responsible for storage, initiating the breakdown of nutrients, and passing the digesta into the small intestine. Within the stomach, food is mixed with the gastric juices and turned into a pulp-like mass called chyme or digesta. The digesta is then emptied into the small intestine in a controlled manner for further digestion and absorption. Thus, the function of the stomach is mechanical and chemical through the motility of the muscular layer and the secretion of the gastric glands. In addition, the stomach is also an important endocrine organ and secretes various peptide hormones with different functions.

WALL STRUCTURE

The wall of the stomach consists of the usual four layers, i.e., mucosa, submucosa, muscularis externa and serosa. The structure and the appearance of the mucosal layer vary markedly among different parts of the stomach. Accordingly, the stomach can be divided into four distinct zones, the pars oesophagea, the cardia, the fundus and the antrum (Figure 2). The pars oesophagea is a continuation of the oesophagus and its

luminal surface is covered with the stratified squamous epithelium. The luminal surface of the other parts of the stomach is covered by simple columnar epithelium. The columnar epithelium invaginates into the lamina propria, forming gastric pits with openings on the luminal surface. Simple columnar epithelial cells that cover the luminal surface and line the gastric pits secrete viscous mucus to protect the gastric lining from the acid and proteolytic enzymes. At the bottom of the gastric pits, there are openings of gastric glands that occupy most of the mucosal layer.

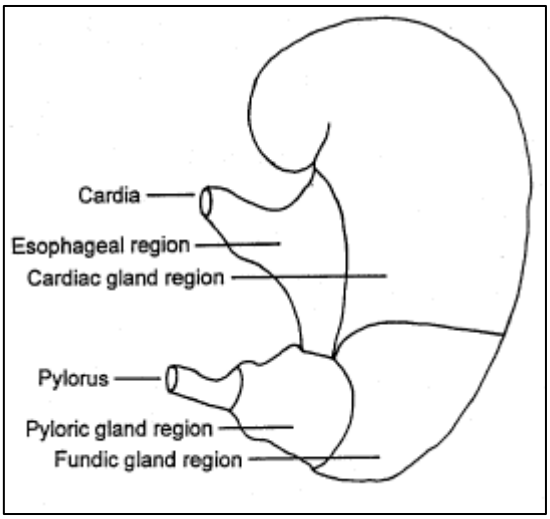

Figura 2. Different mucosal regions in pig stomach.

There are distinct differences in the glandular structures of different stomach zones (Figure 3). The cardiac glands in the cardiac zone are short tubular mucus producing glands and open into shallow, narrow gastric pits. In the fundic zone, the oxyntic glands are long tubular structures opening into wide gastric pits. The pyloric glands in the pyloric region are short tubular glands opening into deep gastric pits. The gastric glandular structures are well developed in piglets at the time of birth but cellular differentiation within the glands continues after birth (Xu et al., 1992b).

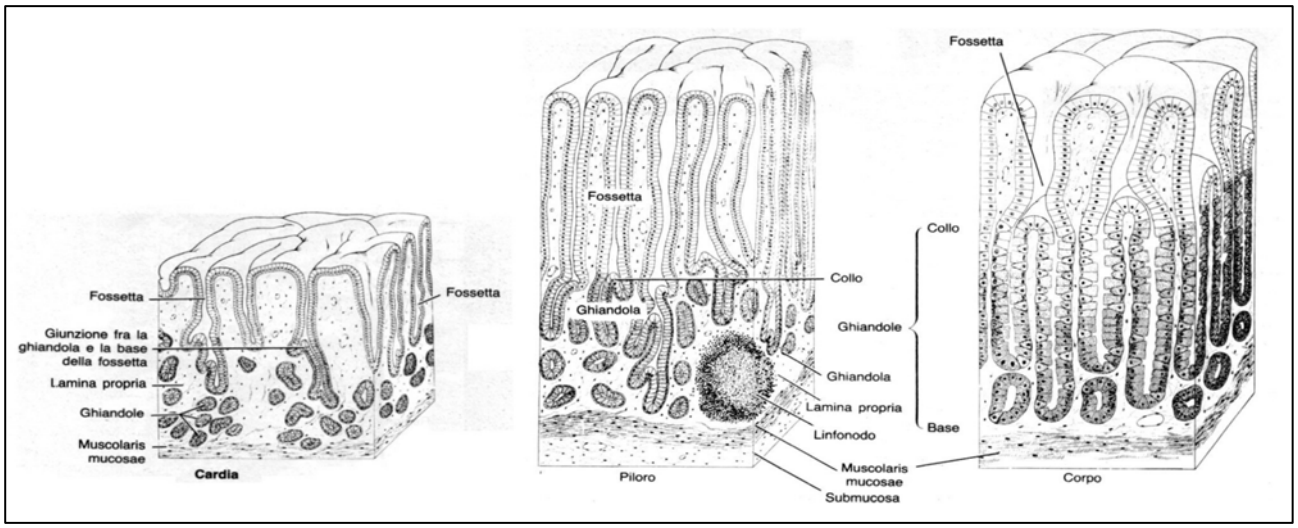

Figure 3. Different glandural stucture in different region of the stomach.

The surface epithelial cells are continuously desquamated and regeneration takes place at the deep part of the gastric pits and at the neck of the gastric glands by the process of mitosis of undifferentiated stem cells. The newly formed cells are slowly pushed upwards through growth pressure to replace the lost cells. At the base of the mucosal layer there is a well-developed muscularis mucosae in all parts of the stomach (Sloss, 1954). Such well-developed muscularis mucosae may help to empty the gastric glands through contraction. The muscularis externa, unlike that in other parts of the gastrointestinal tract, has three layers of smooth muscle: an outer longitudinal layer, a middle oblique layer, and an inner circular layer (Sloss, 1954). This arrangement of muscle fibres allows the stomach to contract in a variety of ways. At the pylorus, the internal and middle layers are greatly thickened to form the pyloric sphincter. The circular sphincter helps to control the passage of digesta into the small intestine. The serosa covering the stomach is part of the visceral peritoneum.

OXYNTIC GLANDS

Oxyntic glands are long branched tubular glands that are located in the fundic (or oxyntic) mucosa of the stomach. Several of these glands open into a single gastric pit (Figure 4). The oxyntic glands contain various types of cells, including **mucous neck cells, parietal cells, chief cells, endocrine cells** and undifferentiated **stem cells** which are found in the neck region of the gland and at the bottom of gastric pits. Stem cells are short and columnar in shape with oval nuclei near the base and are capable of mitosis. The new cells either move upwards to replace desquamated surface mucous cells or move downwards to differentiate into other types of cells**. Mucous neck cells** are present in clusters at the neck region of the gland. They are irregular in shape with the nucleus at the base and secretory granules near the apical surface. The secretory granules contain mucinogens and are stained intensely with PAS. In pigs, scattered mucous cells are also found in the lower part of the oxyntic glands.

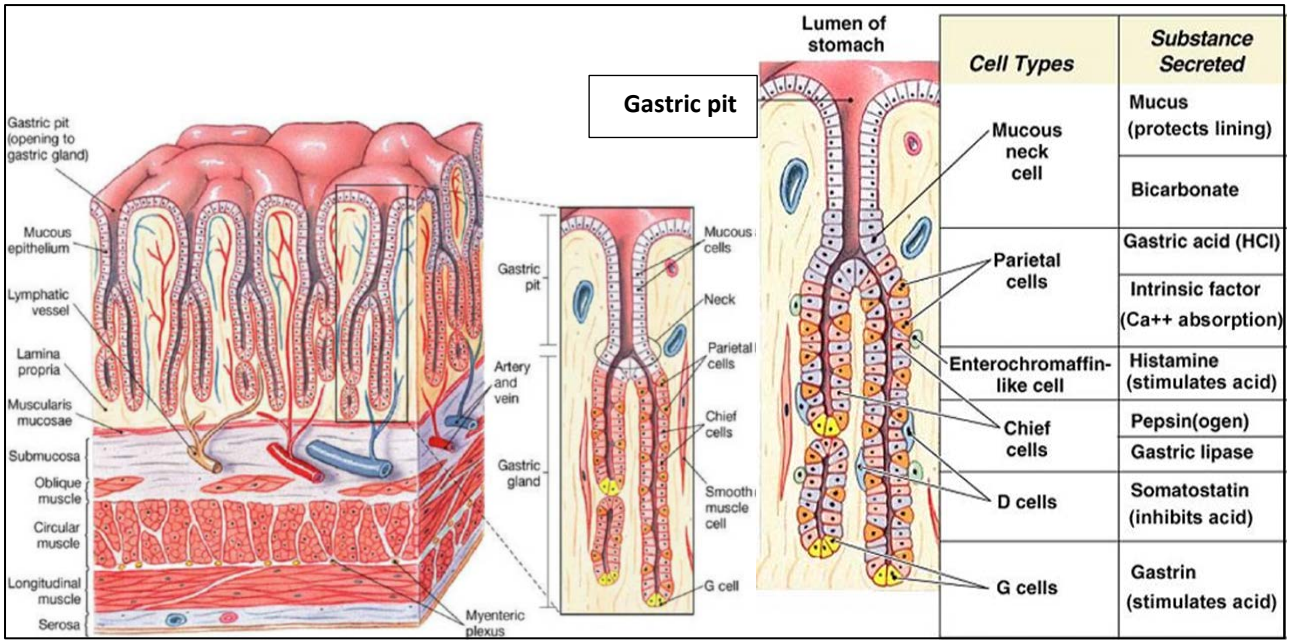

Figure 4. General cell composition and location in a gastric gland. G and D cells a mainly in pyloric glands, while paretial and chief cells in oxyntic (fundic) mucosa. (Basic and Clinical Pharmacology. Mc Graw-Hill, 2012)

Parietal cells produce HCl and are abundant in the oxyntic glands. Parietal cells are more abundant in the upper half of the gland than in the lower half. A cross-section of a parietal cell appears to be triangular in shape, with its base adjacent to the basal lamina and its apex directed towards the lumen of the gland. The nucleus of the parietal cell is spherical in shape, and some cells contain two nuclei. The striking features that are seen at the ultrastructural level are numerous mitochondria and deep, circular invaginations of apical plasma membrane forming the intracellular canaliculus. Hydrochloric acid is secreted initially into the intracellular canaliculus before being discharged into the glandular lumen. The large number of mitochondria indicates that the production of hydrochloric acid is a process with a high-energy requirement. **Chief cells** are enzyme-producing cells. For this reason, they are also called zymogenic cells. Chief cells are numerous in the oxyntic glands and are more abundant in the lower region of the tubular glands. Their cytoplasm is basophilic due to the abundance of rough endoplasmic reticulum. The secretory granules that are located at the apical cytoplasm contain inactive proteolytic enzymes. When released into the glandular lumen, the proteolytic enzymes are activated by hydrochloric acid and converted to active enzymes, proteases. Oxyntic glands also contain various types of endocrine cells that secrete regulatory peptides. Endocrine cells are scattered in the basal region of the epithelial layer between basement membrane and other epithelial cells. Some of the endocrine cells send a thin cytoplasmic extension to the lumen of the gland. Their cytoplasm is filled with small secretory granules that can be stained with silver or chromium salts.

CARDIAC GLANDS

Cardiac glands are short tubular glands that are located in the cardiac region of the stomach. The terminal portion of a cardiac gland is often coiled and has a large lumen. The glands consist mainly of **mucus-secreting cells**. Undifferentiated stem cells are present in the neck or upper region of the gland. Endocrine cells are scattered throughout the gland. Parietal cells are frequently seen in the cardiac glands, particularly in newborn pigs (Xu et al., 1992b).

PYLORIC GLANDS

Pyloric glands are branched short-tubular glands opening into deep gastric pits in the pyloric antrum. The glands are primarily made up of **mucous cells** with scattered parietal and endocrine cells. The dominant type of endocrine cells in the pyloric glands is the **G cell**, which produces the peptide hormone gastrin.
D cells are another endocrine cell type, which produce somatostatin, presents in all these three glands with much more prevalence for pyloric gland. Undifferentiated stem cells are also present at the neck region of the pyloric glands.

INTESTINE

Small intestine

The small intestine is the longest part of the gastrointestinal tract. It measures about 3.5 meters in length in newborn piglets and up to 20 meters in adult pigs. It receives digesta from the stomach and continues the process of digestion in preparation for absorption of nutrients. Most nutrients are absorbed in the small intestine. The small intestine is divided into three regions: duodenum, jejunum and ileum. The duodenum commences at the pyloric sphincter and comprises about one-twentieth of the small intestine. The ileum is short and is the last part of the small intestine; it terminates at the caecum. The jejunum is the mid- and longest portion of the small intestine. Although the histological structure of the wall differs significantly amongst the duodenum, jejunum and ileum, there is no clear boundary between the regions of the small intestine. In most reports, the divisions of the small intestine into the duodenum, jejunum and ileum are arbitrary.

WALL STRUCTURE

The wall of the small intestine consists of the four layers that are characteristic of the gastrointestinal tract. The mucosal layer of the small intestine has a characteristic morphology with a finger-like or leaf-like projections called villi. The projections increase the luminal surface area and make the small intestine more efficient in the digestion and absorption process. In the jejunal region of adult pigs, the layers of mucosa and submucosa are folded to form plicae circulares, further increasing the luminal surface area (Sloss, 1954). The newborn piglets, the villi are approximately 0.5-1.0 mm in length, and they are longer in the jejunum than those in the duodenum and the ileum (Xu et al., 1992a).

The intestinal mucosa contains numerous glands, which are also known as intestinal crypts. Intestinal glands are simple tubular glands that open into the intestinal lumen at the base of the villi (Figure 5). The lining of the intestinal mucosa consists of simple columnar epithelium. Underneath the epithelium is the lamina propria. As in other parts of the gastrointestinal tract, the lamina propria contains numerous diffused leukocytes and isolated nodules of lymphatic tissue. In the ileal region the lymphatic nodules aggregate to form Peyer's patches, which often extend into the submucosal layer (Sloss, 1954). Underneath the lamina propria, there are two thin layers of smooth muscle cells, an inner circular and an outer longitudinal layer, forming the muscularis mucosae. Contractile activity of these muscle cells causes movement of the villi and increases their contact with luminal contents.

The submucosa of the small intestine is made up of loose connective tissue and contains numerous blood and lymphatic vessels. In addition, the submucosa of the duodenum contains clusters of ramified, coiled tubular glands called Brunner's glands. These are mucus-producing glands that secrete mucus into the intestinal crypts. The secretory products of the Brunner's glands are alkaline (pH 8.1-9.3), and act to protect the duodenal mucous membrane against the effect of acidic digesta discharged from the stomach, and help to bring the intestinal contents to the optimum pH for pancreatic enzyme action. The muscularis externa consists of inner circular and outer longitudinal smooth muscle cells. The muscle cells are innervated by the autonomic nervous system. The cell bodies if the parasympathetic neurons appear as clumps of light-staining cells between the two layers of muscle cells. The nerve clumps are called the myoenteric plexus which together with the submucosal nerve plexus, comprise an intrinsic innervation of the intestine. Two kinds of muscular contractions take place in the small intestine. One is a local contraction that displaces intestinal contents both proximally and distally. Local contractions serve to mix the digesta with digestive juice and move it into contact with the mucosa for absorption. The second type of contraction, referred to

as peristalsis, moves intestinal contents distally. Except for part of the duodenum, the small intestine is completely covered by visceral peritoneum forming the layer of serosa. The small intestinal serosa is a thin layer of simple squamous epithelium supported by connective tissue. It contains blood vessels and lymph vessels that join with larger blood and lymph vessels in the mesentery. The blood vessels eventually drain into the hepatic portal vein.

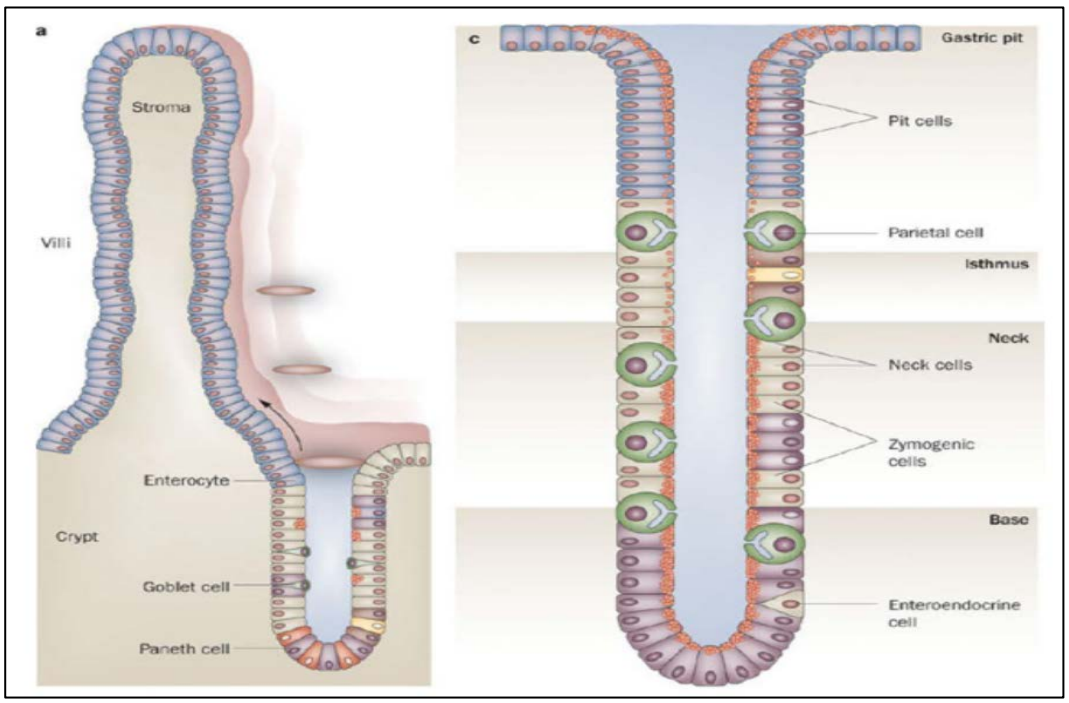

Figure 5. Intestinal villi and crypt gland cells compared to a stomach gland（Nature Publishing Group, 2009).

VILLI AND CRYPTS

Intestinal villi are finger-like or leaf-like structures that project into the intestinal lumen. The villi are lined by a simple columnar epithelium with a core of connective tissue forming the lamina propria. Embedded in this connective tissue are capillaries and lymphatic vessels. Typically, each villus contains a centrally placed lymphatic that begins in the villus as a blind tube and drains into larger lymphatic vessels in the submucosa. Nutrients absorbed by the surface epithelial cells will pass through the wall of the capillaries and the lacteals, and enter the cardiovascular or lymphatic system.

Multi-potent stem cells residing at the base of each villus, in the so-called crypts of Lieberkühn, give rise to four primary epithelial lineages: absorptive enterocytes, mucin-secreting goblet cells, enteroendocrine cells, and Paneth cells (Fig.6A). Each of these cell types will be discussed in detail below. The epithelium is anchored on a continuous sheet of extracellular matrix, or basement membrane, consisting of a mixture of collagen, laminin, and fibronectin (Beaulieu, 1999). The actual composition of the basement membrane varies along the crypt-villus axis and this is thought to provide signals essential for enterocyte proliferation, survival and differentiation (Beaulieu, 1999). The cellular constituents of the crypts, notably stem cells, goblet cells, enteroendocrine cells, and Paneth cells, collectively are responsible for water and ion secretion, as well as exocrine, paracine and endocrine secretions, whereas the intestinal villi are primarily responsible for fluid and nutrient absorption.

Absorptive enterocytes

The epithelium lining the intestinal villi consists of primarily columnar enterocytes. Also known as intestinal absorptive cells or absorptive enterocytes. Scattered between the enterocytes are mucus producing goblet cells. Enterocytes are tall columnar cells, with an oval nucleus located at the basal region. The apical surface membrane of the enterocytes is intensively folded, forming the structure that is called the microvilli . When viewed under a light microscope the apical membrane of the enterocytes appears striated. Thus, the apical membrane of enterocytes with microvilli is also called the striated border membrane or the brush border membrane. The brush border membrane of the enterocyte contains various channels and nutrient transporters, and is the active site for nutrient absorption. The projection of microvilli at the apical membrane increases the absorptive surface area of an enterocyte tremendously. Neighboring enterocytes are joined by junctional complexes to stop the diffusion of harmful substances through the intercellular space. The enterocyte not only has an absorptive function, but also digestive functions. Digestive enzymes are anchored to the brush border membrane and their functional groups extend outward to become part of the glycocalyx. Included amongst the enzymes are saccharidases (e.g., lactase, isomallase, maltase and sucrase) and various peptidases. The brush border membrane also contains the enzyme enteropeptidase, also known as enterokinase. This enzyme converts trypsinogen into active trypsin; the latter can then activate additional trypsinogen and other pancreatic zymogens.

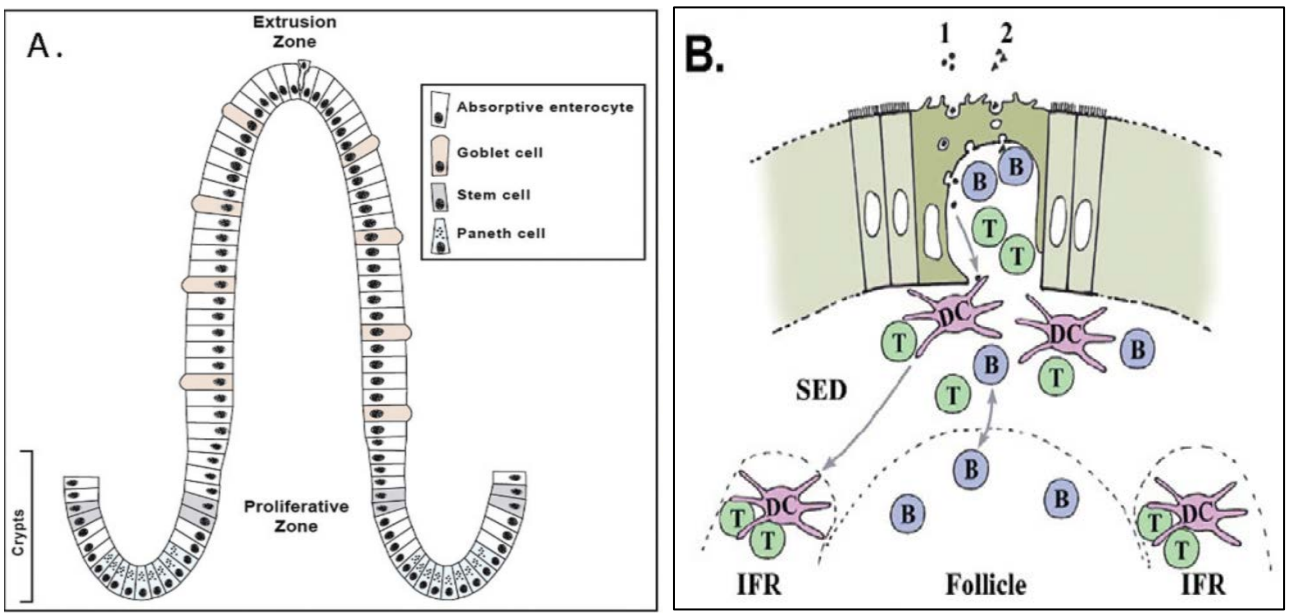

Figure 6A. Gut epithelial cell distrubuition where stem cells riding in a proliferative zone of each villus called crypt of Lieberkuhn, give rise to four primary cells: absorptive enterocytes, mucin-secreting globlet cells, enteroendocrine cells and Paneth cells. Figure 6B.The FAE is found exclusively overlying organized lymphoid follicles situated throughout the small and large intestines. (both: Vajdy M., 2008) (Neutra et al., 2001).

Goblet Cells

Goblet cells, also known as mucin-producing cells, are responsible for the production of the mucus gel that blankets the surface of the intestinal epithelium. Goblet cells, named for their characteristic goblet-like shape, are present in the small and large intestine, and are found along the entire crypt-villus axis. In villi of the small intestine, goblet cells are interspersed among absorptive enterocytes (Fig. 6A)(Karam, 1999). Under normal conditions, individual goblet cells constitutively secrete mucins, high molecular weight glycoproteins that consist of core polypeptides heavily decorated with both N- and O-linked oligosaccharide

side chains. This so-called baseline secretion of mucins is necessary for both maintenance and renewal of the mucus layer, which is important in both epithelial function and defense (Deplancke and Gaskins, 2001; Lievin-Le Moal and Servin, 2006). For example, the mucus layer aids in lubrication and protection of the intestinal mucosa, as well as serving as an important defense mechanism against microbial pathogens and toxins. The viscous mucus gel limits diffusion of macromolecues and impedes motility of parasites and bacterial pathogens. Through heterogeneous N- and O-linked oligosaccharide side chains on mucins, the mucus layer also provides "decoy" ligands for lectin like adhesins expressed by microbial pathogens and toxins, thereby competitively inhibiting these agents from gaining access to their receptors on the apical surfaces of enterocytes. In situations where the intestinal epithelium is exposed to microbial pathogens, toxins or other intraluminal irritants, goblet cells can release additional mucins that are normally stored in apically-residing granules.

Enteroendocrine Cells

Enteroendocrine cells are a specialized sub-population of "sensory" epithelial cells that serve as a link between the intestinal lumen and the enteric nervous system (Flemstrom and Sjoblom, 2005). The primary function of this epithelial cell type is to secrete peptide hormones and transmitters. Enterochromaffin cells, for example, are responsible for the production and secretion of serotonin (5-hydroxytryptamine), a hormone which regulates (among other things) intestinal peristaltic and secretory reflexes. Recently, a subpopulation of enteroendocrine cells have been proposed to serve as "taste receptors," based on the immunohistochemistry and real time PCR analysis demonstrating the presence of taste signaling molecules in the small intestine of mice (Bezencon et al., 2007).

Paneth Cells

Paneth cells migrate to the crypt base where these cells reside for an average of 21–28 days in most mammals (Andreu et al., 2005). Paneth cells possess a well-developed apical secretory apparatus. Their location in the crypt base places them in an ideal position to deliver growth factors to dividing cells in higher compartments of the crypt, and to create gradients of anti-microbial factors which limit, or altogether prevent, microbial colonization of the small intestinal crypts. Key anti-microbial factors produced by Paneth cells include lysozyme, the cryptidins alpha-defensins and other digestive enzymes with known anti-microbial properties (Wehkamp et al., 2006).

Follicle-Associated Epithelium (FAE) and M Cells

The intestinal epithelium maintains a close collaboration with an underlying local immunological network, collectively referred to as the mucosal immune system (Holmgren and Czerkinsky, 2005; Neutra et al., 2001). Nowhere is this collaboration more apparent than in organized lymphoid follicles, which are present throughout the small intestine and colon. In the small intestine, these macroscopic structures consist of aggregates of between 5 and 10 lymphoid follicles known as Peyer's patches. These organized lymphoid follicles contain germinal centers which represent the primary sites of mucosal B cell differentiation and somatic cell hypermutation (Brandtzaeg and Johansen, 2005). As mucosa-associated lymphoid tissues lack afferent lymphatics, germinal center activity is driven exclusively in response to antigens present in the intestinal lumen. Uptake and transepithelial transport of macromolecular antigens from the intestinal lumen to the organized lymphoid follicles is achieved the so-called follicle-associated epithelium (FAE).
The FAE is distinct from the villus epithelium in both structure and function (fig.6B). Whereas the villus epithelium is specialized for digestion and absorption of nutrients and is dominated by absorptive enterocytes, mucin-secreting goblet cells and enteroendocrine cells, the FAE contains few or no goblet or

enteroendocrine cells and has reduced levels of certain digestive enzymes. There are also fewer defensin- and lysozyme-producing Paneth cells in follicle-associated crypts. Enterocytes within the FAE, like their counterparts on villi, have well developed microvilli and are coated with a thick filamentous brush border glycocalyx, but they are not identical to villus cells (Frey et al., 1996). FAE enterocytes express lower levels of the membrane associated hydrolases involved in digestive functions (Savidge and Smith, 1995). It is also apparent that the glycosylation patterns of epithelial cells in the entire FAE differ from those on villi, indicating that glycosyltransferase expression in the FAE is distinct (Mantis et al., 2000). The FAE of Peyer's patches also express chemokines involved in leukocyte homing, which are not expressed elsewhere in the small intestinal epithelium (Zhao et al., 2003b). Finally, the FAE is devoid of pIgR and therefore it is unable to transport IgA from the interstitium to the lumen. Probably the most distinguishing feature of the FAE is the presence of M cells, a unique epithelial cell type that is specialized in the uptake and transepithelial transport of particulate antigens, including particles and macromolecules, viruses, bacteria, and parasites (Amerongen et al., 1991; Jones et al., 1994; Marcial and Madara, 1986; Pappo and Ermak, 1989). Indeed, M cells have been considered the "gateway" to the gut associated lymphoid tissue (GALT). The apical and basolateral surfaces of M cells have distinct features that enable them to rapidly and efficiently deliver mucosal antigens from the lumen to underlying lymphoid follicles (Fig. 6B). Consequently, M cell apical membranes are more accessible to particles, viruses and bacteria than adjacent enterocytes. In mice and humans, the apical surfaces of M cells have a pattern of glycosylation that is distinct from FAE enterocytes and villus enterocytes (Clark et al., 1993; Giannasca et al., 1999). M cells also selectively express Toll-like receptors and pattern recognition receptors on their apical membrane that may facilitate antigen recognition and contribute to signaling in the local environment (Chabot et al., 2006). The M cell basolateral membrane is deeply invaginated to form a large intraepithelial "pocket" containing specific sub-populations of naive and memory B and T cells (Yamanaka et al., 2001), and occasional dendritic cells (Iwasaki and Kelsall, 2001). The pocket brings the M cell basolateral surface to within a few microns of the apical surface, shortening the distance that transcytotic vesicles must travel to cross the epithelium. Antigens transported by M cells are sampled by adjacent DCs.

Large intestine

The large intestine consists of the caecum, the colon and the rectum, and is about 0.7 meter in length in newborn piglets and up to 4.5 meters in length in adult pigs. The caecum is a cylindrical blind sac that is located at the proximal end of the colon. The proximal part of the colon undergoes four complete turns that spiral towards the center of the coil; it is termed the centripetal colon. A central flexure permits the spirals to reverse and the colon undergoes 3.5 turns from the center of the coil, giving rise to the centrifugal colon. The major functions of the large intestine include the absorption of later, electrolytes and certain nutrients that are produced by bacterial fermentation; so it provides a location for numerous microorganisms. The wall of the large intestine as the four layer structure that is characteristic of the gastrointestinal tract. The structural organizations of each layer are similar to those described for the small intestine, with the exception of the mucosal layer. The mucosa of the large intestine has no villous projections but contains numerous straight tubular glands that extend through the full thickness of the mucosa. The luminal surface and the tubular glands are lined with columnar epithelial cells that generally resemble the enterocytes of the small intestine. However, the brush border at the apical surface of the enterocytes is much thinner than that of the small intestinal enterocytes, numerous mucus-secreting goblet cells and scattered endocrine cells are also found in the tubular glands. At the lower half of the glands,

there are numerous stem cells with the capacity for mitotic division. New epithelial cells that arise from the stem cells in the glands migrate upwards and replace the degenerated surface cells.

Although most nutrients are absorbed by the small intestine, the absorption of some nutrients also occurs in the large intestine, especially in its proximal region. In newborn piglets, rudimentary villi are found in the proximal region of the large intestine and these structures gradually disappear during the first few days of postnatal life (Xu et al., 1992a). The enterocytes in the proximal region of the large intestine in a neonatal animal are similar to those cells in the small intestine, having long microvilli at the apical surface, the ability to absorb amino acids and glucose, and digestive enzyme activities at the brush border. It has been suggested that the newborn colon, which is capable of digestion and absorption, may provide an additional capacity to the immature small intestine for nutrient digestion and absorption (Xu, 1996). The lumen of the large intestine is the home for billions of microorganisms. These microorganisms utilize the food residues that are discharged from the small intestine and convert them into useful nutrients such as short-chain fatty acids and vitamins. These nutrients can then be absorbed in the large intestine and be used by the animal. Bacteria that are capable of breaking down cellulose and hemicellulose have been found in the lumen of the large intestine in adult pigs, which indicates that pigs have some capacity to utilize fibrous diets (Varel and Yen, 1997). In addition, bacteria in the large intestine synthesize most of the vitamin K that is needed by the animal.

AMINO ACIDS

Twenty amino acids are the building blocks of proteins (Table 1). Some of them can not be synthesised, or not in sufficient quantities, by animals; they must therefore be supplied through feeding and are referred to as essential amino acids. These are listed in Table 2. To maintain good health and growth performance, feeds must provide the indispensable amino acids in sufficient quantities to cover the requirement of animals.

Amino acid	3-letter	Amino acid	3-letter
Alanine	Ala	Leucine	Leu
Arginine	Arg	Lysine	Lys
Asparagine	Asn	Methionine	Met
Aspartic acid	Asp	Phenylalanine	Phe
Cysteine	Cys	Proline	Pro
Glutamic acid	Glu	Serine	Ser
Glutamine	Gln	Threonine	Thr
Glycine	Gly	Tryptophan	Trp
Histidine	His	Tyrosine	Tyr
Isoleucine	Ile	Valine	Val

Table 1. The 20 amino acids and their abbreviations. (Ajinomoto Eurolysine S.a.S.)

	Pigs
• Indispensable amino acids	
EAA that can not be synthesised *de novo* or at a sufficient rate to maintain the associated biological functions	Lys
	Thr
	Met
	Trp
	Val
	Ile
	Leu
	His
	Phe

Table 2. Essential amino acids in pig. (Ajinomoto Eurolysine S.a.S.).

Some amino acids are essential dietary components in certain situations but not in others; they have been referred to as conditional essential amino acids.

For example, glutamine is normally regarded as nonessential for the pig. However, under total parenteral nutrition or during the early post-weaning period, supplementation with glutamine prevents intestinal mucosal atrophy (Wu et al., 1996). It is known that glutamine is an important energy source for the enterocytes (Wu et al., 1995). Glutamine also serves as a substrate for the endogenous synthesis of arginine and proline. It has also been reported that supplementing the diet of early-weaned piglets with glutamine preserves normal lymphocyte function following *Escherichia coli* infection (Yoo et al., 1997).
The sulphur-containing amino acid cysteine occurs in two forms, either as itself or as cystine in which two cysteine molecules are joined together by a disulphide bridge. Cystine can be synthesized from methionine; consequently when there is an inadequate supply of cystine in the diet, the methionine requirement will increase. An adequate supply of dietary cystine will have a sparing effect on the methionine requirement. Cystine can satisfy approximately 50% of the sulphur amino acid requirement of growing pigs (Chung and Baker, 1992). In practice, the requirement for sulphur amino acids is usually met by a mixture of methionine and cystine. Similarly, phenylalanine can meet the requirement for the two aromatic amino acids phenylalanine and tyrosine, as phenylalanine can be converted to tyrosine. A mixture of these two amino acids is commonly used to meet the dietary requirements of aromatic amino acids.

AMINO ACID BALANCE

The nutritional value of a protein is primarily dependent on its amino acid composition, especially the content of essential amino acids. A protein that has a perfect balance of amino acids to meet all amino acid requirements has been described as an ideal protein. However, the ideal pattern of amino acid composition for maintenance may differ from that for tissue growth. There are also changes in the amino acid content of body tissues at different stages of development. Therefore, there is no ideal protein that can meet all amino acid requirements for the growing pig. Nevertheless, the porcine milk protein is regarded to be close to an ideal protein for neonatal pigs.

An amino acid that is present in the diet in an inadequate amount relative to its requirement is regarded as a limiting amino acid. Supplementation of the diet with this amino acid will significantly improve its nutritional value and subsequently the performance of the animal that is fed on this diet. Information about which amino acids are most limiting in natural feedstuffs is important in formulating pig diets. The most limiting amino acids of cereal grains commonly used in pig diets are presented in Table 3.

Cereal grain	*1st limiting*	*2nd limiting*	*3rd limiting*
Barley	Lysine	Threonine	Histidine
Corn	Lysine and tryptophan		Threonine
Oats	Lysine	Threonine	
Sorghum	Lysine	Threonine	Tryptophan
Wheat	Lysine	Threonine	

Table 3. Limiting amino acids and cereal grain used in diets for pigs. (Adapted from Lewis, 2001)

As can be seen, lysine is the first limiting amino acid in most cereal grains. In contrast, methionine is the first limiting amino acid in soybean meal (Berry et al., 1966). Soybean meal and canola meal have relatively high lysine and low sulphur amino acid contents, whilst cereal grains arc low in lysine and high in sulphur amino acids. A combination of these two types of proteins can markedly improve the nutritional value of a diet; this phenomenon is known as complementation.

Amino acids that are limiting in common feedstuffs can be produced by chemical synthesis. Currently, four crystalline amino acids (lysine, methionine, tryptophan and threonine) are commercially available as feed additives. Supplementation of the diet with crystalline amino acids requires caution. Crystalline methionine and tryptophan are highly toxic, and improper mixing or using an incorrect dose can cause poisoning. In newly weaned piglets, dietary addition of 4% methionine causes a 52% reduction in body weight gain and a significant decrease in feed intake (Edmonds et al., 1987). Antagonistic effects have been observed between arginine and lysine and amongst leucine, isoleucine and valine, possibly due to competition for carrier proteins during the absorption and metabolism process. When there is an excessive amount of one amino acid in the antagonistic group in the diet the requirement for other competitive amino acids increases.

Published balances of amino acids relative to lysine have been well reviewed (Urynek and Buraczewska, 2003) (Table 4). Since ARC (1981), the change that has affected commercial diets the most has been the increase in levels of threonine (usually the second- limiting amino acid in pig feeds) relative to lysine. Fuller *et al.* (1987) suggested a level of 72%, having separated out requirements for maintenance and growth and feeding semi-purified diets. Work by D.J.A. Cole and L. Bong (1989) has found the value to be between 65 and 70%. It is unusual to find commercial diets with tryptophan at a level greater than 17-18% of lysine despite the fact that tryptophan, being a precursor for serotonin, is known to play a role in stimulating feed intake. More recently studies demonstrated that pigs between an average of 8 and 25 kg responded to an increasing level of dietary tryptophan up to 21% of lysine.

Source	Lysine	Methionine and cystine	Threonine	Tryptophan
Cole (1978)	100	50	60	18
Fuller *et al.* (1979)	100	53	56	12
ARC (1981)	100	50	50	15
Fuller *et al.* (1987)				
Maintenance	100	150	142	29
Growth	100	53	69	18
Both	100	56	72	19
van Lunen and Cole (1996)	100	50–55	65–57	18

Table 4. The ideal amino acid balance of commonly weaner feeds.

Dietary amino acids are major fuels for the small intestinal mucosa, as well as important substrates for syntheses of intestinal proteins, nitric oxide, polyamines, and other products with enormous biological importance. Recent studies support potential therapeutic roles for specific amino acids (including glutamine, glutamate, arginine, glycine, lysine, threonine, tryptophan and sulfurcontaining amino acids) in gut-related diseases. Results of these new lines of work indicate trophic and cytoprotective effects of amino acids on gut integrity, growth, and health in animals and humans.

In particular, these amino acids affect the gut because it is an important organ responsible for digestion, absorption and metabolism of dietary nutrients. It contributes to 9–12% of whole-body protein synthesis and is the most important place of entry for foreign antigens, including food proteins, natural toxins, commensal gut flora, and invading pathogens (Li et al., 2007). The intact intestinal tract is lined by a continuous monolayer of intestinal epithelial cells, of which a primary function is to act as a physical barrier, interacting with a complex external environment. The intestinal tract is also one of the largest lymphoid organs in the body, and consists of immune cells in organized gut associated lymphoid tissues (Field et al., 2002). Amino acids are not only important substrates for the synthesis of proteins and other nitrogenous compounds, but also key regulators through major metabolic pathways (Meijer, 2003). Recent studies with animals and humans indicate additional roles for amino acids in maintaining gut health (Wang et al., 2008).

In the contest of the studies presented further here is discussed in particular the importance of threonine and tryptophan.

Threonine

Threonine is the second limiting amino acids in pigs. Among the essential amino acids, threonine is particularly important for mucin synthesis and maintenance of gut barrier integrity (Bertolo et al., 1998) because it is a major component of intestinal mucin and plasma G-globulin in animals. The retention of dietary threonine by the intestine is (up to 60%) high and animal feeding studies indicate that changes in components of the immune system are sensitive to dietary threonine intake (Stoll et al., 1998). It has been shown in piglets that only 38% of dietary threonine appears in portal blood; the remainder is retained by the gut. Nearly 90% of the gut-retained threonine is either secreted as mucosal proteins, threonine-rich mucin, or metabolized. It is known that in intestinal mucosa, a major fate of threonine is incorporation into mucins, which are major glycoproteins protecting the epithelium from injury (Le Floc'h and Seve, 2005; Schaart et al., 2005). A study with rats demonstrated that dietary threonine restriction dramatically and specifically impaired the synthesis of mucins in all segments of the small intestine, reaching the largest reduction of 40% in the duodenum (Faure et al., 2005). In addition, Wang et al. (2007) reported that both a deficiency and an excess of dietary threonine reduced the synthesis of intestinal mucosal protein and mucins in young pigs. The implications of threonine for intestinal health and nutritional requirements have been highlighted in several recent studies. For example, the threonine utilization for synthesis of small intestinal proteins is increased substantially in response to sepsis, representing over two-fold of the threonine intake (Faure et al., 2007). Thus, under inflammatory conditions, threonine availability may become limited for the synthesis of intestinal mucins, which leads to an impairment of gut barrier function. Consequently, an increase in dietary provision of threonine and other amino acids can promote mucin synthesis and rebalance the gut microbiota to favor intestinal protection and mucosal healing (Faure et al., 2006). There are evidences in threonine effects on immune system defence. Parenteral feeding leads not only to atrophy of mucosal epithelial cells, but also to atrophy of the goblet cells, which secrete a large amount of threonine-rich mucin (Bengmark and Jeppsson, 1995). Through protein synthesis and cellular signalling mechanisms, addition of 2mM threonine to the culture medium prevented apoptosis, stimulated cell growth and promoted antibody production in lymphocytes (Duval et al., 1991). Also a dietary supplementation with threonine increased serum levels of IgG in sows (Cuaron et al., 1984). Further, increasing dietary threonine intake increased antibody production, serum IgG levels and jejunal mucosal concentrations of IgG and IgA, while decreasing jejunal mucosal concentrations of IL-6 in young pigs

challenged with Escherichia coli. These findings provide support for a role of dietary threonine in modulating immune function in livestock and perhaps humans.
For these reasons it is conceivable that the neonatal small intestine needs high requirement for threonine.

Tryptophan

In European diets, tryptophan is the fourth limiting amino acid for growth. This means that when the tryptophan supply is low compared to the other essential amino acids, the protein synthesis and accretion and, finally, the growth rate will be limited.
Tryptophan has the particularity to be involved in several biological functions such as appetite regulation, stress and immune responses. This explains why the young pigs exhibiting a high growth rate potential are especially sensitive to low and inadequate tryptophan dietary supply. Consequently, requirement and practical recommendations can be different according to the biological function to be optimized. In young pigs, the main effect of tryptophan on growth rate is associated to an increase in feed intake when tryptophan is supplied to a level close to recommendations for a maximum growth rate. However, some factors also impact directly on tryptophan metabolism and thus limit its availability for growth and any other functions in which tryptophan is involved.
So, besides being a nutrient that is fundamental to allow maximum growth of the pigs, tryptophan also has other physiological functions of great interest.
Tryptophan has positive effects on voluntary feed intake. This characteristic makes it a very important nutrient in feeds for animals with low appetite and limited feed intake capacity, as it is the case for lactating sows and weaned piglets. This greater feed intake leads to increased growth performance and improved feed conversion ratio in piglets, and to lower body weight losses in sows during lactation. The effect can be very noticeable when extreme levels of tryptophan are used, but even in commercial ranges (22% vs 18% of Trp/Lys) significant improvements can be obtained in piglets. Although recent studies suggest the potential mediation of hormones such as melatonin (pineal gland), insulin (pancreas) or ghrelin (stomach), the effect of tryptophan on feed intake seems to be fundamentally related to its role as a precursor of serotonin, a neurotransmitter synthesized in the brain and in the gastrointestinal tract, which plays a role in regulating feed intake. It has been observed that the amount of dietary tryptophan needed to achieve a certain level of feed intake is greater in diets with higher protein content. High protein diets contain a greater quantity of large neutral amino acids (valine, isoleucine, leucine, phenylalanine and tyrosine), which compete with tryptophan for the same transporter during the intestinal absorption and also to pass the blood-brain-barrier, which reduces the quantity of tryptophan available for the synthesis of serotonin. Tryptophan can modulate aggressive behaviors and improve the stress responses in swine. It has been observed in various species, including humans, for which tryptophan has been used as an anti-depressant in the 1980s. It has also been reported that tryptophan may reduce the negative effects on meat quality caused by stress during transport and before slaughter. The mechanism of coping stress is not well described, but it is most likely due to tryptophan being served as precursor for serotonin synthesis.
However, clear effects have been observed only when using levels of tryptophan that are either much lower or much higher than the usual levels found in commercial feeds.
The level of tryptophan in the blood and the quantity of tryptophan available for body protein synthesis are decreased during inflammatory or disease challenge conditions. This is due to the lower level of feed intake during inflammatory states, the increased demand for tryptophan for the synthesis of acute-phase proteins, which are rich in tryptophan, and also due to the greater catabolism of tryptophan via kynurenine

pathway caused by the action of the enzyme IDO (indoleamine 2,3 dioxygenase), which is stimulated in the presence of cytokines (inflammatory states) (Fig 7).

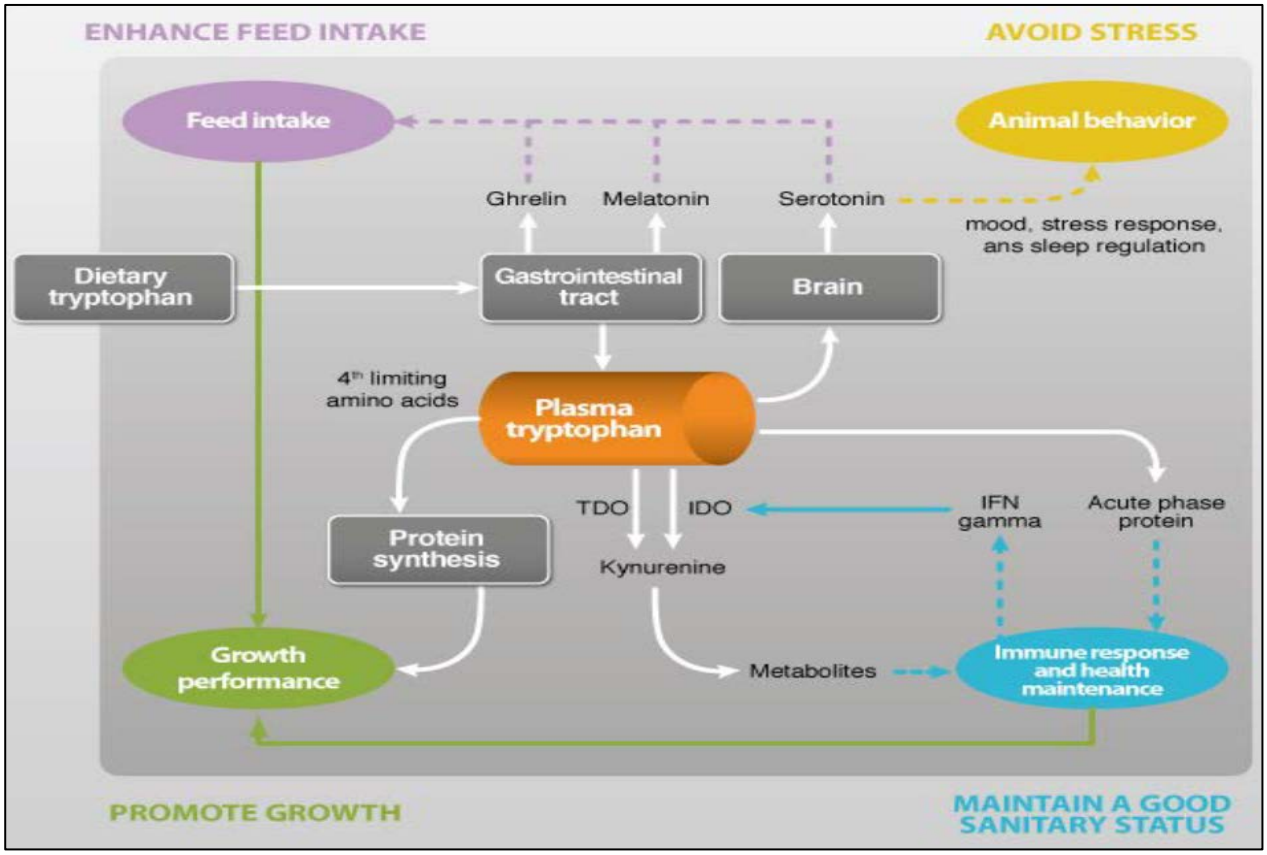

Figure 7. Global pattern of the biological roles of tryptophan and their implications in animal growth. (Ajinomoto S.a.S)

An adequate inclusion of tryptophan is especially advisable in low health conditions due to its effect on ingestion capacity and also to counteract the greater demand for tryptophan for non-productive functions. Moreover, in some studies it has been observed that the inclusion of tryptophan in the diet is capable of modifying the inflammatory response. According to these studies, a diet with an adequate level of tryptophan produces a lower inflammatory response than a diet that is deficient in tryptophan, with lower levels of plasma haptoglobin (acute phase protein) and a lower activity of enzyme IDO.

The products of tryptophan catabolism include serotonin, N-acetylserotonin, melatonin and anthranilic acid (Kim et al. 2007). Tryptophan catabolism is increased to generate anthranilic acid through the indoleamine 2,3-dioxygenase (IDO) pathway during inflammation or stimulation by LPS or certain cytokines (Platten et al., 2005). Serotonin, melatonin and N-acetylserotonin can enhance host immunity by inhibiting the production of superoxide, scavenging free radicals and attenuating the production of TNFa (Perianayagam et al., 2005). In addition, N-acetylserotonin is an inhibitor of sepiapterin reductase, an enzyme for the synthesis of tetrahydrobiopterin (Shi et al. 2004). By modulating inducible NO synthesis, this tryptophan metabolite can affect both innate and acquired immunity systems. Excitingly, anthranilic acid was recently found to inhibit the production of proinflammatory Th1 cytokines and prevent autoimmune neuroinflammation (Platten et al. 2005). Because there is a progressive decline in tryptophan concentrations in plasma of animals with inflammation, its catabolism plays a critical role in the functions of both macrophages and lymphocytes (Melchior et al. 2004). Early work indicated that tryptophan

starvation resulting from IFNg treatment was associated with the antiproliferative effect of this cytokine on intracellular parasite and tumours (Ozaki et al. 1988). Interestingly, progressively increasing concentrations of IFNg were required for its growth inhibition in the presence of elevated tryptophan concentrations (Pfefferkorn, 1984). Available evidence suggests that tryptophan catabolism plays a role in immune responses by producing a local immunosuppressive environment that is able to control T-cell homeostasis and self-tolerance during inflammation (Platten et al. 2005). A deficiency of dietary tryptophan impaired the immune response in chickens (Konashi et al., 2000). Conversely, oral administration of 300 mg of tryptophan to rats enhanced phagocytosis by macrophages and the innate immune response (Esteban et al., 2004). Dietary supplementation with 0,22% L-tryptophan also increased resistance to bacterial and parasitic infections in rats fed a 20% zein diet (Watson & Petro, 1984). At present, a potential use of crystalline tryptophan for animal health management is not fully developed.

In the literature, recommendations are usually expressed in digestible basis and in relation to lysine, following the concept of ideal protein. In table 1 the values recommended for piglets by different institutions are shown. Traditionally Trp:Lys ratios of 17-18% have been recommended, but there is currently a great deal of evidence that suggests that higher levels (≥ 22%) can be more profitable, especially in low health conditions. In the same way, the levels traditionally recommended for lactating sows oscillate between 18 and 20 % according to the sources, but there are also studies that have shown improvements in feed intake and reduced weight loss with a Trp:Lys ratio of at least 22%.

So, tryptophan is an important amino acid in swine diet formulations due to its positive effect on feed intake, growth performance and health .

WEANING, PWD and GUT MICROBIOTA

Weaning

Over the past 50 years, the weaning age of piglets has been decreased from 10–12 weeks to current ages of 3–5 weeks (Nabuurs, 1998), and in our case regards 4 weeks (28 days). Weaning is a stressful time in a pig's life where it has to adapt rapidly to major changes in environment and nutrition.

The weaning transition is commonly accompanied by adverse changes in intestinal morphology, including reduced villus height, increased villus width, increased crypt depth and reduced absorptive capacity and brush-border enzyme activity (McCracken et al., 1999). It has been even reported that the switch from liquid to solid diets reduces feed intake in neonatal piglets and may subsequently reduce the growth rate.

Furthermore, an adequate nutrient intake is essential in providing both the nutrients and hormonal signals required to stimulate gut mucosal growth (Burrin et al., 2000). It has also been shown that weaning of piglets on to solid diets causes anorexia and local intestinal inflammation (McCracken et al., 1999). In contrast, piglets weaned to a liquid diet (e.g., bovine or ovine milk) maintain a normal intestinal structure after weaning (Pluske et al., 1996). These foundings have lead to a sweeping change in the industry practice towards liquid feeding systems for nursery pigs. The length of time it takes piglets to adapt to weaning appears to be quite variable.

The weaning process is known to decrease digestive function in several ways. Although weaning has little effect on gastric lipase activity, the process dramatically decreases exocrine pancreatic lipase activity. Weaning also dramatically reduces both the hydrolytic capacity and the specific activity of major exocrine pancreatic proteases. It is known that brush-border enzyme activities decline along the villus-crypt axis towards to the crypt (Fan et al., 2001). Thus, weaning associated villus atrophy may markedly reduce intestinal digestive and absorptive capacities. These changes are assumed to impair the ability of the small intestine to digest and absorb nutrients and hence to predispose the piglet to malabsorption and diarrhoea.

Several biological explanations have been suggested for weaning associated diarrhoea.

Firstly, porcine milk is rich in IgA and glutathione; IgA provides passive mucosal immunity whereas gl**u**tathione is an important antioxidant essential for the maintenance of intestinal mucosal integrity (Reeds et al., 1997). Weaning removes these luminal protection agents and increases the susceptibility to enteric infections and diarrhoea.

Secondly, weaning decreases goblet cell on villus-crypt units (Dunsford et al., 1991), thus reducing the protective mucus secretion. It has been proposed that the mucus barrier reduces colonization by pathogens of the intestinal villus membrane. Weaning also disturbs intestinal mucosal active immunity by altering mucosal T-cell subsets, causing intestinal mucosal inflammation and subsequently increases paracellular translocation of pathogenic microbes and their toxins (Spreeuwenberg et al., 2001).

Thirdly, weaning causes villus atrophy and crypt hyperplasia and subsequently alters the ratio between the villus absorptive cells and the crypt secretory cells. Thus, weanling piglets may be physiologically vulnerable to diarrhoea.

The abrupt change from milk to starter solid diets in pigs weaned at about 3-4 weeks is associated with an underfeeding period and a weight lost immediately after weaning. The resulting growth check can have a major impact on the subsequent performance. Feed and alternative strategies limiting growth problems in this sensitive period are important to improve the performance further.An important aim for the pig industry is to formulate economically viable growth-promoting diets to ease the transition from sow's milk to nursery diets. In addition to satisfying the nutritional requirements of weaned pigs, such diets are

increasingly assessed for their ability to modulate microbial succession, stabilize the commensal microbiota, improve immune function and enhance disease resistance in the young animal.

Post-weaning disease - PWD

The gastrointestinal tract performs two major functions: assimilation of nutrients, fluid and electrolytes; and maintenance of a protective barrier to prevent uncontrolled passage of toxins and infectious agents into the systemic circulation. Hence, when gastrointestinal disorders occur, one or both of these functions are compromised, which may lead to severe growth retardation or death.

The gastrointestinal tract of the neonatal pig undergoes two major functional changes that are associated with birth and weaning. At birth, the gut encounters severe challenges when it suddenly takes over the responsibility for nutrient acquisition from the placenta, and it is exposed to a diversity of pathogenic and non-pathogenic microorganisms. Furthermore, during this early period the newborn pig is especially susceptible to gastrointestinal infections because of its immature immune system. The piglet may succumb to infections if it fails to acquire an adequate supply of immunoglobulins through the consumption of colostrum immediately after birth.

The gut encounters a second challenge at the time of weaning. Weaning usually involves a shift from a liquid milk diet to a solid, typically grain-based, diet that has a significantly higher content of carbohydrates and a lower content of protein and lacks many of the growth stimulating and protective substances that are present in milk. In addition, the grain-based diet may not be as palatable as sows milk and may contain substances that have anti nutritional effects. This change in food source, along with other stresses related to weaning such as separation from the sow and littermates, relocation to a new environment and mixing with unfamiliar piglets, often leads to a reduction in feed intake and growth rate and also to gastrointestinal disorders and diarrhoea.

Diarrhoea is a condition characterized by increased frequency and fluidity of bowel movements. The faeces are watery, soft and may contain abnormal amounts of fat, mucus, blood or fibrin, depending on the aetiology of the diarrhoea. The high concentration of water and electrolytes in the faeces during diarrhoea may result from increased gastrointestinal secretion, reduced digestive and absorptive capacity and/or abnormal intestinal motility (O'Loughlin et al., 1991). The common enteric pathogens that cause diarrhoea in young pigs are rotavirus, transmissible gastroenteritis virus, *Clostridium perfringens, Escherichia coli, Isospora suis* and *Cryptosporidium parvum*.

The diarrhoea is initially noninfectious, but at a later stage is often aggravated by opportunistic viruses, bacteria and protozoa. The most common infectious agents of post-weaning diarrhoea are enteropathogenic strains of *E. coli.* Post-weaning diarrhoea is a complex, multifaceted and poorly understood syndrome that involves the interactions of psychological stress, dietary reactions, enteric pathogens and genetic susceptibility. Changes associated with weaning, e.g., in small intestinal morphology (Mosenthin, 1998; Pluske et al., 1996), enzyme activity (Hampson and Kidder, 1986) and absorption capacity (Miller et al., 1984) are well documented in literature. The changes, which include villous atrophy, crypt hyperplasia, reduced brush border enzyme activity and absorptive capacity, are believed to predispose the weaning pig to post-weaning diarrhoea.

Colibacillosis

Escherichia coli is an enterobacteria Gram- that forms part of the normal intestinal microbiota of healthy animals. Usually, *E. coli* present in animals are communal antipathogenic strains and they even play a beneficial role, since they compete in several ways with the pathogenic strains in the ecological niche of the lumen.

The term *E. coli* includes around 200 different serotypes that are classified by their antigens of the cell wall (O antigens), of the capsule, (K antigens) of the fimbriae (F antigens) and of the flagella (H antigens).

One of the main characteristics of *E. coli* is its great capacity to adapt to environmental conditions and experiment change. This is why it acquires a resistance to antibiotics much easier than other bacteria.

Colibacillosis is a major cause of illness and death in recently weaned pigs. Usually it is a consequence of enterotoxigenic strains of *E. coli* (ETEC) producing enterotoxins (heat-labile toxin LT) or VTEC types producing heat-stable toxins (STa/Stb) which act totally in the gut and stimulate hypersecretions of mucus and electrolytes. LT is a heavy molecular toxin and easily induces the formation of specific antibodies, which makes it an important component in vaccines. The toxin acts on the enterocytes, activating the adenylate cyclase which stimulates the production of Cl-, Na+ and HCO_3- ions and, consequently, diarrhea due to hyper secretion. This in turn, provokes dehydration, acidosis, and in serious cases, death. The feces of affected piglets have an alkaline pH. The organism also produces fimbrial adhesions, which mediate the adherence of bacteria to the mucosal surface (Table 5).

Sero-group	Fimbriae	Enterotoxin	Haemolysins	Group of pigs
0149	K88	LT and/or STa or STb	+	Nursery and weaned
0157	K88 or F18	LT and/or STa or STb or Stx2E	+/–	Nursery and weaned
08	K88 or K99	LT and/or STa or STb	+	Nursery and weaned
0138	F18	LT and/or STa or STb or Stx2E	+	Weaned
0138	F18	LT and/or STa or STb or Stx2E	–	Weaned
0141	987P	STa	–	Nursery
020	987P	STa	–	Nursery
09	987P or K99, F41	STa	–	Nursery
0101	K99, F41	STa	–	Nursery
045	–	–	–	Weaned

Table 5. Serogroups, fimbriae, enterotoxins and haemolysins of nursery and weaned piglets (Varley M.A. and Wiseman J., 2001).

Fimbriae produced include K88 (F4), K99 (F5) and 987P (F6); F41 and F18 (F107 and 2134P) are less common but some may produce a shiga toxin stx 2E. Age and genetic background seem to determine the inherent susceptibility of piglets to *E. coli*. Pigs are resistant to F18+ *E. coli* at birth but become susceptible after several weeks (Imberechts et al., 1997), whereas K99 oresistance is substantially complete by 2 weeks (Runnels et al., 1980). Resistance is achieved by failure to produce the receptor on epithelial brush-border membranes to which the fimbriae adhere (Francis et al., 1998).

Heritable resistance to colibacillosis caused by K88 and F18 ETEC is well documented but has not been reported with regard to *E. coli*, which produces other adherence fimbriae. ETEC expressing K88 and F18 fimbrial adhesions account for nearly all cases of post-weaning colibacillosis in pigs. Genes for F4 and F18 were identified in 92.7% of all ETEC from PWD (Frydendahl, 2002). F18 fimbriae are typically associated with diarrhea of weaned pigs, whereas F4 fimbriae are associated with diarrhea in nursing pigs as well as in weaned pigs. About 50% of pigs in common breeds inherit resistance to K88 organisms. Pigs genetically susceptible to *E. coli* K88+ account for a high proportion of all colibacillosis and so selective breeding for resistance to K88+ and perhaps F18 could have a significant effect on the economic losses due to *E. coli* infections. Susceptibility to *E. coli* infection is dominant over resistance (Francis *et al.*, 1998)(Francis et al., 1998). Sows with the virulent phenotype do not produce K88 antibody subsequent to oral exposure with K88+ ETEC or K88 antigens but they probably produce circulating IgG anti-K88 antibody following parenteral vaccination(Van den Broeck et al., 1999).

F4 (K88) fimbriae

F4 are flexible fimbriae that occur as F4ab, F4ac, or F4ad variants, but the F4ac variant is by far the most common type that is seen. The 'a' antigenic region is conserved and a second antigenic region is variable and designated 'b', 'c', or 'd'. F4ab used to be detected at relatively high frequency and F4ad was reported to appear in Europe in 1973 (Guinee and Jansen, 1979), but F4ac is now the dominant type, worldwide. For example, in one study examined 44 F4-positive E. coli isolates from pigs with diarrhea for the presence of genes for F4ab, F4ac, and F4ad fimbriae (Choi and Chae, 1999). They found that 96% carried the F4ac fimbrial genes and 4% carried the F4ab fimbrial genes.
Others , Alexa et al. used PCR to show that the F4ac variant was present in 98% of 237 F4-positive porcine ETEC from PWD; F4ab was present in 0.8%, and F4ad in 1.3%. All F4 antigenic types bind carbohydrates on glycoconjugates present on intestinal epithelial cells, intestinal mucus, or red blood cells (Grange et al., 2002). The three varieties represent not only antigenic variants but also exhibit differences in binding specificity as some pigs are susceptible to all three types, some are susceptible to two types (ab and ac or ab and ad), and some are susceptible to a single type (ad or ab) and some are resistant to binding by all three types (Francis et al., 1999).

GUT MICROBIOTA

The gut of an adult pig harbours a wide spectrum of microorganisms with anaerobic bacteria being the predominant species (Maxwell and Stewart, 1995). The number of bacteria in the stomach and the proximal small intestine is relatively few (10^3-10^5 per gram of digesta) due to low pH or short retention time of digesta in these regions (Gaskins, 2001). The number of bacteria in the distal small intestine increases up to 10^8 per gram of digesta. The large intestine including the caecum and the colon is the main site of microbial colonization and its content contains 10^{10}-10^{11} bacteria per gram (Gaskins, 2001).
However, the microbiota within the GI tract of mammals can be considered a metabolically active organ with its wide biodiversity in terms of species and the high number of cells that can reach 10^{14} (Backhed et al., 2005; Macfarlane and Macfarlane, 2004; Murphy et al., 2005). Under normal circumstances, commensal bacteria are an essential health asset with a nutritional function and a protective influence on the intestinal structure and homeostasis.

Studies on the survey of the gut bacterial communities in 60 species of mammals based on 16S rRNA analysis showed that diet, host phylogeny and gut morphology influence the microbial ecology of the gastrointestinal tract (Ley et al., 2008a; Ley et al., 2008b). If mammals are classified as monogastric and polygastric, their microbiota clusters into groups that correspond to these categories. However, the composition of the fecal microbiota is also a strong predictor of the host physiology status. The major microbial groups in monogastric animals (such as pig, chicken, rabbit and man) are Bacteroides, Clostridium, Bifidobacterium, Eubacterium, Lactobacillus, Enterobacteriaceae, Streptococcus, Fusobacterium, Peptostreptococcus and Propionibacterium.

In mammals, the percentage of the different microbial groups varies between individuals, depending on age (Mueller et al., 2006) and on the health/pathological status (Abt and Artis, 2009). Diet is an additional factor influencing the gut microbiota; herbivores contain a higher number of bacterial phyla, while carnivores the fewest number, and omnivores are at an intermediate level.

Resident bacteria may exert a dual function, the stimulation of mucosal mechanisms of defence and the maintenance of the homeostasis of the immune response. Thus there is evidence of a correlation between the composition of the colonizing microbiota and variations in immunity (O'Hara and Shanahan, 2006). The gut microbiota, with its metabolic, trophic and protective functions, is able to affect positively the integrity of the intestinal barrier. Loss of the integrity (i.e., intestinal barrier dysfunction) leads to a progressive increase of intestinal permeability, inducing a switch from “physiological” to “pathological” inflammation that is characteristic of diseases such as intestinal bowel disease (IBD) (Frank et al., 2007; Lambert, 2009). Intestinal pathogens produce toxins and other classes of substances i.e. mucinases, adhesins and invasins, which interfere with epithelial metabolism. All together, the pathogenic phenotype is likely to directly trigger uncontrolled pathological inflammation. Increasing evidence indicates that changes in gut microbiota, with an increase of pathogenic bacteria and a decrease of health-promoting bacteria, such as bifidobacteria and lactobacilli, play an important role in promoting and maintaining intestinal inflammation in IBD (Andoh and Fujiyama, 2006). Physiological and psychological stressors leading to dysfunction of the intestinal barrier and to increase of intestinal permeability, have an impact on gut microbial composition and susceptibility to enteric pathogens (Gareau et al., 2009). Beneficial bacteria, such as lactobacilli and bifidobacteria, have been shown to decrease when stressing factors occur (Si et al., 2004).

Moreover, stress situations generally result in a poor growth rate and productivity in livestock and poultry. As an example, in piglets, weaning is a critical period that involves stressful factors, such as withdrawal from the mother, lack of antibodies originating from the sow's milk and dietary changes. In poultry production systems, birds are routinely subjected to stressors such as feed withdrawal, temperature fluctuations, and confinement during transportation (Humphrey, 2006; Spreeuwenberg et al., 2001; St-Pierre et al., 2003).

AGPs and ALTERNATIVES

Antibiotics are natural substances that are produced by yeasts or moulds, and they inhibit the growth of microorganisms. Antibiotics are traditionally used as therapeutic drugs against microbial infections. It is now well recognized that antibiotics have a growth promoting effect when used as feed additives in domestic animals. Moore, Evenson, Luckey, McCoy, Elvehjem and Hart (1946) discovered this effect when they found that feed supplementation of antibiotics promoted growth in broiler chickens. A similar effect was subsequently observed in pigs. Since the 1950s, antibiotics have been used widely as feed additives in the pig industry.

From this discover antibiotics have been included in animal feed at sub-therapeutic levels, acting as growth promoters (Antibiotic Growth Promoters, AGPs), (Dibner and Richards, 2005). However, worldwide concern about development of antimicrobial resistance and about transference of antibiotic resistance genes from animal to human microbiot (Mathur and Singh, 2005; Salyers et al., 2004) led to banning the use of antibiotics as growth promoters in the European Union since January 1, 2006 (EC, 2001, 2003) The removal of these compounds from animal diets has put tremendous pressure on the livestock and poultry farms, one of the main consequences being a substantial increase in the use of therapeutic antibiotics (Casewell et al., 2003). There is evidence that AGPs have long been effective in prevention of necrotic enteritis (NE) in poultry flocks and that the incidence of NE has increased in countries where AGPs have been stopped (Van Immerseel et al., 2004).

There is the need to look for viable alternatives that could enhance the natural defence mechanisms of animals and reduce the massive use of antibiotics (Verstegen and Williams, 2002). One way is to use specific feed additives or dietary raw materials to favorably affect animal performance and welfare, particularly through the modulation of the gut microbiota which plays a critical role in maintaining host health (Tuohy et al., 2005). A balanced gut microbiota constitutes an efficient barrier against pathogen colonization, produces metabolic substrates (e.g. vitamins and short-chain fatty acids) and stimulates the immune system in a non-inflammatory manner.

Mode of action

Although antibiotics have been used extensively for decades as feed additives to promote animal growth, the mode of action for this effect is still unclear. Possible modes of action, as suggested by Cromwell (2001), include metabolic effects, nutritional effects, and disease control effects. The underlying mechanism is likely to be the alteration of the balance of microflora in the gastrointestinal tract of the host Numerous studies have shown that certain antibiotics influence the metabolic processes in pigs, e.g., the effect of chlortetracycline on water and nitrogen excretion and the inhibitory effect of tetracycline on fatty acid oxidation in liver mitochondria. However, the metabolic effect is unlikely to be the major mechanism by which antibiotics promote growth. The tissue concentration of antibiotics is unlikely to reach an effective level of the dosage of antibiotics that are given as feed additives. Furthermore, many antibiotics that promote am mal growth are not absorbed from the gut. The nutritional effect of antibiotics may be attributable to several aspects. Some microbes that live in the gastrointestinal tract synthesize vitamins and amino acids that are essential to animals, whilst others compete with the host for essential nutrients. The alteration of the microflora population following antibiotic feeding may improve the availability of essential nutrients to the host animal. It is also well documented that antibiotic feeding is associated with a

significant reduction in intestinal wall thickness and rate of passage of food through the small intestine (Ravindran et al., 1984), which may improve nutrient absorption. The most prominent effect of feed supplementation with antibiotics is probably the improvement of animal health. Pigs that are raised in modem intensive piggeries are continuously exposed to harmful stress and suffer from subclinical diseases. It has been reported that feed supplementation with antibiotics reduces the ammonia concentration both in animal tissues and in stock houses (Visek, 1984), and that the supplementation is more effective in a poor and dirty environment than in a clean environment (Cromwell, 2001). However, Hays (1987) reported that antibiotics promoted growth in both conventional pigs and germ-free animals, which suggests the presence of a mechanism other than interaction with intestinal microbes.

Although the use of antibiotics as feed additives has become an integral part of the nutritional strategy for neonatal pigs and other farm animals, safety concerns have been raised since 1968 (Swann, 1969). The major concern is whether the widespread usage of antibiotics in animal feeds will endanger public health through the development of drug resistant enteric bacteria. The drug resistant bacteria may pass on the resistance to pathogenic microbes through plasmid transfer. Such concern has lead to the restriction and even ban of the use of antibiotics in animal feeds in some countries, e.g., Denmark since January 2000. However, it has been argued that banning antibiotic usage as a feed additive does not reduce the total amount of antibiotics that are used in animal production, and only shifts the usage of antibiotics to therapeutic treatments (Mudd et al., 1998). Furthermore, there is no direct evidence that links a definite health hazard in humans with the usage of antibiotics in animal feeds (Animals et al., 1999). Another concern over the usage of antibiotics in animal feeds is the presence of residues of the drug in animal tissue. Certain antibiotics require withdrawal from animal feed for a prescribed period prior to slaughter to avoid the occurrence of residues in edible tissue. Many newly developed antibiotics for usage in animal feeds, such as flavomycin and apromycin, can rarely be absorbed in the intestine, and have little chance of being deposited in animal tissues (Afifi and Ramadan, 1997).

AGPs are mild antibiotics and one of the potential pitfalls in their prolonged use is that they apply genetic selection pressure on the population of non-pathogenic gut bacteria normally resident in the gut lumen and bound to the enterocytes. Over time, this selection pressure will favour those bacterial genotypes that are best able to survive the antibiotic environment. Over long time spans, there is the possibility that extremely virulent pathogenic strains will arise out of this forced evolution and the antibiotics will no longer work as bacteriocides. In such a scenario, presumably the antimicrobial product would also lose its ability to enhance digestion and growth. Another possible route for problems is with the phenomenon of plasmid transfer of bacterial resistance to the antibiotic to other unrelated strains of bacteria that also exist in the general environment.

Withdrawal consequences

The consequences of the withdrawal of growth promoters discussed in this chapter can be summarized as follows: (i) reduced profitability of local farming enterprises; (ii) increased feed requirements to produce the same weight of animal; (iii) increased number of animals to maintain same output of meat; (iv) increased need for drinking water, much of which would be from the mains; (v) increase in slurry output and area of land needed for spreading; (vi) increased need for fossil fuels to transport feeds, greater numbers of animals and slurry; (vii) increased area of tillage to grow extra grain; (viii) increased imports of expensive protein sources; and (ix) increased environmental pollution.

Alternative Tools

There are alternatives to the use of antibiotic growth promoters for creating conditions where animals can exploit their full genetic potential for growth. Some of these have been around for many years and have been well researched. If conventional antimicrobials were to be precluded from use in the animal industries then there may be a significant increase in demand for such products. These alternatives include: (i) improved husbandry (ventilation, stocking density, grouping, all-in/allout production flows); (ii) improving on-farm hygiene status; (iii) use of effective vaccination schemes; (iv) in-feed enzymes to promote digestion; (v) improved nutritional specification to increase nutrient and energy retention; (vi) organic acids to optimize conditions in the gut lumen microenvironment; (vii) use of herb and spice formulations, some of which have been shown to have natural growth-enhancing properties; (viii) probiotics (lactobacillus cultures that can control the conditions and microflora on the gut wall to ensure pathogenic exclusion and improved digestion); (ix) immuno-potentiating agents (either natural nutrients such as vitamin E or other compounds to improve natural mucosal immunity); and (x) prebiotics, amongst which are non-digestible sugars such as oligosaccharide molecules, which can also enhance the processes of digestion.

DEFENCE and IMMUNITY

Mucins

A key component of the mucosal barrier is the production of **mucin** or mucus. These glycoproteins line the surface epithelium from the nasal cavity/oropharynx to the rectum (Belley et al., 1999; Deplancke and Gaskins, 2001). Mucus-producing goblet cells continuously produce a thick barrier covering adjacent epithelium. Particles, bacteria, and viruses become trapped in the mucus layer and are expelled by the peristaltic processes of the gut, nasal tract and lungs. Hence, mucus prevents potential pathogens and antigens from gaining access to the underlying epithelium, a process called non-immune exclusion. Mucins or mucus also serve as a reservoir for secretory immunoglobulin A (**SIgA**). This antibody traverses the epithelium and is secreted into the lumen. The SIgA, present in the mucus layer, binds pathogens and prevents their attachment to the epithelium

Mucin production may therefore be considered a key innate defense mechanism of intestinal epithelial cells. A negative feature of enhanced mucin production is that it may slightly reduce nutrient absorption (Satchithanandam *et al.*, 1990)(Satchithanandam et al., 1990).

So, a defensive firmly adherent mucous gel forms over the gastric surface, composed of 95% water and 5% extensively crosslinked mucin glycoproteins that are products of mucin (MUC) genes (Corfield et al., 2001). It also contains products from acid, bicarbonate, and other secreted proteins, resulting in viscous properties. This mucous gel layer at a minimum serves as an important physical defense against luminal constituents, but may also have a strong role in other pre-epithelial defenses of the stomach and duodenum (Atuma et al., 2001; Wallace and Granger, 1996). The physical structure of the mucus gel is well established.

The predominant proteins and the major constituents of intestinal mucus are high molecular-weight proteoglycans called mucins, glycoproteins that are highly cross-linked. Mucins consist of a central protein core with large numbers of oligosaccharides (accounting for up to 60–80% of the molecular mass) attached to specific regions of the core. The oligosaccharides are variable and complex, and the degree and type of glycosylation of mucins is central to their function. The patterns of glycosylation are tissue specific within the gastrointestinal tact. Mucins can be divided into two groups: secreted, gel-forming mucins (mainly in a form encoded by the *MUC2* gene) and membrane-bound mucins (predominantly encoded by *MUC4*). Goblet cells of the surface epithelium store mucin in apically located granules. Mucin is secreted at a low baseline rate to maintain the mucous coat over the epithelium.

To describe mucins more precisely, B. Jan-Willem van Klinken et al. proposed a definition based on amino acids composition including the following aspects: 1) mucins contain high amounts of proline (P), threonine (T), and/or serine (S) residues [P, T, and S together constitute 20-55% of amino acid (aa) composition], concentrated in one or several major regions of the polypeptide, i.e., PTS regions; and 2) the serine and threonine residues within the PTS regions are heavily O-glycosylated, resulting in 40-80% O-linked oligosaccharides by weight in the mature mucin. The many different oligosaccharide chains explain the inherent heterogeneity of mucin. The functions of all mucins depend highly on their O-glycosylated state (Van Klinken et al., 1995). The dietary composition and microbial flora, as well as interactions between the dietary constituents and the flora, influence the composition and functional characteristics of intestinal mucins (Sharma et al., 1995). Degradation of the carbohydrate chains of mucin glycoproteins involves glycosidases and glycosulphatases produced by specialized strains of normal enteric bacteria, resulting in the release of component monosaccharides that can be used as a source of nutrition by other, larger

populations. Such functional specialization provides an ecological niche for enzyme-producing specialists and is likely to be a contributing factor to microbial diversity in enteric bacterial ecosystems. Bacterial/mucosal cross-talk may lead to changes in gene expression for mucin peptides. For example, the ability of selected probiotic strains of *Lactobacillus* to inhibit the adherence of attaching and effacing bacteria is mediated through their ability to increase expression of MUC2 and MUC3 intestinal mucins (Mack et al., 1999).

Increased production of intestinal mucins occurs in weaned pigs compared with the levels detected in pre-weaned animals. Age-related intestinal glycosylation changes play an important role in modifying the properties of intestinal receptors for dietary constituents as well as commensal and pathogenic bacteria and glycosylation is a major factor governing the adherence of bacteria to intestinal mucins.

Many of the intestinal glycosylation patterns associated with microbial attachment have their basis in only small changes in oligosaccharide chain termination by linked sialic acid, galactose, *N*-acetylgalactosamine or fucose. Terminal galactosyl moieties are common constituents of all intestinal glycoproteins and glycosphingolipids in newborn and suckling pigs . Several of the enterotoxigenic bacterial strains associated with outbreaks of diarrhoea in pigs, including *E*. *coli* K88 and *E. coli* 987P, express fimbriae and/or heat labile toxins that specifically interact with galactosyl structures on intestinal mucosal surfaces (Kelly et al., 1994). Enterotoxigenic *E. coli* infections are common contributing factors to postweaning scours in pigs. Predisposing factors in such infections include: the removal of protective levels of IgA and other beneficial factors present in sow milk; inadequate feed and water intake; inadequate gastric acid secretion; unstable microbiota; and expression of membrane and mucin glycoconjugates that serve as binding sites for enteropathogens (Kelly and Coutts, 2000).

Innate immunty

During their evolutionary development, vertebrates and invertebrates were subjected to selection pressure conveyed by infectious pathogens, which resulted in the early development of the non-specific, or innate immune system (Mushegian and Medzhitov, 2001). Functioning independent to prior exposure to bacterial pathogen, innate immunity can respond to bacterial invasion extremely quickly, and may be considered the ”first line of defense” against bacterial infection. The predominant leukocites that mediate the action of the innate immune system are natural killer cells, mast cells, macrophages and neutrophils, which are derived from myeloid descendants of hemapoietic stem cells that reside in bone marrow. Constituting approximately 50% of the leukocytes found in blood, neutrophils are considered the most active of the cells involved in innate responses, and circulate constantly in the blood.

In the gut, epithelial cells provide the first point of contact for both bacterial and dietary antigens. These cells play a pivotal role in initiating inflammatory immune responses by secreting chemokines and cytokines that promote the activation and recruitment of myelolymphoid effector cells to sites of infection or damage. An important receptor class in bacterial recognition is the toll-like receptor (TLR) (Cario et al., 2000); these receptors recognize signaling pathogen-associated positive patterns (PAMPS) such as gram negative lipopolysaccharide (LPS) and gram positive peptidoglygan and trigger downstream cascade signalling that activate epithelial transcription factors which drive inflammatory gene expression. Gene products including IL-8 and MIP-2α are chemotactic for neutrophils and macrophages (Hang et al., 1999). Epithelial cells also produce antimicrobial peptides referred to as beta-defensins, an important constituent of the innate immune system, that kill bacteria thus limiting their translocation across the epithelial barrier during infection and invasion (O'Neil et al., 1999).

Intestinal inflammation leads to expression of adhesion molecules on endothelial cells lining the tissue capillaries, to which blood-borne neutrophils bind by virtue of complimentary cell-surface receptors that they express (Butcher, 1991). Bound neutrophils infiltrate the tissue via the capillary wall by a process known as diapedesis, which allows the cell to fit through a pore much smaller than its size. After entering the infected tissue, neutrophils also recognize PAMPs via specific cell-surface pattern recognition receptors including TLRs. Neutrophils migrate towards the source of these antigens by a process known as 'chemotaxis' and non-specifically engulf the invading bacteria. Recognition of bacterial antigens activates the neutrophil, resulting in an 'effector' phase characterized by activation of complement system, and secretion of inflammatory agents as such as chemikines and cytokines including interleukin IL-1, IL-6, IFN-γ, tumor necrosis factor TNF-α and reactive oxygen metabolites, all of which have direct or indirect anti-bacterial actions. However although present in low numbers at birth, blood-borne neutrophils do not reach adult levels until 21 days after weaning, also chemotactic mechanism of neutrophils (and macrophages) is reported to be impaired in young pigs, and the complement system may not reach adult concentration until 4 weeks of age (Stokes et al. 1992).

Another component of the innate immune system is the mast cell. Mast cell are present in the lamina propria of the intestine and respond to antigen and non-antigen.dependent stimulation, releasing a broad of bioactive mediators which serve to recruit further leukocytes such as neutrophils, and promote the development of the intestinal inflammatory response (Malaviya and Abraham, 2001; Yu and Perdue, 2001). Mast cells are of particular importance in the pathogenesis of allergic reaction, in which they play a central role.

Adaptive immunity

In addition to the innate immune system, which provides a generic response to repeated bacterial invasion, there is the adaptive immune system, providing what is known as 'acquired' immunity. It consists of two arms: the humoral and the cellular immunity. They are functionally distinct, and their activation depends on the nature of the antigen involved.

Humoral immunity

The activation of the adaptive immune response begins with processing and presenting intracellular antigens to either humoral or cellular arm of the immune system. In the case of humoral immunity, bacterial or other soluble antigens are taken up by specialized antigen-presenting cells (APCs) such as tissue macrophages and dendritic cells, which use proteolitic enzyme to degrade and process the antigen into immunogenic peptides(Kagnoff and Murray, 1987). These peptides are presented on the surface of the APC, associated with specialized antigen-receptor molecules referred to as maior histocompatility complex (MHC) class II molecules. The MHC class II-antigen complex is subsequently recognized by antigen-specific helper T cells. T cells are commonly identified by specific 'cluster of differentiation' (CD) molecules which are expressed on their cell surface – in the case of helper T cells this is CD4, and on this basis helper are often referred to as CD4+ T cells. Antigen recognition by helper T cells allow them to secrete specific lymphokines. These lymphokines stimulate antigen-specific B cells to undergo clonal multiplication and differentiation, producing large numbers of antibody-secreting plasma cells. The immunoglobulins secreted by plasma cells recognize and bind specific antigens associated with the pathogenic agent that initiated the immune response, and effect removal of the agent through such processes as opsonisation and

complement-mediated direct cytotoxicity. The humoral immune system therefore provides a potent, antigen-specific response to extracellular infection.

Cellular immunity

Viral infection, which interfere the cellular machinery of host cell to enable viral replication, necessitates the destruction of the infected cell, using the cytotoxic actions of the so-called 'cellular' immune response. Most somatic cells are susceptible to viral infection, and most are therefore also able to process and present viral antigen to the cellular arm of the immune system. The process of the antigen presentation begins with the intracellular processing of a subset of viral antigens into immunogenic peptides, which are then presented on the cell surface as MHC class I antigen complexes (Jackson and Peterson, 1993). In contrast to the humoral immune system employs MHC class I molecules in antigen presentation, which mediate recognition of antigen by antigen-specific cytotoxic T lymphocyte. Cytotoxic T lymphocytes express the CD8 surface molecule, and are therefore often referred to as CD8+T cells. Recognition of the antigen MHC class I complex 'activates' the cytotoxic T lymphocyte, causing it to multiply by clonal expansion, and to synthesize and secrete bioactive factors that destroy the infected cell. As in the case of humoral B lymphocytes, once activated, the cytotoxic action of the T cell is antigen-specific, meaning it will only kill cells expressing the stimulating antigen in conjunction with the same MHC class I molecules involved in induction of the immune response (Kagnoff and Murray, 1987). The cell-mediated immune response therefore specifically targets and destroys only the infected cells that are the source of viral replication, effectively removing the intracellular pathogenic threat while leaving healthy cells unperturbed.

Toll-like receptors

The fruit fly, *Drosophila melanogaster,* possesses a protein,Toll (named after the German word *toll* meaning "awesome," based on the phenotype of the flies lacking this protein), which was originally discovered as a key regulator in embryonal development (Haller and Jobin, 2004), but was later found also to be required for mounting fly host defense against fungal pathogens (Hugot et al., 2001). Homologues of the *Drosophila* Toll protein with host defense-related functions have been observed in almost all multicellular organisms, attesting to the evolutionary importance of these proteins (Celsus, 1935; Ogura et al., 2001; Stagg et al., 2003). TLRs are single-spanning (type I), integral membrane glycoproteins and members of a receptor superfamily that also includes the receptors for interleukin-1 (IL-1) and IL-18, as well as several cytoplasmic adaptor molecules important for TLR and IL-1 receptor (IL-1R) signaling (Fukata et al., 2005). The group members share a structurally conserved "Toll/IL-1R" (TIR) motif in the cytoplasmic domain, but they differ markedly in their extracellular domain, because TLRs contain numerous leucine-rich repeat (LRR) motifs, whereas IL-1Rs contain three immunoglobulin-like domains (Fig. 42-2). Humans and pigs have similary 10 TLRs (*TLR1-10*), whereas mice appear to have 11 *Tlr* genes (*Tlr1-9, Tlr11,* and *Tlr12*) (Stagg et al., 2003). TLRs fall into several groups based on their degree of sequence homology, including the TLR2 subfamily (TLR1, TLR2, TLR6, and TLR10) and the TLR9 subfamily (TLR7-9) (Fig. 8). The different TLRs are variably expressed by different cell types in the gastrointestinal tract and most other organs (Bilsborough and Viney, 2004), and they can recognize distinct microbial signature structures and activate innate host defense pathways. Individual TLRs are discussed in the order of their historical discovery and the available body of knowledge.

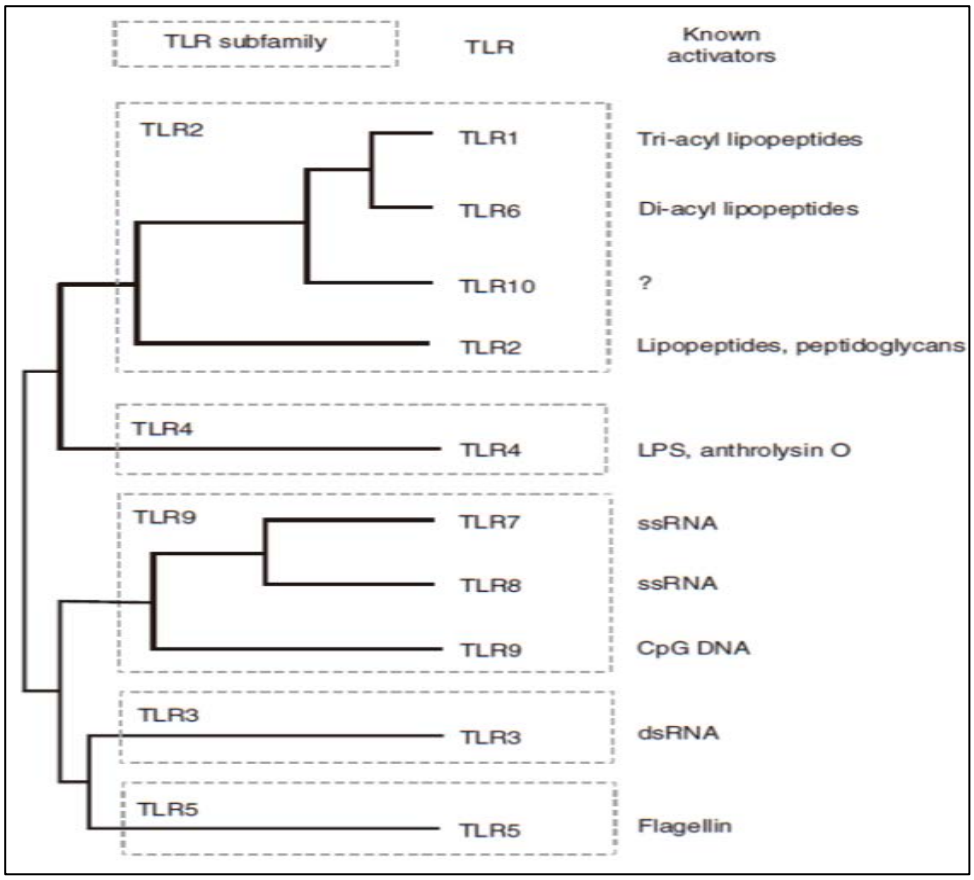

Figure 8. The Toll-like receptor (TLR) family, grouped in five sub family. The 10 TLRs are activeted by different molecules (Johnson L.R., 2006).

TLR4

The prototypic and best-studied mammalian TLR is TLR4, the functional importance of which was first recognized through its identification as the gene responsible for genetic hyporesponsiveness to LPS in certain strains of inbred mice (Turnbull et al., 2005). TLR4 is predominantly expressed by hematopoietic cells, particularly macrophages, dendritic cells, and B cells, throughout the body, although intestinal macrophages have only low TLR4 levels under normal conditions (Hayden and Ghosh, 2004; Ivashkiv and Hu, 2004). Stromal cells in different organs can also express the receptor. For example, endothelial cells isolated from the small intestine, colon, and liver produce functional TLR4 on their surface (Inagaki-Ohara et al., 2003; Mudter and Neurath, 2003), as do intestinal myofibroblasts (Bacon et al., 2002). Intestinal and gastric epithelial cells have been studied extensively for their TLR4 phenotype. Most commonly used human colon epithelial cell lines produce little TLR4 messenger RNA (mRNA) or protein, whereas a few other lines display significant constitutive expression (Papadakis, 2004; Yu et al., 2004). *In vivo,* human intestinal epithelium has low levels of TLR4 normally (Berkes et al., 2003; Hayden and Ghosh, 2004; Inagaki-Ohara et al., 2003), but expression is increased under inflammatory conditions (Ivashkiv and Hu, 2004). Consistent with this, interferon-γ (IFN-γ), whose expression is increased in regions of inflammation, can induce TLR4 expression in certain colon epithelial cell lines (Berkes et al., 2003; Papadakis, 2004) (Berkes et al., 2003; Papadakis, 2004). In the gastric epithelium, TLR4 appears to be expressed constitutively in antrum and corpus, and levels are increased during acute gastritis associated with *Helicobacter pylori* infection (Mumy and McCormick, 2005). TLR4 is required for recognition of LPS, the major constituent of the outer leaflet of the outer membrane of gram-negative bacteria (Fig. 9). LPS is a complex phospholipid composed

of lipid A, core oligosaccharides, and sets of repeating oligosaccharides that make up the O-antigen characteristic of different groups of bacteria. Lipid A, the biologically active component of LPS also termed *endotoxin,* is a phosphorylated disaccharide of N-acetylglucosamine acylated with four to seven hydroxy fatty acids and serves as the hydrophobic membrane anchor of LPS (Fig. 42-5). TLR4 alone is insufficient for the recognition of lipid A and requires a complex with the secreted protein, MD-2 (Liu et al., 2004). Cells that lack either TLR4 or MD-2, or both, are unresponsive to LPS. This point is illustrated in human colon epithelial cell lines, which produce little TLR4 or MD-2. These cells cannot be stimulated with LPS, but forced transgenic expression of TLR4 and MD-2 in combination, but not individually, renders them LPS-responsive (Yu et al., 2004). Although TLR4 and MD-2 are necessary and sufficient for specific LPS recognition, two other proteins, LPS-binding protein (LBP) and CD14, are involved in highly sensitive LPS detection. LBP is a liver-derived serum protein that binds LPS with high specificity and serves to concentrate it and convert it from an aggregate to a monomeric form (Playford and Ghosh, 2005). Only the latter is recognized by the TLR4/MD-2 receptor. LPS bound to LBP is delivered to CD14, which is produced by most macrophages and neutrophils. LBP, acts as a sentinel to concentrate LPS and enhance its recognition by TLR4/MD-2. CD14 is not present in intestinal epithelial cells and is in only a small fraction (<10%) of macrophages in the normal intestinal lamina propria, which is paralleled by a general lack of LPS responsiveness in these cell populations (Meyer-Hoffert et al., 2004; Yu et al., 2004). However, acute colonic inflammation is accompanied by an increased number and proportion of CD14-expressing macrophages in the mucosa, which is probably caused by the influx of CD14+ cells from the circulation (27–29) and is accompanied by enhanced LPS responses of those cells (Quinn and Gauss, 2004). These results indicate that intestinal epithelial cells, as well as intestinal macrophages, are hyporesponsive to LPS in the normal state, which is an attractive concept because it can explain why luminal exposure of the epithelium to LPS from the commensal microbiota is not associated with mucosal inflammation. Although intestinal epithelial cells express low levels of TLR4 under normal conditions, those cells positive for TLR4 express significant quantities of the protein intracellularly in the Golgi apparatus (Pavlick et al., 2002). Accordingly, binding of LPS and activation of cell signaling occurs at the cell surface in macrophages, but appears to require internalization in intestinal epithelial cells (Oz et al., 2005). Engagement of TLR4/MD-2 leads to activation of two major signaling pathways; one pathway is dependent on the central signal adapter molecule, myeloid differentiation factor 88 (MyD88), whereas the other is not (Fig.10). TLR4-mediated activation of MyD88 requires another adapter molecule, TIR homology domain–containing adapter protein (TIRAP; also termed MyD88-adaptor–like, Mal), and proceeds through interleukin-1 receptor–associated kinases (IRAK) 1 and 4 and tumor necrosis factor receptor–associated factor (TRAF) 6, culminating in activation of inhibitor of κB kinase (IKK) and nuclear factor (NF)-κB, as well as mitogen-activated protein kinase (MAPK) and activator protein 1 (AP-1) (Stagg et al., 2003). In a second pathway, TLR4 links to TIR homology domain–containing adapter inducing IFN-β (TRIF; also termed TIR homology domain–containing adapter molecule-1 [TICAM-1]) via the adapter molecule, TRIF-related adapter molecule (TRAM), which leads to activation of interferon regulatory factor 3 (IRF-3), and ultimately production of IFN-β, as well as TRAF6 leading to activation of IKK/NF-κB and MAPK/AP-1 (Facorro et al., 2004). Accordingly, LPS stimulation of TLR4/MD-2–expressing cells up-regulates expression of a wide range of NF-κB and AP-1 target genes, including multiple proinflammatory and chemotactic cytokines, such as IL-8, tumor necrosis factor-α (TNF-α), and IL-6, as well as the type I IFN, IFN-β. These LPS responses are strongly enhanced by the presence of LBP and CD14. In addition to sensitizing cells to LPS activation, CD14 is required for sensing certain LPS chemotypes by TLR4/MD-2, because activation of TRIF and type I IFN production by the minimal LPS structure, lipid A, is CD14 dependent, whereas that of MyD88 and NF-κB is not (Kouloulias et al., 2004). In addition to host defense against specific gram-negative bacterial pathogens, TLR4 may play a role in the regulation of inflammatory responses promoted by poorly defined, and possibly multiple, commensal

bacteria. Several studies have demonstrated that TLR4 deficiency exacerbates experimentally induced colitis in murine models, in which the intestinal microbiota contributes to the development of disease (Vergnolle, 2005; Vergnolle et al., 2004).

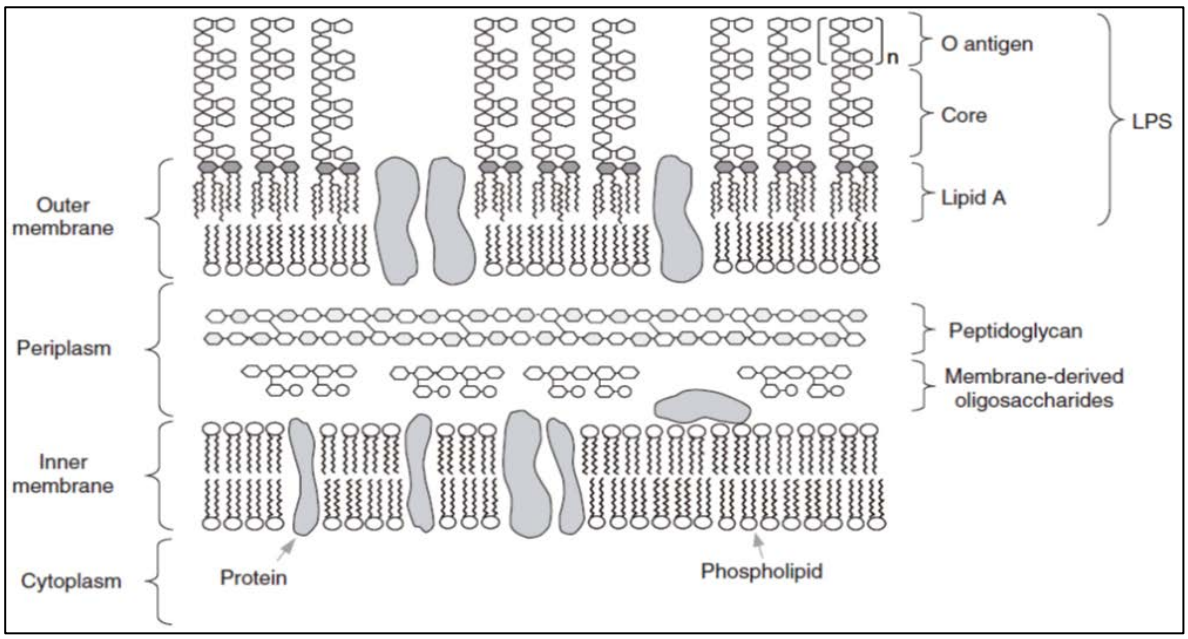

Figura 9. Structure of gram-negative bacterial cell wall. (Johnson L.R., 2006). Lipopolysaccharide (LPS) is composed of three domein: the inner lipid A, core and the most hydrophilic region O antigen.

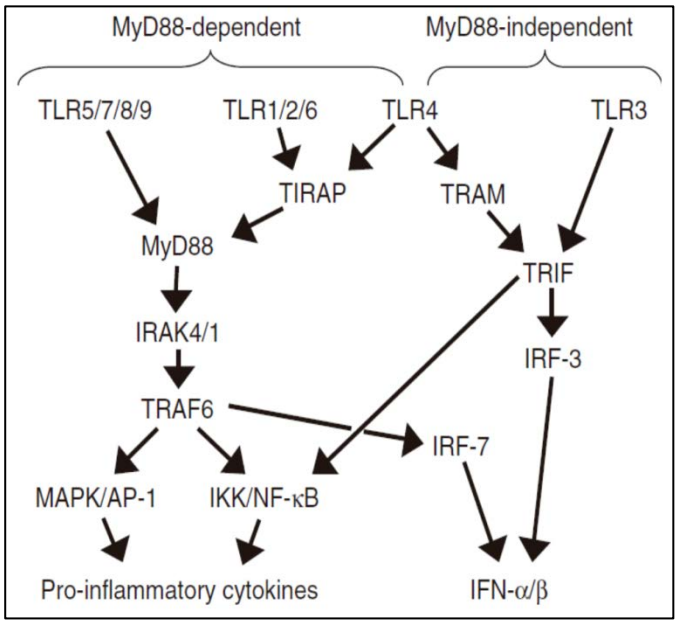

Figure 10. Toll-like receptor (TLR) signalling pathways: MyD88 dependent and no-dependent pathway. (Johnson L.R., 2006)

Immunity and immunoblobulins in pigltes

Piglets are born with little immunity, as the placenta in this species does not allow the passage of maternal antibodies to the fetus. As described by Bailey *et al.* (2001), newborn piglets have essentially no active mucosal immunity . Few clusters of lymphocytes are present in the intestinal mucosa at the time of birth, which subsequently develops into Payer's patches. It is evident that the neonatal pig is highly susceptible to infectious diseases.

During the birth process and early postnatal life, microbes from the mother and surrounding environment colonize the gastrointestinal tract of the infant. Exposure to this microbiota is a major predisposing factor in the anatomical and functional expansion of the intestinal immune system. Bacterial antigens play a very significant role in the proliferation and development of the gut-associated lymphoid tissue (Brandtzaeg, 1996; Helgeland et al., 1996). The mechanisms by which microbes influence the phenotype and function of lymphoid cells are largely unknown but are likely to involve complex events that are probably triggered following the 'normal' route of antigen uptake and processing

While commensals are required for many physiologic processes, including mucosal lymphoid tissue organogenesis and IgA production, the microbiota outnumbers the host cells by 100-fold, presenting a serious risk of the commensals invading and infecting the host. It seems paradoxical that the host is dependent on its intestinal microbiota for the development of mucosal lymphoid tissues in the gut, because the major function of these tissues is to protect mucosal surfaces against invading microbes. This situation might have arisen evolutionarily from the need for the host and its intestinal microbiota to strike a peaceful coexistence, or homeostasis. It has become clear that the principal effector molecule of mucosal immunity, IgA, is also a major arbiter of host-microbial homeostasis in the gut. By binding to commensals, secretory IgA (SIgA) prevents their translocation into host tissues and subsequent initiation of infection and host immune responses (Kramer and Cebra, 1995).

The initial phase of passive immune protection involves the uptake of high levels of colostral immunoglobulins in the early hours of life. The predominant immunoglobulin isotype in colostrum is IgG. Although maternal IgG is protective against many systemic pathogens, most pathogens encountered by the piglet are found at the mucosal surfaces, where IgG antibodies are rare and largely ineffective. A second, longer phase of passive protection, occurring as colostrum formation ends and lactation proceeds, sees IgG concentrations decrease quickly as IgA becomes the major immunoglobulin in sow milk. This maternal IgA provides short-term intestinal protection by neutralizing viruses, inhibiting bacterial attachment and opsonizing or lysing bacteria (Porter, 1986; Gaskins, 1998). Although suckling piglets receive partial protection against those antigens to which the sow has previously developed immunity, they have little or no protection against new infectious agents that may be introduced to rearing units.

During the suckling period, piglets continue to receive a local passive immune protection through milk-borne SIgA. With growing age, the neonate increasingly produces its own SIgA, induced in GALT by intestinal commensals. There is a tendency to distinguish between different types of IgA produced on the intestinal mucosal surfaces of neonates. This somewhat arbitrary categorization is based on experimental detection or non-detection of IgA/antigen interactions. Antigen-specific IgA is easily defined but the use of the term 'natural IgA' is more confusing. In essence, natural IgA designates all IgA produced for which specificity cannot be determined. The production of most natural IgA is thought to be stimulated, not necessarily specifically, by the presence of members of the normal gut flora (Cebra, 1999).

The induction of both high-affinity, organism-specific SIgA and "natural" poly-reactive IgA provides broad protection against bacterial translocation and colonization of host tissues, and thus helps maintain a peaceful coexistence between commensals and their host.

Commensal enteric bacteria engender a self-limiting humoral mucosal immune response while permanently colonizing the intestine. It has been proposed that successful SIgA responses to commensal bacteria can attenuate chronic stimulation of Peyer's patch germinal center reactions even though the bacteria persist in the gut (Talham et al., 1999). The remaining bacteria in the gut are continuously coated with IgA. In the suckling pig it is likely that IgA coating of gut commensal bacteria is supplemented with maternal antibodies. Indeed, maternally derived IgA coating may protect the neonatal immune system from microbial antigen to the point where it delays active development of natural IgA responses (Cebra, 1999).

Bacterial induction of host IgA synthesis is a good example of how mutualism is achieved and maintained between commensal bacteria and their host. The induced IgA maintains homeostasis between the host and the intestinal microbiota by preventing commensal translocation into host tissues and the initiation of host immune responses against the bacteria. This IgA helps maintain a peaceful coexistence between the host and the intestinal microbiota so that both benefit from their interaction. The intestinal commensals gain a stable, nutrient-rich environment, and among the many benefits gained by the host, commensal-induced IgA provides a potent defense against opportunistic pathogens.

IgA and pIgR

Besides microbiota, the mucosal surfaces lining the gastrointestinal tract is continuously bombarded by potentially infectious agents such as bacteria, viruses, fungi, and parasites, in addition to soluble dietary and environmental substances. The first line of specific immunological defense against these environmental antigens is secretory immunoglobulin A (SIgA) (Brandtzaeg et al., 1997; Lamm, 1997), which is produced by selective transport of polymeric IgA (pIgA) across epithelial cells lining mucosal surfaces (Kaetzel CS and K., 2005; Norderhaug et al., 1999). SIgA is the major immunoglobulin in intestinal secretions. SIgA is generated by the concerted action of two distinct cell types: polymeric IgA (pIgA)–producing plasma cells and pIgR-expressing mucosal epithelial cells. About 40 years ago, Tomasi et al.(1965) isolated SIgA and demonstrated that it comprised a dimer of IgA subunits, joined by a small polypeptide called the 'J chain', and covalently bound to a glycoprotein of about 80 kDa [originally designated the 'secretory piece' and now called secretory component (SC)].The J chain is an inherent part of dimers and larger polymers of IgA (collectively called pIgA), as well as pentamers of IgM, but it becomes degraded intracellularly when coexpressed with other immunoglobulin classes (Johansen et al., 2000). Transport of polymeric immunoglobulins (IgA and to a lesser extent IgM) across mucosal epithelial cells is mediated by a transmembrane glycoprotein called the polymeric immunoglobulin receptor (pIgR). Brandtzaeg and Prydz (1984) provided direct evidence for an integrated function of J chain and pIgR in epithelial transport of immunoglobulins, by demonstrating that only polymeric IgA and IgM containing J chain could bind to the surface of intestinal epithelial cells expressing pIgR. Therefore, the pIgR expression level determines the epithelial capacity for pIgA and pentameric IgM export. The pathway of pIgR-mediated transport of pIgA across polarized epithelial cells has now been characterized thoroughly (Fig. 11).

So, pIgA and pentameric IgM are unique antibody isotypes because in addition to their heavy and light chains, they contain the short polypeptide J chain (Fig. 12).

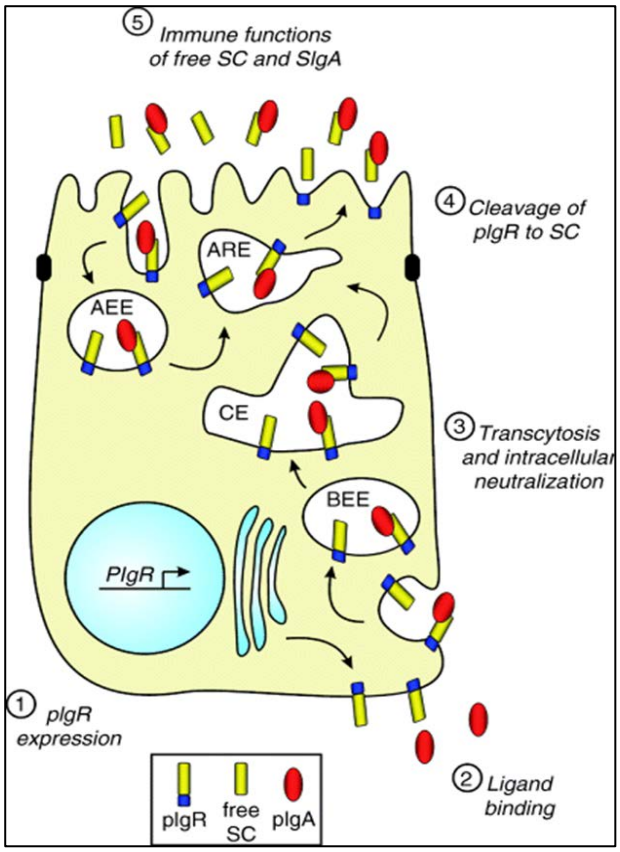

Figura 11. **Schematic rappresentation of the pathway of the polymeric immunoglobulin receptor (pIgR) and its secretory component (SC) through an epithelial cell. At the mucosal surface and in external secretions, free SC and secretory IgA (SIgA) contribute to innate and adaptive immune defense. (pIgA, polymeric IgA; BEE, basolateral early endosome; CE, common endosome; ARE, apical recycling endosome; AEE, apical early endosome). (Kaetzel C.S., 2005).**

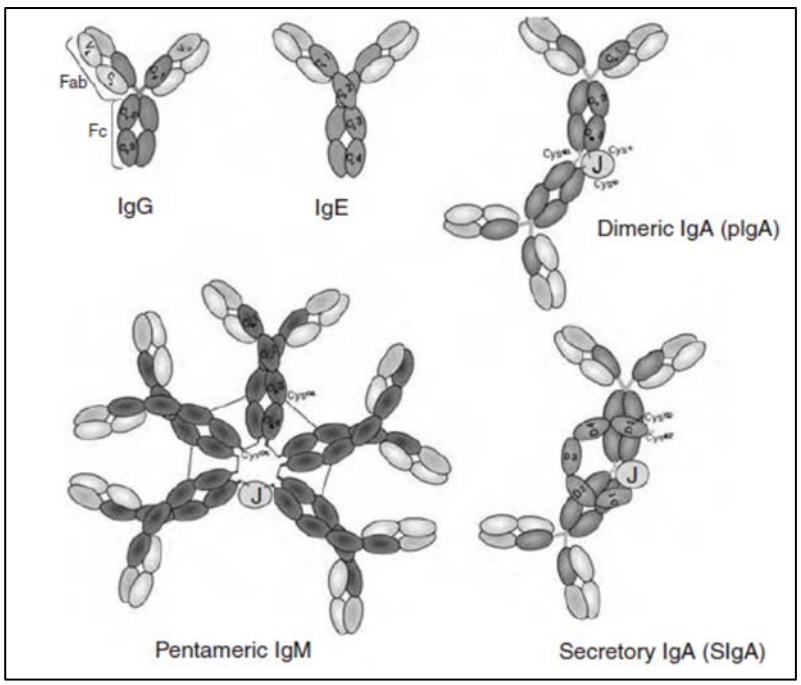

Figure 12. **Rappresentation of the different immunoblobulins. Only pIgA and pIgM contain a J chian polypeptide (Johnson L.R., 2006)**

The J chain is not necessary for polymer formation but regulates the structure and function of the polymers formed. The ability of IgA and IgM to polymerize is determined by features of their Fc region, as well as enzymes that catalyze disulfide exchange reactions. IgA forms mostly dimers when J-chain expression is high, although J-chain–containing trimeric and tetrameric IgA may be generated as well (Vaerman et al., 1998). The number of J-chain molecules per polymer remains a disputed issue. It has often been assumed that pIgA and pentameric IgM contain only one J chain. However, immunochemical studies have indicated that dimeric IgA contains two and pentameric IgM three to four J chains (Johansen et al., 2000). Transcytosis of pIgA by pIgR promotes intracellular neutralization and transcellular excretion of antigens and pathogens and ensures continuous delivery of SIgA to the epithelial surface and external secretions. With each round of pIgA transport, epithelial cells 'sacrifice' the extracellular domain of pIgR as cleaved SC, either free or complexed to pIgA (Fig. 11).

Upregulation of pIgR expression thus would increase the capacity of mucosal epithelial cells to transport dimeric IgA. Regulation of pIgR expression in mucous membranes involves complex interactions among host, microbial, and environmental-derived factors (Table 6). The extensive literature on this topic recently has been reviewed (Johansen and Brandtzaeg, 2004; Kaetzel, 2005).

	Nature of regulation
Cytokine	
IFN-γ	Upregulates pIgR in diverse mucosal epithelia; synergistic with TNF, IL-1, and IL-4
TNF	Upregulates pIgR in diverse mucosal epithelia; synergistic with IFN-γ, IL-1, and IL-4
IL-1	Upregulates pIgR in diverse mucosal epithelia; synergistic with IFN-γ and TNF
IL-4	Upregulates pIgR in diverse mucosal epithelia; synergistic with IFN-γ and TNF
Hormones	
Estradiol	Upregulates pIgR in human and rat endometrium Downregulates pIgR in rat mammary gland
Progesterone	Downregulates pIgR in human and rat endometrium and rabbit mammary gland
Androgens	Upregulate pIgR in male reproductive tract and lacrimal gland in rats, and in human breast and cervical cancer cell-lines
Glucocorticoids	Upregulate pIgR in human breast and colon cancer cell-lines and rat liver and intestine Downregulate pIgR in rabbit mammary gland
Prolactin	Upregulates pIgR in rabbit and sheep mammary gland
Dietary factors	
Retinoic acid (vitamin A)	Enhances upregulation of pIgR by IL-4, IFN-γ, and TNF in a human intestinal epithelial cellline
Microbial factors	
Butyrate	Upregulates pIgR in a human intestinal epithelial cell line
Lipopolysaccharide	Upregulates pIgR in a human intestinal epithelial cell line
Bacterioides thetaiotaomicron	Colonization of germ-free mice with *B. thetaiotaomicron* upregulates intestinal expression of pIgR
Double-stranded RNA	Upregulates pIgR in a human intestinal epithelial cell line
Reovirus	Upregulates pIgR in a human intestinal epithelial cell line
Saccharomyces boulardii	Treatment of rats with *S. boulardii* upregulates intestinal expression of pIgR

Table 6. Regulator of pIgR expression. (IFN, interferon; IL, interleukin; TNF, tumor necrosis factor) (Johansen, 2004).

The observation that pathways for PIGR gene regulation by host and microbial factors appear to converge provides evidence for the coordination between innate and adaptive immune responses in mucosal epithelia. Upregulation of pIgR levels by LPS is significant in light of the recent discovery that pIgR can participate in intracellular neutralization of LPS by epithelial cells (Fernandez et al., 2003). Enhanced uptake and neutralization of LPS by pIgR-pIgA may be an important mechanism by which intestinal inflammation is controlled in the presence of commensal bacteria.

A direct role for intestinal bacteria in pIgR regulation suggests that the innate immune system may 'active' epithelial cells for transport of pIg produced during the adaptive immune response. Host cells mediate

innate immune responses to microbial components through Toll-like receptor (TLR) signaling (reviewed in (Barton and Medzhitov, 2003; Takeda and Akira, 2003) and can activate nuclear translocation of the nuclear factor-κB (NF-κB) transcription factor, which, in turn, activates proinflammatory genes. Intestinal epithelial cells have been shown to express a wide variety of TLRs, the expression of which is upregulated during intestinal inflammation (Cario and Podolsky, 2000; Hausmann et al., 2002).

A prerequisite for intracellular neutralization would be the physical interaction between specific IgA antibodies and the antigen within the epithelial cell.

Emerging research on the complex biology of pIgR highlights the key role of this receptor in bridging innate and adaptive immune responses at mucosal surfaces (Fig. 13). Free SC, produced by transcytosis of pIgR in the absence of pIgA ligand, is an important component of innate anti-microbial defense. Commensal and pathogenic microorganisms can upregulate pIgR expression by signaling through TLRs and probably other receptors, thus enhancing the innate immune response of SC and the capacity of epithelial cells to transport antigenspecific pIgA. Intracellular neutralization of LPS by pIgR-pIgA can limit access of this inflammatory mediator to cells in the lamina propria. Thus, the pIgR plays a central role in innate and adaptive immune defense and maintenance of homeostasis at mucosal surfaces that are constantly exposed to environmental pathogens, antigens, and commensal microorganisms.

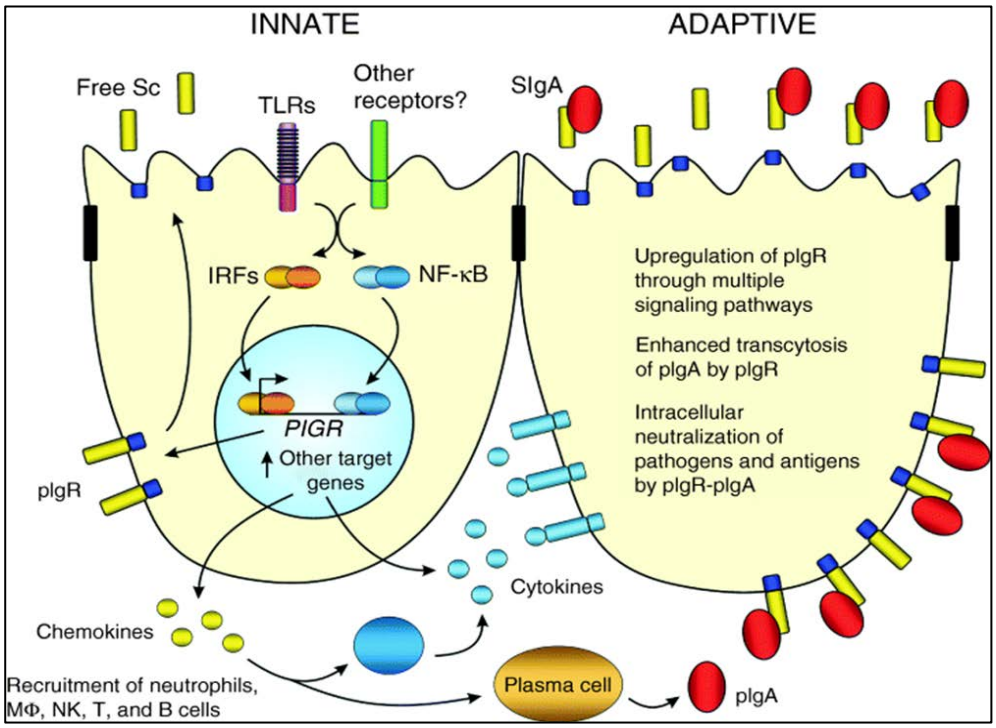

Figura 13. The pIgR bridges innate and adaptive immunity. SC functions as innate immunty component while SIgA as adaptive immune component. (IRFs, interferon regulatory factors; MF, macrophage; NF-kB, nuclear factor-kB; NK, natural killer cell). (Kaetzel C.S., 2005).

Gut associated lymphoid tissue (GALT)

To mount effective immune responses to potentially harmful enteric microorganisms or to maintain tolerance to commensal bacteria and food antigens, the intestinal tract has evolved a complex network of lymphoid tissue within the gut that is involved in antigen transport, processing, and presentation to T cells within the intestinal tissue gut-associated. The mucosal immune system can be divided into two

functionally distinct arms (Brandtzaeg et al., 1999)(Fig.14): organized mucosa-associated lymphoid tissue, which constitutes the inductive sites where antigens sampled from mucosal surfaces stimulate cognate naive T and B lymphocytes, and effector sites, where effector cells perform their action, such as pIgA production by lamina propria plasma cells (Brandtzaeg et al., 1999; Brandtzaeg and Johansen, 2005).

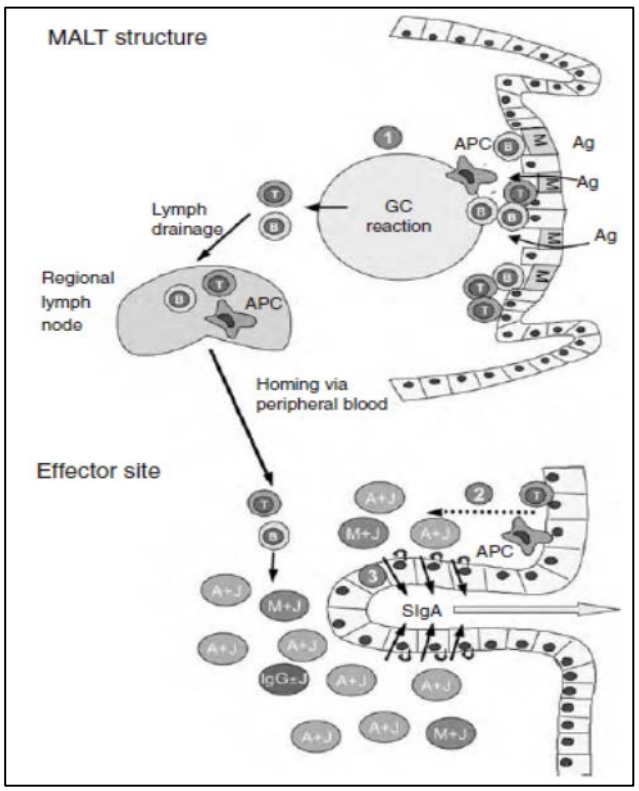

Figura 14. Schematic steps in secretory immunoglobulin A (SIgA) generation. (APC, antigen presenting cell; GC, germinal center; A+J, polymeric IgA;

The gut is the largest antibody-producing organ in humans, and most of its plasma cells are primed initially in the gut-associated lymphoid tissue (GALT). This lymphoid tissue (GALT) is composed of several discrete inductive and effector sites that include the macroscopically visible Peyer's patches and mesenteric lymph nodes (MLNs), as well as microscopic lymphoid aggregates that are scattered throughout the wall of the small and large intestine scattered solitary called isolated lymphoid follicles (ILFs) (Fig. 15) (van Ijzendoorn et al., 2002). The Peyer's patches are "nodules" of lymphoid aggregates immediately underlying the epithelium and are found throughout the entire length of the small intestine. These macroscopically visible structures consist of collections of large numbers of T cells separated by well-defined B-cell follicles. In addition, Peyer's patches are separated from the intestinal lumen by a single layer of columnar epithelial cells called follicle-associated epithelium, or FAE. The space immediately underlying the FAE is referred to as the subepithelial dome (SED). The FAE can be distinguished from the epithelial cells that cover intestinal villi by its less pronounced brush border, reduced levels of digestive enzymes, and the large number of infiltrated lymphocytes (both T and B cells) and antigen-presenting dendric cells. The most characteristic feature of the FAE is the presence of microfold (M) cells (see Fig. 15). The M cells are specialized enterocytes that contain neither surface microvilli nor a layer of mucus that covers all other epithelial cells. The M cells play a critical role in binding invasive microorganisms and particulate antigens, as well as transport of luminal antigens to the SED.

The MLNs are the largest lymph nodes in the body and function to collect and concentrate antigens draining the intestinal mucosa. Naive T cells continuously recirculate from the blood into the MLNs in

search of their cognate antigen. The first step in mounting an effective immune response against enteric microorganisms is the uptake and transport of enteric antigens from the lumen to the Peyer's patches, gut interstitium, and MLNs in the intestine. Transfer of enteric antigens may occur by at least three different pathways:

1. The first and best-characterized pathway involves the uptake and transfer of antigen by the M cells from the lumen to the underlying SED region of the Peyer's patches (van Ijzendoorn et al., 2002). There, the antigen is endocytosed by antigen presenting cells (APCs) called dendritic cells (DCs), followed by migration of the DCs to the B- and T-cell regions of the Peyer's patches. Interaction of antigen-loaded DCs with the different B- and T-cell populations activates these cells to undergo immunoglobulin class switching from expression of IgG to IgA for B cells, and T-cell polarization to different Th subsets. Activated lymphocytes within the Peyer's patches may then migrate to the MLNs via lymphatic vessels, where they may reside for some time during which they differentiate further before entering the blood circulation via the thoracic duct.

2. A second pathway by which luminal antigens may be transported to the gut interstitium or MLNs, or both, is by direct endocytosis of luminal antigens by DCs located in the basolateral area of the villi epithelium. *In vitro* and *in vivo* studies by Rescigno and colleagues (Giffroy et al., 2001; Natvig et al., 1997) have demonstrated that DCs may migrate to the basolateral space between epithelial cells where they send out their pseudopodia apically/luminally between epithelial cells to "sample" the luminal environment. The authors proposed that these APCs can then migrate to the MLNs via the lymphatics, where they present their antigen to naive T cells.

3. Finally, luminal antigens may gain access to the intestinal tissue by simple paracellular diffusion processes from where these antigens may gain access to the systemic circulation and other lymphoid tissue such as the spleen, or they may be endocytosed by interstitial DCs, or both. As described earlier, antigen-loaded DCs will most likely migrate to the MLNs, where they will interact with naive T cells.

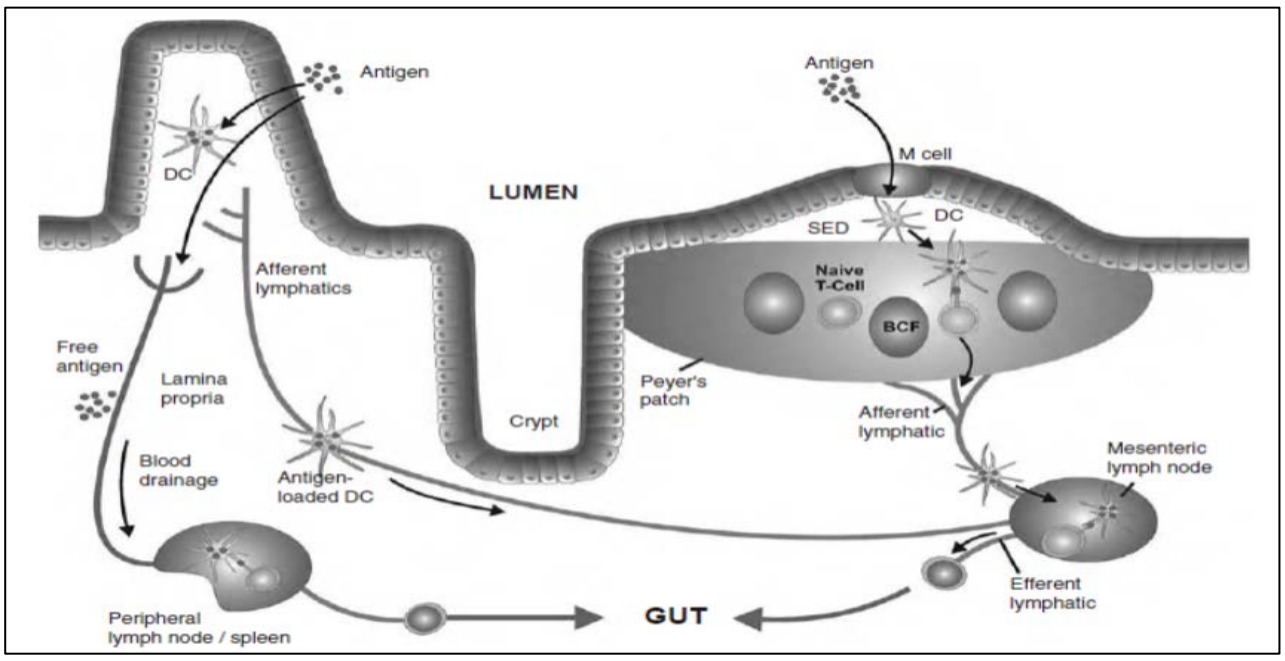

Figura 15. GALT and intestinal immune responses for whom three different pathaways are proposed.

GUT SENSING

Taste percepition

Palatability, in the sense of stimulus that trigger meal initiation, may be an important aspect of feed formulation to improve voluntary feed intake particularly during critical periods such as at weaning and at dietary changes. Food palatability and hedonic value play central roles in nutrient intake. However, post-ingestive effects can influence food preference independently of palatability (Uematsu et al., 2009).
Recently, it was evaluated feed ingredient preferences in piglet diets and showed that the nature of the feedstuffs included in a diet affected feed palatability and voluntary feed intake (Sola-Oriol et al., 2009). Oro-sensorial perception of feedstuffs, such as odour and taste, has evolved to trigger preference for nutritious or aversion for toxic compounds ((Goff and Klee, 2006). Voluntary feed intake of pigs determines nutrient intake levels and has a great impact on efficiency of pork production. Food selection results from the balance among taste, smell and texture perceptions and other factors, including nutritional status, physiology and environment (Garcia-Bailo et al., 2009). The animals, including pigs, learn to associate the sensory properties of foods with internal signals generated after consumption and use this learned information as a key part of determining intake of foods and selection between them. Omnivores have developed preferences or aversions for some nutritional or toxic compounds identified as innate. Even within uniform groups of animals, individuals vary morphologically and physiologically and they also manifest differences in their diet selection and dietary breadth (Villalba et al., 2009). Humans and most non-primate mammals studied to date (except cats; (Li et al., 2005), share significant similarities in the nature and mechanisms of the five primary taste activities defined as sweet, bitter, sour, salty and umami. Sweet taste identifies carbohydrates (energy), umami recognizes amino acids (protein), salt taste targets proper dietary electrolyte balance, and sour and bitter warn against the intake of potentially noxious and/or poisonous chemicals (Bachmanov and Beauchamp, 2007; Bachmanov et al., 2011). Other candidate taste families are being studied such as a taste for complex carbohydrates (Sclafani, 2004), calcium ((Conigrave et al., 2007; Conigrave et al., 2000), hypo-osmotic fluids (Galindo-Cuspinera and Breslin, 2006) and lipids (Gilbertson et al., 2010).
Taste perception variability among animal species may contribute to understand species differences in nutritional and dietary requirements. The recently discovery of taste receptors family could help to better understand food components preferences and consequences on pig physiology related to nutrition.

Gastrointestinal sensing and GPCRs

In general, taste refers to five basic oral perceptions: sweet, salty, sour, bitter, and the taste of starch and umami (or savoury). Taste contributes not only to the selection of foods to be eaten and to palatability (Yeomans, 1998), but also to satiation, thermogenic effects (Brondel et al., 1999) and the 'reward value' of food (Berridge, 2009). Another important role of the taste system is to determine whether a food is nutritious and should be ingested or is potentially toxic and should be rejected (Breslin and Huang, 2006).
The idea that the GI tract may have 'taste' cells that detect nutrients has been around for at least 25 years (Newson et al., 1982). In 1996, Höfer *et al*. showed that α-gustducin, a taste-signalling protein, is located in gut epithelial cells (Hofer et al., 1996). More recently, several investigators reported the expression of bitter, sweet and umami taste receptors in the gut (Dyer et al., 2005; Wu et al., 2002). Bezençon *et al*.

showed that T1R1, T1R2, T1R3, α-gustducin and Trpm5 are expressed in the stomach, small intestine and colon of mice and humans, with the exception of T1R2, which was not detected in the mouse and human stomach or mouse colon (Bezencon et al., 2007).

Recently, several receptors that respond to nutrients or their immediate breakdown products, i.e. fatty acids, sugars, amino acids and proteolytic products, have been identified within the GI tract and serve as chemosensors for food intake (Conigrave and Brown, 2006; Egan and Margolskee, 2008; Engelstoft et al., 2008)(Fig. 16). Interestingly, all these receptors seems to share a common seven α-helical transmembrane structure and a protein G coupled intracellular activation pathway. The current consensus is that luminal contents activate these receptors in the enteroendocrine cell membranes, ultimately leading to increased intracellular Ca2+ levels and subsequently to peptide hormone release (Sternini et al., 2008). For this reason this receptors are also called G protein-coupled receptors (GPCRs). GPCRs constitute a large and diverse family of proteins whose primary function is to transduce extracellular stimuli into intracellular signals (Kroeze et al., 2003). This diversity is dictated both by the multiplicity of stimuli to which they respond, as well as by the variety of intracellular signalling pathways they activate. GPCRs can be classified in six families based on sequence homology and functional similarity ((Attwood and Findlay, 1994; Foord et al., 2005; Kolakowski, 1994)), namely: family A (rhodopsin-like), family B (secretin receptor family), family C (metabotropic glutamate/pheromone), family D (fungal mating pheromone receptors), family E (cyclic AMP receptors) and family F (frizzled/smoothened).

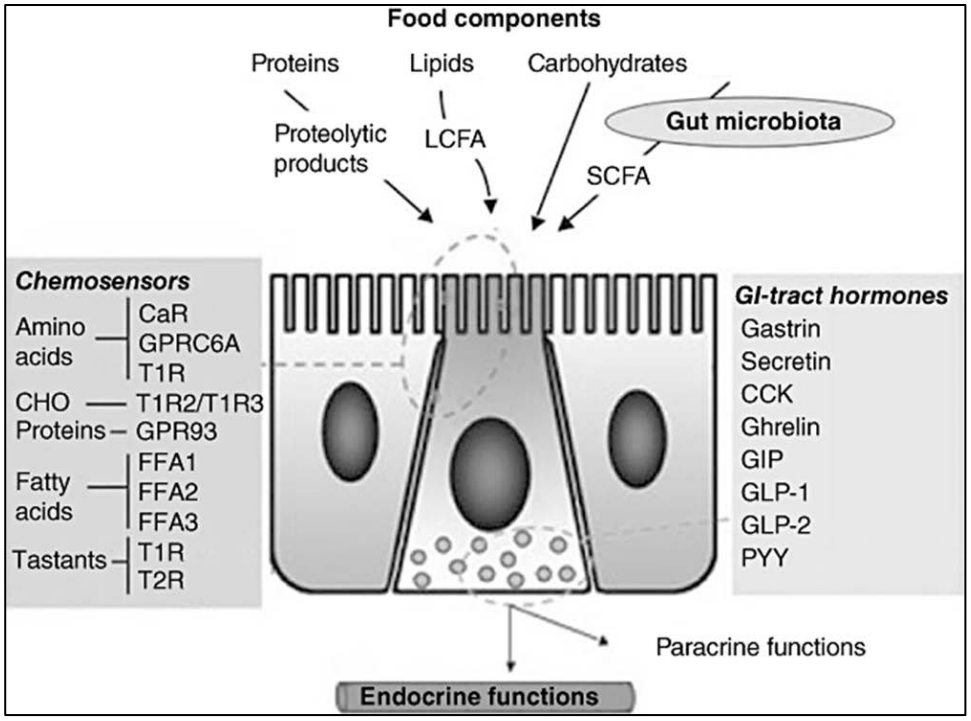

Figura 16. Overview of gastrointestinal nutrient sensing and secretory functions supposed to be related with. (CaR, calcium-sensing receptors; CCK, cholecystokinin; FFA, free fatty acid; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide; LCFA, long chain fatty acid; PYY, peptide YY; SCFA, short chain fatty acid). (Geraedts M.C.P. et al., 2010)

Family A and C have been shown to play a role in nutrient sensing (Brauner-Osborne et al., 2007; Choi et al., 2007a).The so called taste Receptors (TR) belong to class C family and are divided in two sub families : T1Rs, which response to umami and sweet stimuli, and T2Rs, which response to bitter stimuli.

The family A receptors are diverse in their ligand preferences, and several respond to nutrients derived from food, notably protein degradation products and free fatty acids (FFAs). Therefore, the family A receptors can be divided in several subgroups, namely the protein receptors (ex GPR93) (Choi et al., 2007b) and fatty acid receptors (FFAR). Free fatty acids are known to exert a wide range of physiological effects, typically on cellular metabolism and stimulate the release of satiety hormones. A variety of medium- and long-chain FFAs have been identified as ligands for the FFA1 (previously termed GPR40), GRP84 and GRP120 receptors (Briscoe et al., 2003; Hirasawa et al., 2005; Kotarsky et al., 2003; Wang et al., 2006), whereas short-chain FFAs activate FFA2 and FFA3 (previously termed GPR43 and GPR41, respectively) (65–67)(Brown et al., 2003; Le Poul et al., 2003; Nilsson et al., 2003).

The family C receptors consists of several members, such as the metabotropic glutamate (mGlu) receptors, the γ-aminobutyric acid type B (GABAB) receptors, calciumsensing receptors (CaSR), taste receptors (TRs) and GPCR family C, group 6, subtype A (GPRC6A).

These receptors have diverse functions, ranging from neurotransmission by the mGlu and GABAB receptors, regulation of calcium homeostasis by CaSR and often are sensible to more than one agonist.

So, the classification of mammalian promiscuous nutrient-sensing 7TM receptors into different classes is based on sensitivity to the breakdown products from major organic nutrients protein, lipid, and carbohydrate. Because all endogenous agonists for family C receptors are in fact nutrient-like molecule, such as amino acids, sugars and bitter compounds the receptors can be divided into amino acid-sensing receptors and taste receptors. More over family C 7TM receptors function either as homodimers (CaSR and GPRC6A) or heterodimers (T1R1/T1R3 and T1R2/T1R3).

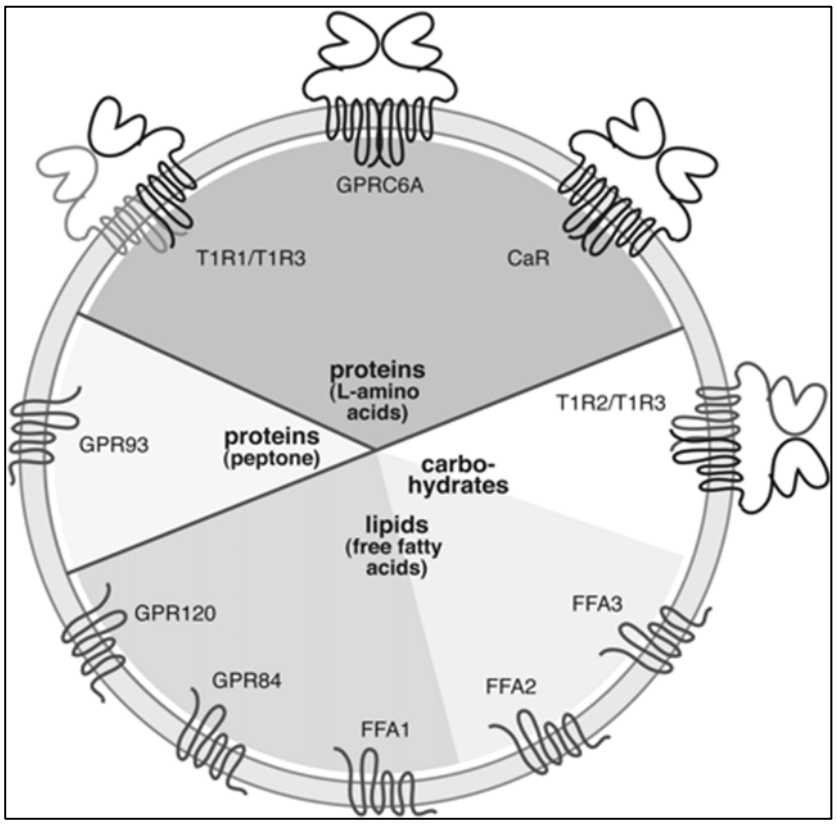

Figure 17. Classification of mammalian nutrient-sensing 7TM receptors into different classes based on sensivity to the major nutrient amino acids, lipid and carbohydrate.

SWEET and UMAMI receptors

The T1R family contains three genes and belongs to the class C family of GPCRs. According with the typical characteristic of all GPCRs receptors, T1Rs share 7 α-helical transmembrane domains, an intracellular C-terminal domain, which allow the signal transduction, and an N-terminal extracellular domain.

The T1Rs receptor family (T1R1, T1R2 and T1R3) possesses a large N-terminal extracellular domain and generates at least two heteromeric receptors: the T1R1/T1R3 and the T1R2/T1R3, for umami and sweet tastes, respectively (Bachmanov and Beauchamp, 2007; Hoon et al., 1999; Nelson et al., 2001). The distinct ligand specificities of T1R1/T1R3 and T1R2/T1R3 receptors imply that T1R1 and T1R2 play more substantial roles in ligand binding of umami and sweet taste receptors than does T1R3 (Nelson et al., 2002). Moreover, T1R3 subunit might also be involved in the taste of calcium and magnesium (Tordoff et al., 2008).

In mammals, there is only one known receptor for sweetness, the heterodimer T1R2/T1R3 (Li et al., 2002; Margolskee, 2002; Matsunami et al., 2000). The sweet TR heterodimer recognizes a large collection of diverse chemical structures like sugars, some D-amino acids, artificial sweeteners and some proteins that have been described as sweet compounds in human, rat and mouse (Li et al., 2002; Nelson et al., 2002). The T1R2/T1R3 double knockout mice do not display behavioural and nerve responses to sweet stimuli, supporting the functionality of T1R2/T1R3 as the sweet receptor. Nevertheless, other studies in single and double knockout mice of T1R2 and/or T1R3 suggested that T1R2 and T1R3 may act as a monomer under high concentrations of natural sugars (Zhao et al., 2003a) or other sweet taste receptors may exist (Delay et al., 2006).

The umami taste, also known as the monosodium glutamate (MSG) taste, is perceived by the heterodimer T1R1/T1R3 and other metabotropic glutamate receptors, mGlu1 and mGlu4 (Chaudhri et al., 2008; Yasuo et al., 2008). The umami receptor is formed by the heterodimeric link of TAS1R1 and TAS1R3. (Li et al., 2002; Nelson et al., 2002). Besides this receptor previously have been proposed additional umami taste receptors. These are truncated forms of metabotropic glutamate receptors mGluR1 and mGluR4, that have been identified in taste sensing tissues of rodents (Chaudhari et al., 2000; Toyono et al., 2003). In any case, the heterodimer formed by T1Rs plays a predominant role in the perception of umami. It has also been demonstrated that heterodimer activation by the L-glutamate is enhanced by the presence of 5'-ribonucleotides (Nelson et al., 2002). Both for the sweet receptors and the umami the range of reactive molecules changes depending on the species. Although in human is perceived only the L-glutamate, the rodents perception is extended to several L-amino acids (Zhao et al., 2003). Mouse models reported that umami responses were abolished or reduced in T1R3 and T1R1 knockout mice (Damak et al., 2003; Zhao et al., 2003a). The main substance eliciting umami taste in humans is L-glutamate, an amino acid widely present in food. In general, the umami taste is mainly related to protein, peptides and L-amino acids, and it is enhanced by 5'-ribonucleotide monophosphates such as inosine (IMP) and guanosine-5'-monophosphate (GMP) (Conigrave and Hampson, 2010).

There is a genetic variation in taste that influences food selection, natural allelic variation and common polymorphisms of T1R's may be responsible of variation in taste responses that may explain sensory differences, within and between populations. Several polymorphisms in T1R3, T1R1 and T1R2 have been described in human (Kim et al., 2006; Raliou et al., 2009) and mouse (Inoue et al., 2007) that may explain differences in dietary preferences. Although the porcine genome is not still available, have recently reported the sequence of the porcine umami T1r3 genes but only partially T1r1 and not T2r (Kiuchi et al., 2006). The porcine umami TRs show higher nucleotide and amino acid sequence homologies with carnivore mammals (e.g. the cat) than with human and mice. Therefore, the extrapolation of umami taste perception from humans and mice may not be a good model for an understanding of the pig taste.

In pigs, the T1R3 (pT1R3) has been shown in several tissues with a similar expression profile than mouse, except for kidney (Fig. 18). Kiuchi et al. (2006) showed that tongue expressed the pT1R3 at a much higher rate than other tissues. Stomach also expressed this gene significantly more than intestine and liver. In general, tissues reported that had the highest level of expression of pT1R3 were tongue (in circumvallate and fungifrom papillae, mucosal epithelial cells, lymphocytes in submucosal tissues of the lingual tonsil and follicular B lymphocytes), testis (spermatogenic cells), and kidney. The presence of this gene in non-taste tissues of pigs suggests that the taste receptors may be involved in the chemosensory function of these organs participating in several behavioural, digestive and metabolic processes.

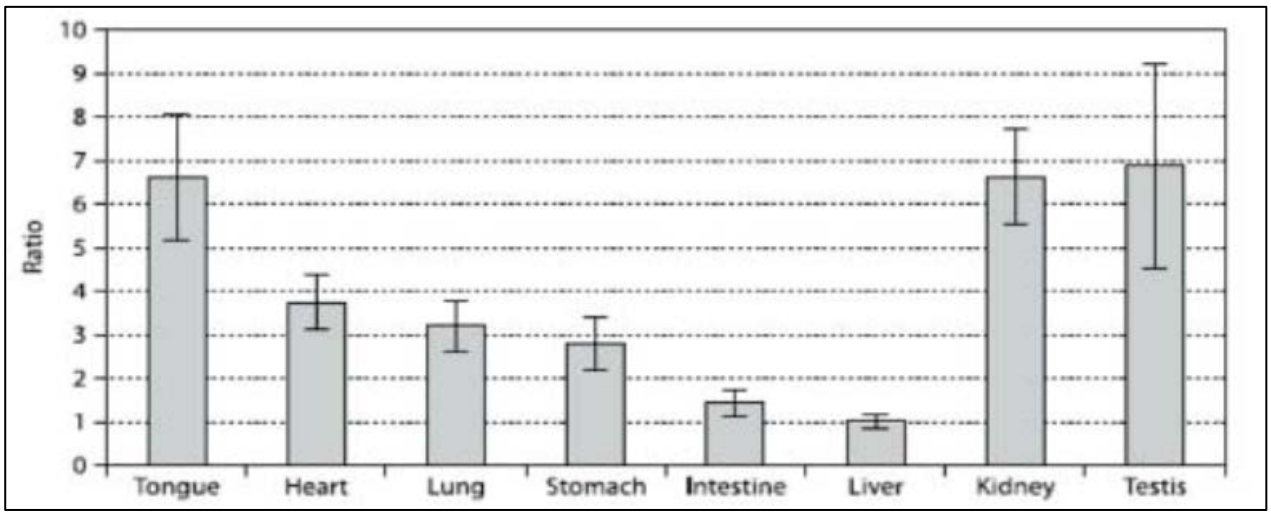

Figure 18. Measurements of T1R3 gene expression in porcine tissue. (Kiuchi et al., 2006)

A mechanism involving TR coupled to G proteins and increases in intracellular Ca+2 induction, has been postulated that would result in a release of peptides that regulate a wide variety of GI functions, and also control food intake through the gut-brain axis (Sternini et al., 2008). (Figure 19).

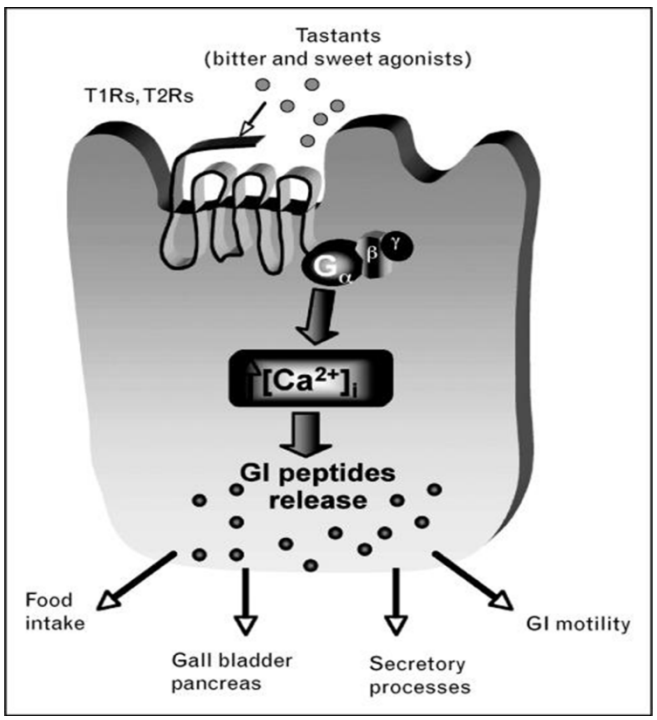

Figure 19. General mechanism involving sweet and bitter taste receptors on enteroendocrine cells. (Sternini et al, 2008)

BITTER receptors

The bitter receptors are a family of multi-GPCR genes called TAS2R (taste receptor, type 2), or T2RS as well (Adler et al. 2000; Chandrashekar et al. 2000; Matsunami et al. 2000, Mueller et al., 2005). So, the T2R family is defined as bitter taste receptors and the number of genes involved differs largely among animal species (Mombaerts 2000; Matsunami and Amrein 2003; Shi and Zhang 2006; Roura et al., 2008). But the pig T2R sequences are not published yet, so there is still no information about them but probably they will be available soon. It has been estimated by gene sequencing studies in rats and humans, that there are about 30 different genes of receptors for bitterness and some pseudo-gene, and that they have a variable amino acid identity from 21 to 90% (Bufe et al. 2002). A complete and updated nomenclature of human and murine TAS2Rs is presented by a recent review (M. Behrens and W. Meyerhof, 2011). The TAS2Rs have the important role to prevent the intake of all dangerous substances that are usually bitter. Given the extensive chemical diversity of compounds detected, it is clear that these receptors recognize a wide variety of different chemical groups. It has not yet been understood how so few receptors can be sufficient to monitor the presence of the many bitter compounds existing in nature. From studies of "deorphanization" of the ligand it seems to be clear though, how the same compound can be detected by different receptors and that some receptors can recognize a wide range of molecules while others have a more selective target (Meyerhof et al., 2010). Furthermore, also the sensitivity level of the detection turns out to differ enormously, with EC50 values induced by quantities of compounds that range from nanomolar levels (Kuhn et al., 2004) to millimolar levels (Bufe et al., 2002). The presence of the taste receptors family in the gastrointestinal tract, bitter together with that used for the "sensing" of sweet and umami is related to the composition of the intestinal lumen content, while their presence in the airways could be related to different function.

Calcium-Sensing Receptor (CaSR)

As the name implies, the primary physiological agonist for CaR is extracellular Ca2_, which is sensed by tissues relevant for maintaining calcium homeostasis such as the kidney and the parathyroid gland (Brown, 2007; Tfelt-Hansen and Brown, 2005), where it helps maintain extracellular calcium homeostasis . However, organs not involved in regulating calcium homeostasis also express CaSR, including nutrient- sensing organs such as the stomach, the lower gastrointestinal (GI) tract, liver and pancreas. The gastric mucosal CaSR is localized to the basolateral membrane of parietal cells and mucous cells (Rutten et al., 1999), the apical and basolateral surface of antral G cells, and most recently its presence was detected on D cells (Ray et al., 1997). Although the function of CaSR in the gastric mucosa is a subject of ongoing research, the multiple substances that modulate its activity, including cations, amino acids, and pH, make it an intriguing candidate for gastric chemosensing. Several reports have demonstrated a role for CaSR in protein/amino acid sensing, including CaSR-mediated L-amino acid-stimulated gastric acid release from stomach parietal cells (Busque et al., 2005), cholecystokinin (CCK) release from duodenal enteroendocrine cells (Hira et al., 2008), and inhibition of parathyroid hormone release from human parathyroid cells (Conigrave et al., 2004). Also just recently, CaSR expression was described in taste tissue of the tongue, suggesting that the receptor may sense calcium and even amino acid taste (San Gabriel et al., 2009).

The most potent amino acids at CaSR in vitro are the aromatic amino acids such as L-phenylalanine and L-tryptophan, followed by aliphatic and polar amino acids, whereas acidic, basic, and branched-chain amino acids are weak or inactive (Conigrave et al., 2000). It is noteworthy that amino acids work as allosteric

enhancers at CaSR, requiring a certain level of extracellular Ca2+. Experiments have detailed that in vivo L-amino acid sensing per se is enabled at physiological concentrations of Ca2+ (around 1 mM) (Conigrave and Hampson, 2006). Gastric CaSR is activated, not only with Ca2+, but even with other cations like Mg2+, which enhances histamine induced, H+/K+ ATPase-mediated acid secretion. Using the potent stimulant Gd3+, CaSR activation can also occur without histamine (Dufner et al., 2005).The stimulation of CaSR in human cultured antral G cells releases gastrin. CaSR knockout mice lack gastrin release in response to luminal Ca2+, peptone, or rise of pH, supporting CaSR as a luminal acid sensor.According with the other GPCR, the receptor relies on extracellular calcium binding and phospholipase C mediated opening of nonspecific cation channel and elevating intracellular calcium (Buchan et al., 2001). For the same reasons, it is believe that this intracellular calcium wave acts as hormone activator in gastrointestinal tract. CaSR can also be allosterically modulated by small synthetic molecules acting at the 7TM domain. Both positive modulators, termed calcimimetics (e.g., NPS R-568, cinacalcet, and calindol), and negative modulators, termed calcilytics (e.g., NPS 2143 and Calhex 231), have been described previously (Kessler et al., 2006). Compounds of this sort may present novel drugs for affecting nutrient sensing by CaSR and subsequent regulating exocrine secretion of various hormones. Because CaSR has been identified on the apical surface of G cells by immunohistochemical staining, it is possible that luminal sensing of calcium by CaSR triggers the increase in gastrin and acid secretion noted after ingestion of calcium-containing antiacids. The CaSR has the ability to detect nutrient amino acids, and thus may serve as the mediator of the gastric phase response, or interfere with other gastrointestinal hormone release.

SIGNAL TRASDUCTION

There is a common transduction pathway for sweet, umami and bitter taste that starts with a ligand binding to the GPCR receptor. Although various components of the taste signaling cascade are predominantly expressed in taste receptor cells, the overall composition is typical for GPCR mediated signal transmission. Activation of taste receptors has been shown to promote rapid changes in the level of second messengers through a bifurcating signaling pathway involving the G protein (Fig. 20).

So, the first molecule activated is the G protein coupled with the receptor which is composed by three subunits called α, β, γ. The activated taste receptor proteins interact with a heterotrimeric G protein complex consisting of a Gα-subunit such as Gα- gustducin, Gα-transducin, Gα14, as well as Gβ3 or Gβ1, and Gγ13 (McLaughlin et al., 1992; Ruiz-Avila et al., 1995; Tizzano et al., 2008). Mice lacking Gα showed reduced responses to bitter, sweet and umami tastants, suggesting that Gα plays a role in taste transduction. The G protein gustducin is proposed to be the more taste-specific signalling molecule (Ruiz et al., 2003).

The α-Transducin (Tα) is another taste-specific signalling molecule expressed in TRs at much lower level, but it seems to be only involved in response to umami compounds (He et al., 2004). After dissociation of the G protein subunits, the Gβγ-subunits activate the phospholipase Cβ2, which, in turn, produces diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3) whereas the α-subunit reduces the intracellular level of cAMP through activation of phosphodiesterases. IP3 activates of the type III IP3-receptor residing within the endoplasmic reticulum membrane results in an increase of the intracellular calcium ion level, which, in turn leads to the opening of a transient receptor potential channel, TRPM5, located in the plasma membrane (Damak et al., 2006; Zhang et al., 2003). The activation of this non-selective cation channel finally results in the entry of monovalent cations, membrane depolarization and generation of action potential of taste cells (Fig. 20).

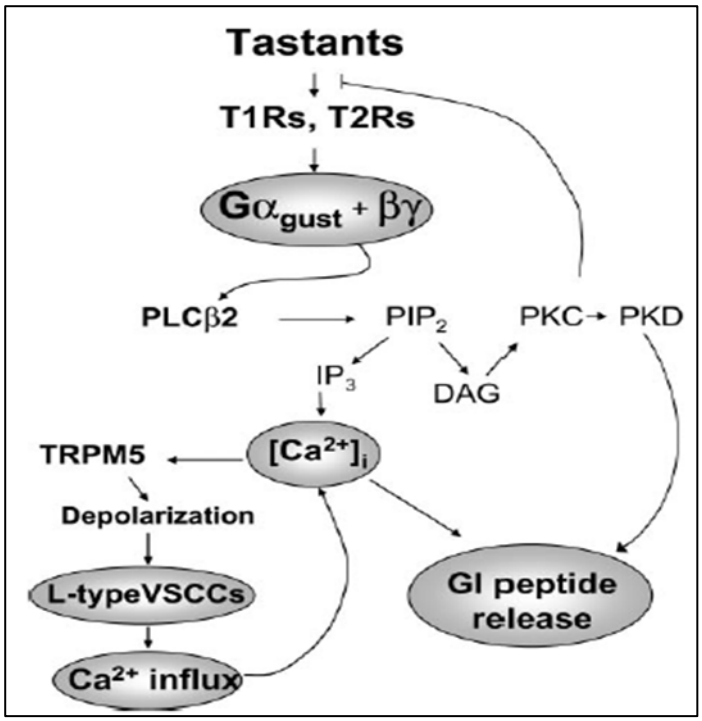

Figure 20. **The overall taste receptor signal trasduction pathway. (Rozengurt E., 2006)**

So, the complex pathways that mediate taste signaling in taste receptor cells are becoming increasingly understood but the link between taste receptors and potential biological effects have to be still completely understood. The expression and function of taste receptors in specific cells of the lining of the GI mucosa and the unraveling signal-transduction pathways that mediate their biological effects open new way for understanding molecular sensing in the GI tract. An hypothetical scheme of correlation between TR and emerging hypotetical GIT biological responses is shown in Fig. 21.

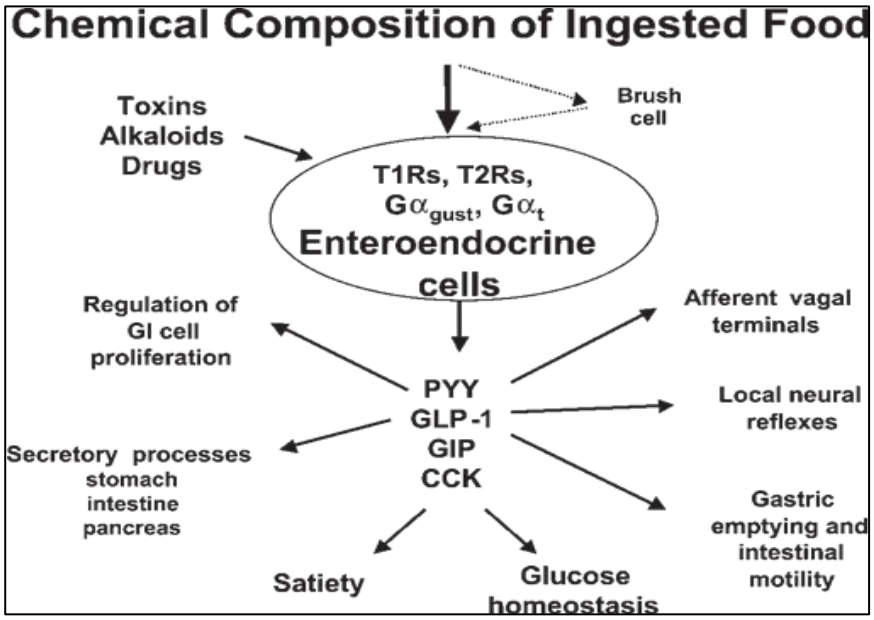

Figure 21. **A scheme showing the potential biological effects elicited by taste receptor activation. (Rozengurt E., 2006)**

OREXYGENIC SIGNAL

GHRELIN

The name *ghrelin* is based on “ghre,” a word root in Proto-Indo-European languages for “grow,” in reference to its ability to stimulate GH release. Ghrelin is a 28-amino acid peptide, in which the serine-3 (Ser3) is *n*-octanoylated, and this modification is essential for ghrelin’s activity. Ghrelin is the first known case of a peptide hormone modified by a fatty acid.

The nonacylated form of ghrelin, des-acyl ghrelin, also exists at significant levels in both stomach and blood (Hosoda et al., 2000). In blood, des-acyl ghrelin circulates in amounts far greater than acylated ghrelin. It is often observed that not only active, but also inactive, forms of peptide hormones exist in our body.

In mammals, ghrelin homologs have been identified in human (Kojima et al., 1999), rhesus monkey (Angeloni et al., 2004), rat (Kojima et al., 1999), mouse (Tanaka et al., 2001), mongolian gerbil (GenBank accession no. AF442491), cow (GenBank accession no. AB035702), pig (GenBank accession no. AB035703), sheep (GenBank accession no. AB060699), and dog (Tomasetto et al., 2001) (Fig. 22). The amino acid sequences of mammalian ghrelins are well conserved; in particular, the 10 amino acids in their NH2 termini are identical. This structural conservation and the universal requirement for acyl-modification of the third residue indicate that this NH2-terminal region is of central importance to the activity of the peptide.

Figure 22. Ghrelin sequence comparison among vertebrate. On the left the pre-proghrelin sequence, on the right the ghrelin sequence(Kojima M., 2005).

The ghrelin receptor is most homologous to the motilin receptor (Feighner et al., 1999; McKee et al., 1997). Accordingly, the amino acid sequence of ghrelin has homology with that of motilin, another gastric peptide with gastric contractile activity (Asakawa et al., 2001; Depoortere, 2001). Alignment of the 28- amino acid peptide ghrelin and the 19-amino acid motilin reveal that they share eight identical amino acids. In fact, after our discovery of ghrelin, Tomasetto et al. (Tomasetto et al., 2000) reported the identification of a gastric peptide, motilinrelated peptide (MTLRP). In 2005, Zhang et al. (Zhang et al., 2005) used a bioinformatics approach to identify a 23-amino acid peptide derived from the ghrelin peptide precursor; this discovery brought exciting new insights to the gut peptide field. The authors named this peptide 'obestatin' because obestatin has the ability to inhibit food intake in mice by intraperitoneal or intracerebroventricular injection. In addition, the authors reported that peripheral injection of obestatin inhibited jejunal contraction, suppression of gastric emptying and decreased body-weight gain (Gourcerol et al., 2006; Holst et al., 2007; Yamamoto et al., 2007). However, their findings could not be reproduced by several groups, and must therefore be interpreted with caution.

Posttraslational modification system

Ghrelin is found in mammalian species as well as non-mammalian species and it is highly conserved throughout vertebrate species (Dieguez and Casanueva, 2000; Kojima et al., 2008). Ghrelin, like many other peptide hormones, is generated from a precursor protein called pre-pro-ghrelin. Human prepro-ghrelin contains 117 amino acids (Kojima and Kangawa, 2005), while porcine ghrelin is derived from a 118-residue prepro-peptide.After removing the signal sequence by cleavage of amino acid-25, the resulting pro-ghrelin peptide sequence (94 amino acids) has an N-terminal glycine residue. The prohormone convertase PC1/3 then cleaves pro-ghrelin after arginine-28, generating the mature 28-amino acid ghrelin peptide (Zhu et al., 2006)(Fig. 23).

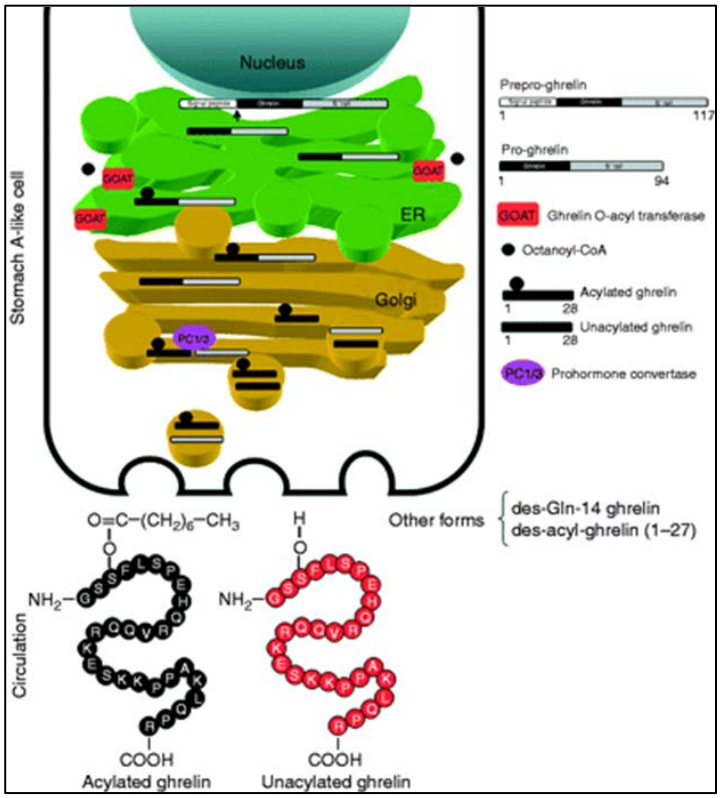

Figure 23. Posttraslational processing and acylation of the pro-ghrelin peptide. (Romero A., 2010).

Recent studies based on different cultured cell lines with different patterns of processing protease expression have demonstrated that not only PC1/3, but also both PC2 and furin can process pro-ghrelin to the 28-amino acid ghrelin (Takahashi et al., 2009). Therefore, ghrelin is the N-terminal fragment generated by cleavage of pro-ghrelin, whereas the C-terminal fragment is called 'obestatin'. To date, there is no clear evidence supporting a role of obestatin in the regulation of energy balance (Chartrel et al., 2007; Gourcerol et al., 2007; Nogueiras et al., 2007), even though previous reports claimed that obestatin had an anorexigenic effect (Zhang et al., 2005). Therefore, the potential role of the C-terminal fragment of pro-ghrelin remains unknown.

So, porcine ghrelin is the 28 amino-acids peptide resulting from the 25‑52 peptide segment of prepro-ghrelin by pro-hormone convertase post-translational cleavage (Fig. 24).

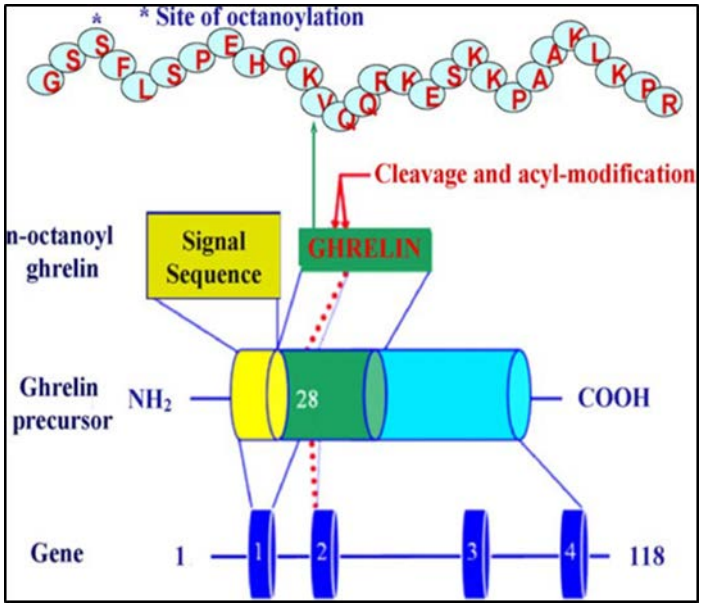

Figure 24. Porcine aminoacid sequence of ghrelin. (Dong X., 2009).

Another posttranslational modification involves ghrelin precursor protein, when serine-3 is acylated with an eightcarbon fatty acid (octanoate). Octanoylation of ghrelin is a specific modification that is required for ghrelin to bind to its receptor GHS-R1a and to exert most of its biological properties, first of all GH release.

The enzyme that catalyzes the acyl-modification of ghrelin was discovered in 2008 by Yang et al. (Yang et al., 2008), using an innovative combination of bioinformatics and cell biology.

Using position-specific iterative BLAST and previously reported sequences of membrane-bound O-acyltransferases (MBOATs) from diverse species including prokaryotes, plants, humans and mice, they identified 16 MBOATs encoded by the mouse genome. They then isolated clones of all of these and tested them for their ability to catalyse octanoylation of ghrelin expressed in heterologous cell lines. Only one of these enzymes, MBOAT4, was found to be able to octanoylate ghrelin, and this enzyme was renamed ghrelin O-acyltransferase (GOAT). Distribution of this enzyme is limited to the gastrointestinal tract and testis, the peripheral tissues that express ghrelin. The optimum temperature of GOAT is 37°C, and its optimum pH range is pH 7 (Ohgusu et al., 2009).

The origin of the modified medium-chain fatty acids (MCFAs) has not been determined. However, it is known that orally ingested MCFAs are directly utilized for acyl-modification of ghrelin (Nishi et al., 2005). Ingestion of either MCFAs or medium-chain triacylglycerols (MCTs) specifically increases production of acyl-

modified ghrelin without changing the total (acyl- and des-acyl-) ghrelin level. When mice ingest either MCFAs or MCTs, the acyl group attached to nascent ghrelin molecules corresponds to those of the ingested MCFAs or MCTs. Moreover, n-heptanoyl (C7:0) ghrelin, an unnatural form of ghrelin, can be produced in the stomach of mice following ingestion of n-heptanoic acid or glyceryl triheptanoate. Thus, it is clear that ingested fatty acids are directly utilized for acyl-modification of ghrelin (Kojima and Kangawa, 2005).
Recently, Barnett et al. (Barnett et al., 2010) described the design, synthesis and characterization of GO-CoA-Tat, a peptide-based bisubstrate analog that antagonizes GOAT. GO-CoA-Tat potently inhibits GOAT in vitro, in cultured cells and in mice.(Zhang et al., 2005)
So, ghrelin is a peptide of 28 amino acids which has two major endogenous forms: a des-acylated form (des-acyl ghrelin) and a form acylated at serine 3 (acyl-ghrelin). Acylation is indispensable for ghrelin to bind its receptor GHS-R1a and serine 3 residue is n-octanoylated with the n-octanoylation at Ser-3 which is essential to stimulate GH release (Kojima et al., 1999)

Distribution

In all vertebrate species, ghrelin is mainly produced in the stomach (Ariyasu et al., 2001). In the stomach, ghrelin-containing cells are more abundant in the fundus than in the pylorus (Date et al., 2000; Yabuki et al., 2004). In situ hybridization and immunohistochemical analyses indicate that ghrelin-containing cells are a distinct endocrine cell type found in the mucosal layer of the stomach (Rindi et al., 2002). Four types of endocrine cells have been identified in the oxyntic mucosa with the following relative abundances: ECL, D, enterochromaffin (EC), and X/A-like cells (Capella et al., 1969; Davis, 1954; Grube and Forssmann, 1979; Solcia et al., 1975). The rat oxyntic gland contains 60–70% ECL cells, 20% X/A-like cells, 2–5% D cells, and 0–2% EC cells; in the human, the corresponding percentages are 30, 20, 22, and 7%. The major products in the granules have been identified as histamine and uroguanylin in ECL cells, somatostatin in D cells, and serotonin in EC cells. However, the granule contents of X/A-like cells were unknown until the discovery of ghrelin. The X/A-like cells contain round, compact, electrondense granules that are filled with ghrelin (Fig. 7) (Dornonville de la Cour et al., 2001). These X/A-like cells account for _20% of the endocrine cell population in adult oxyntic glands. However, the number of X/A-like cells in the fetal stomach is very low and increases after birth (Hayashida et al., 2002).
Ghrelin-immunoreactive cells are also found in the duodenum, jejunum, ileum, and colon (Hosoda et al., 2000; Sakata et al., 2002). In the intestine, ghrelin concentration gradually decreases from the duodenum to the colon. As in the stomach, the main molecular forms of intestinal ghrelin are *n*-octanoyl ghrelin and des-acyl ghrelin.
Ghrelin mRNA is expressed in the kidney, especially in the glomeruli (Gnanapavan et al., 2002; Mori et al., 2000). This result suggests that the kidney is an important site for clearance and/or degradation of ghrelin.
The distribution of Ghrelin mRNA was discovered in hypothalamus, stomach, duodenum, jejunum, ileum, liver, kidney, heart and pancreas of pig aged 90 days and postnatal pig (Yang LY et al, 2004)
Ghrelin mRNA expression was up-regulated 10 days after weaning in the gastric fundus of piglets (Du et al., 2007). Following their initial reports, Yang and his colleagues extracted a 282 bp ghrelin mRNA fragment by RT-PCR from the tissue of pigs, and they cloned this ghrelin gene (Yang LY et al, 2005). Due to the wide expression of ghrelin in human and animal bodies, the ghrelin could be considered as a multifunctional peptide under many physiological conditions

Functions

Ghrelin secreted by the stomach has paracrine or endocrine effects on feeding behavior and GI motility or it circulates in the blood and acts on other target tissues.
Administration of ghrelin to pigs has been implicated in the regulation of food intake, body weight, gastrointestinal (GI) motility and growth hormone (GH) secretion, glucose release, cardiovascular functions, enzyme release, cell proliferation and reproduction in vitro or in vivo (Table 7).

Hormone secretion	
Growth hormone release	↑
ACTH release (weak)	↑
Cortisol release (weak)	↑
Prolactin release (weak)	↑
Insulin release	↑ ? ↓
Anabolic effects	
Appetite	↑
Adipocity	↑
Blood glucose	↑
Gastric function	
Gastric acid secretion	↑
Gastric movement	↑
Turnover of gastric and intestinal mucosa	↑
Cardiovascular function	
Cardiac output	↑
Blood pressure	↓

↑, Stimulate; ↓, decrease.

Table 7. Effects of ghrelin on physiological functions. (Kojima M., 2005).

The most known factor for the regulation of ghrelin secretion is feeding (Cummings et al., 2001). Plasma ghrelin concentration increases when fasting, and decreases after food intake. The factors involved in the regulation of ghrelin secretion have not yet been identified. Blood glucose level may be a most probable candidate: oral or intravenous administration of glucose decreases plasma ghrelin concentration (Shiiya et al., 2002). Because gastric distension by water intake does not change ghrelin concentration, mechanical distension of the stomach alone clearly does not induce ghrelin release (Dzaja et al., 2004; Yildiz et al., 2004). Plasma ghrelin concentration exhibits a nocturnal increase. Plasma ghrelin concentration is low in obese people and high in lean people (Bellone et al., 2002; Rosicka et al., 2003; Tschop et al., 2001). Exogenous GH decreases stomach ghrelin mRNA expression and plasma ghrelin concentration, but does not affect stomach ghrelin stores (Qi et al., 2003).

Appetite regulation

Ghrelin is only a hunger orexygenic signal from peripheral tissues. Intravenous and subcutaneous injections of ghrelin increase food intake; likewise, peripherally injected ghrelin stimulates hypothalamic neurons and food intake (76_81)(Hewson and Dickson, 2000; Nakazato et al., 2001; Ruter et al., 2003; Tschop et al., 2000; Wang et al., 2002; Wren et al., 2001). Because the rate at which peripheral ghrelin passes the blood_brain barrier has shown to be very low, peripheral ghrelin must activate the appropriate hypothalamic regions via an indirect pathway.

The localization of ghrelin receptors on vagal afferent neurons in the rat nodose ganglion suggests that ghrelin signals from the stomach are transmitted to the brain via the vagus nerve (Date et al., 2002; Zhang et al., 2004). As noted above, vagotomy actually inhibits the ability of ghrelin to stimulate food intake. A similar effect is also observed when capsaicin, a specific afferent neurotoxin, is applied to vagus nerve terminals to induce sensory denervation. Moreover, fasting-induced elevation of plasma ghrelin is completely abolished by sub-diaphragmatic vagotomy or atropine treatment (Williams et al., 2003). In summary, ghrelin is secreted primarily from stomach in response to hunger and starvation, circulates in the blood and serves as a peripheral signal, informing the central nervous system (via vagus nerve) to stimulate feeding.

In weaned piglets, ghrelin acted on gastric mucosal cells to stimulate gastric acid secretion in vivo and in vitro. After birth, while the gastrointestinal tract of piglets undergoes substantial developmental changes in structure and function resulting in adaptation to new dietary conditions, GI tract development is often disturbed (Hedemann et al., 2003; Kotunia and Zabielski, 2006; McCracken et al., 1999), so the contribution of ghrelin to gastrointestinal tract development is of value.

If ghrelin can decrease the period of weaning anorexia and increase body weight gain during the weaning period, pigs will potentially be able to improve resistance to pathological and environmental challenges during this period, and fewer days will be required to reach the slaughter weights (Carroll et al., 1998).

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Chapter 1

The effect of a threonine-enriched diet on the growth performance, health, immunity and gastrointestinal functionality, of weaning pigs susceptible or not to *Escherichia coli* K88ac orally challenged with this bacterial strain[1]

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[1] This work was supported in part by Ajinomoto Eurolysine S.A.S.

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Abstract:

Threonine is an important component of mucin and immunoglobulins (**Ig**) which can be affected by *E. coli* infection. The presence of intestinal receptors for the adhesion of the enterotoxigenic *E. coli* K88 **(ETEC**) is genetically controlled in pig. The effect of the susceptibility to ETEC on the Threonine (**Thr**) requirement of ETEC-challenged pigs was studied by a 2 × 2 factorial design. Forty-two weaned pigs were divided into 2 groups using the Mucin-4 (**MUC4**) gene as a marker for ETEC-susceptibility (2 $MUC4^{-/-}$ and 2 $MUC4^{+/+}$ pigs per litter). Within genotype, pigs were fed two diets differing in the standardized ileal digestible (**SID**) Thr : Lysine (**Lys)** ratio: 65% (**Thr-**) *vs*. 70% (**Thr+**). Pigs were orally challenged with 1.5 x 10^{10} CFU ETEC on d 7 and slaughtered on d 12 or d 13. Data were subjected to an analysis of variance with diet, genotype, their interaction, and litter effects. In the 1st week, the Thr+ group consumed more feed ($p < 0.05$). The Thr+ diet alleviated the loss of gain to feed induced by challenge ($p = 0.087$), and increased growth ($p = 0.087$) and gain to feed ($P = .0056$), in the overall trial, compared to the Thr- diet. Before the challenge, the Thr+ group excreted less *E. coli* in feces ($p < 0.05$) while in the post-challenge period the diet did not affect the number of days with diarrhea and the fecal shedding of ETEC. The $MUC4^{+/+}$ pigs responded to the challenge with more days with diarrhea. ETEC fecal shedding, and anti-K88 IgA in blood and jejunal secretum ($p < 0.001$, Vs. $MUC4^{-/-}$ pigs). Among them the Thr+ group had a higher increase of anti-K88 IgA values ($p = 0.089$ in blood and $p = 0.097$ in secretum), than the Thr- group. Total IgA and IgM in blood serum and in jejunal secretum were not affected. The diet did not affect the morphometry of jejunal villous and crypts. The mucin secreti\ng Goblet cells were more numerous in each villus with the Thr- diet than with the Thr+ ($p < 0.05$). No statistically significant difference was seen for the Goblet cells count in the colon and for the quantification of mucins in the scrapings of jejunum and of colon. The trend of improved growth in the whole trial may result from the combination of different partial effects of Thr: a better initial feed intake, an improved immune response and a better control of gut microbiota. It was concluded that a SID Thr : Lys

ratio at 70% is advisable for pigs in the first two weeks after weaning, whatever the genotype for ETEC susceptibility.

Key words: diet, enterotoxigenic *Escherichia coli* K88, pig, threonine, weaning

Introduction

Post-weaning is a stressful condition and requires optimal nutrients supplies to maintain the piglet performance. The appropriate tuning of essential or conditional amino acids can contribute to keep efficient the gut barrier (Lallès et al., 2009), but in this period the pig is more prone to several intestinal pathogen microbes. Nevertheless the development of the disease from some *Escherichia coli* requires appropriate receptors for the adhesion of their fimbriae, that depend on pig genotype (Gibbons et al., 1977; Frydendahl et al., 2003; Yang et al., 2009). This group includes the enterotoxigenic *Escherichia coli* F4ac (**ETEC**), the pathogen most frequently associated with pig colibacillosis. The exact nature of its receptor is not known, but a polymorphism of the Mucin-4 (**MUC4**) gene, linked to the gene of the susceptibility to ETEC, allows the screening of pigs (Jørgensen et al., 2003). The MUC4$^{-/-}$ genotype is not susceptible, while the two other genotypes are susceptible (Jensen et al., 2006).

This gives the opportunity to improve knowledge on the amino acid requirements of post-weaning pigs and on implications of the interaction between different genotypes and the dietary amino acids. There is already evidence that susceptible ETEC challenged pigs have a higher tryptophan requirement, as compared with not-susceptible challenged subjects (Bosi et al., 2009). Threonine (**Thr**) is a relevant amino acid for the maintenance a the gut barrier. Particularly Thr is the first constituent of the mucins produced by the intestinal mucosa (Van Klinken et al., 1995), and thus contributes to the formation of mucus gel, favouring lubrication and physical covering against microbes . Secondly Thr is a major component of plasma g-globulin of several animal species (Tenenhouse and Deutsch, 1966). Our goal was to assess the effect of two Thr dietary additions on growth performance and health of newly weaned pigs differing for the MUC4 gene and were orally challenged with ETEC.

Material and methods

Animals

The protocol of Jensen et al. (2006) was used to screen fifty-six sows and their mating boars for the MUC4 polymorphism. Briefly, the DNA extracted from the bulb of bristles of each pig, amplified by PCR reaction, was digested with XbaI. The resistant allele ($^{-}$) remains indigested by XbaI, while the susceptible allele ($^{+}$) presents two digested fragments. The litters from heterozygous parents were then considered and sampled, to select homozygous pigs of the resistant and sensitive types (2 $^{-/-}$ and 2 $^{+/+}$ pigs within each litter) with the same procedure. The selected forty-eight pigs were weaned at 24 $\pm$ 2 d (7.70 $\pm$ 0.93 kg live weight), assigned to two different diets within each genotype per litter, and housed in weaning rooms with controlled temperature and ventilation. Pigs were individually penned in cages, except the two first days when they were kept in groups of three, to improve their adaptation and feed intake.

Diets and Experimental Design

The piglets were fed the same base diet (standard prestarter diet) without antimicrobials, zinc oxide, or any kind of growth promoter (Table 1). The diet was varied for Thr to Lysine (**Lys**) ratio (65% or 70%, standardized ileal digestible basis- **SID**), by a supplementary addition with L-Thr. The analyzed content of amino acids of the two diets is presented in table 2.

Thus the design of the experiment was factorial, considering, two different genotypes ($MUC4^{-/-}$ Vs. $MUC4^{+/+}$) and two SID Thr to Lys ratios in the feed.

Experimental Procedure

The trial was performed in 2 batches (24 pigs per batch). About one month elapsed between the first and the second batch. All the pigs were supplemented for 3 days (d 0 to d 3 morning) with colistin, just in order to ensure similar health conditions in the gut. Feed and tap water were continuosuly accessible during the trial. On d 7 (31-d-old pigs), all the pigs received 1.5 mL suspension containing 10^{10} CFU of *E. coli* F4ac O149/mL. The inoculum, provided by the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia, was isolated from pigs with colibacillosis, grown in Luria broth and further prepared as described by Bosi et al. (2004)..

Pigs were sacrificed on d 12 (+ 5 after challenge) or d 13. The animals were deeply anaesthetized with sodium thiopenthal (10 mg/kg body weight) and sacrificed by an intracardiac injection of Tanax® (0.5 mL/kg BW) (Intervet Italia, Peschiera Borromeo, Italy).

The procedures complied with the Italian law pertaining to experimental animals and were approved by the Ethic-Scientific Committee for Experiments on Animals of the University of Bologna.

Experimental Observations and Measurements

Pigs were weighed individually at the start of the trial, on day 7 and at sacrifice. Individual feed intake of each pig was registered. Severity of diarrhea was daily characterized by using the fecal consistency five-point scoring system five-point scoring system, where 1 = hard, 5 =watery faeces.

Per each pig, a sample of blood was obtained on d7 and at the end point. The serum was obtained by centrifugation (3,000 × *g* for 10 min), incubated at 56°C for 30 min and stored at −20°C until the analyses for the quantification of anti k88-Immunoglobulin A and M levels (as reported by Bosi et al., 2004) and total IgA and IgM (Evans et al., 1980), by ELISA method. An individual fecal sample was obtained also on d7 and d10 for the determination of total *E. coli* and K88 plate counts, as reported by Bosi et al. (2004); for ETEC, the serially diluted samples were grown on a violet red bile agar medium (containing 0.1 g/L of 4-methyl-umbelliferyl-β-glucoronide), and the the presence of the K88 antigen was assessed by agglutination test with rabbit immune sera against ETEC.

At sacrifice, stomach and intestine were removed and weighted full and empty.

Per each pig, samples of the whole mucosa were for histological measurements, and mucosal scarpings for total mucin quantification (according to Law et al., 2007 controllare con Luisa), phenotype for adhesion of k88 to the villi (see Bosi et al., 2004) and the quantification of IgA and IgM in gut secretum, were obtained on a segment of the jejunum at 75% of the small intestine length An individual sample of colon (picket in the central flexure) was also collected for the same histological measurements and for total mucin quantification.

Histological Analyses

Samples of jejunum were prepared and measured for intestinal morphometry, as described by Bosi et al.(2009). Briefly, formalin-fixed and paraffin wax-embedded samples were deparaffinized in xylene and stained with hematoxylin-eosin; the height of 10 villi and the depth of 10 crypts were measured per each sample. The mucosal-to-serosal amplification ratio M was calculated as ndicated by Kisielinski et al. (2002). The number is based on mean values of villus surface (calculated using length and width of the villus), mucosal unit bottom (determined by villus and crypt width), and villus bottom (determined by villus width):

M = (villus surface + unit bottom – villus bottom)/unit bottom

Where, villus surface = π · (villus length · villus width), unit bottom = π · (villus width/2 + crypt width/2)2, and villus bottom = π ·(villus width/2)2.

Colon samples were processed as jejunum samples.
For histological observation of the goblet cells in the jejunum and in the colon, Periodc acid Schiff (PAS) method (Mc Manus & Mowry, 1960) was used. For the each subject, the number of goblet cells was counted in 20 villous and in 20 intestinal glands, respectively for jejunum and colon; only villi and intestinal gland perpendicular to the mucosal surface were considered suitable for morphometry.

Statistical Analysis

All the individual data were analyzed by analysis of variance using the GLM procedure of SAS (SAS Inst., inc., Cary, NC) with a factor design, including batch and sows within batch, diet, and genotype for MUC4. Data of rectal body temperature were analyzed with the MIXED procedure of SAS, with the option for repeated measures. In cases of *p*-values <= 0.10 for the interaction, the Tukey test for multiple contrasts was used.

Table 1 Ingredients (%) and predicted composition of the basal diet

Feed component	%	Item	% or otherwise
Barley	28.402	Crude protein	17.86
Corn extruded.	33	Crude fat	5.75
Soybean debittered	15	Crude fiber	3.10
Soybean meal 48% CP	5	Ash	5.85
Spray-dried milk whey	11	NDF	11.45
Soybean oil	1	Ca	0.696
Lard	2.5	P tot	0.628
Calcium sulphate	1	P available	0.363
Monocalcium phosphate	1		
Mineral and vitamin premix	1.00	DE, Mcal/kg	3.530
HCl-Lysine	0.52	NE, Mcal/kg	2.579
DL-Methionine	0.222		
L-Threonine	0.226		
L-Valine	0.120		
L-Tryptophan	0.010		
	100		

[1] Provided per kilogram diet: vitamin A, 15,000 IU; vitamin D_3, 2,000 IU; vitamin K_3 2 mg; vitamin E, 50 mg; vitamin B_1, 2 mg; vitamin B_2, 4 mg; vitamin B_6, 4 mg; vitamin B_{12}, 0.04 mg; niacin, 55 mg; biotin, 0.15 mg; d-pantothenic acid, 30 mg; folacin, 2 mg; choline chloride, 200 mg; iron (as $FeSO_4$), 150 mg; zinc (as $ZnSO_4$), 100 mg; copper (as $CuSO_4$), 150 mg; manganese (as $MnSO_4$), 70 mg; iodine (as KI), 1 mg; selenium (as Na_2SeO_4), 0.3 mg.

Table 2. Analyzed amino acid contents of the diets (as-fed basis, g/100 g)

Item	Basal diet	High-Thr diet
Lys	1.26	1.30
Trp	0.306	0.293
Met	0.426	0.425
Cys	0.261	0.268
Thr	0.85	0.90
Free Thr	0.23	0.28
His	0.39	0.40
Ile	0.69	0.71
Leu	1.29	1.32
Phe	0.80	0.81
Tyr	0.56	0.58
Val	0.89	0.90

Results

After the challenge, 7 and 8 MUC4+/+ pigs died, respectively for the basal and high Thr groups. All of them were showing the symptoms of colibacillosis before the death. These subjects were not included in the results.

Growth Performance and in vivo ***Measures***

In the first week the high Thr group voluntary consumed more feed ($p = 0.016$), without significant effect on ADG and G:F (Table 3). ADFI and ADG were powerfully depressed by the *E. coli* K88 infection in ETEC susceptible pigs ($p < 0.0001$) after the challenge, while the higher Thr supplementation did not significantly improve growth, but partially prevented the loss of G:F induced by the challenge ($p = 0.087$). The effect of challenge marked negatively all the pig performance in $MUC^{+/+}$ pigs, for the whole period of trial. More dietary Thr tended to increase overall ADG ($p = 0.087$) and G:F ($P = 0.056$), without effect on ADFI. No interaction between the dietary treatment and the polymorphism for MUC4 was seen for performance parameters.

Rectal body temperature before the challenge was not changed by the experimental factors (Table 4). Values on 5 h and 10 h after the challenge were analyzed with the procedure for repeated measures and covariated for the measure before the challenge. A time x Thr interaction was seen ($p = 0.026$). No effect of the diet was seen on the 5 h measure, and rectal body temperature was close to the one measured before the challenge. Conversely a raise was seen for the 65% group after 10 h ($P < 0.05$), while this variation was prevented in the 70%. Thus rectal temperature was higher in the 65%, than in the other ($p < 0.01$)

Average faecal score and days with diarrhea during the first five days post-challenge were hardly affected by the polymorphism for MUC4 ($p < 0.001$), but not by the diet (Table 5). On the day 7, before the ETEC challenge, the higher Thr supplementation reduced the counts of total E. coli in faeces ($p = 0.024$), while no E.coli K88 was detected in the samples, whatever was the experimental treatment. The counts of total. E. coli and of E. coli K88 were hardly increased in ETEC susceptible pigs on d 3 after the challenge, compared with not susceptible ($p < 0.0001$), but no effect of the diet was detected.

Immunoglobulin in Blood Serum and Jejunal Secretum

Anti-k88 IgA content in the blood serum after one week and immediately before the challenge was not affected by the diet (Table 6). After the challenge $MUC^{+/+}$ pigs responded strongly with a rapid raise of the anti-k88 IgA both in the blood serum and in the jejunal secretum ($p < 0.001$). However it was also seen an interaction between the genotype and the diet: The 70% SID Thr to Lys ratio increased anti-k88 values in the blood serum and in the jejunal secretum of $MUC4^{+/+}$ pigs ($p = 0.089$ and $p = 0.097$, respectively) while no effect of the diet was seen on $MUC4^{-/-}$ pigs.

Anti-k88 IgM content in the blood serum and in the jejunum secretum were never affected by the diet, nor the values in blood serum were changed by the MUC4 genotypes. Anti-k88 IgM values in jejunum secretum were strongly higher in $MUC4^{+/+}$ pigs ($P < 0.0001$), as compared with $MUC4^{-/-}$. Concerning total IgA and total

IgM, no effect of the diet and of the genotype was seen on blood samples obtained before and after the challenge and at sacrifice on jejunal scrapings.

The weight of the gastric content calculated on live weight was reduced in $MUC4^{+/+}$ pigs (p =0.002), but not changed by the diet (Table 7). There was an interaction for the percentage of empty intestine: within 70% diet, $MUC^{+/+}$ pigs tended to have relatively heavier intestine, than $MUC^{+/+}$ pigs, while no difference was seen for the diet 65%. The percentage of the intestine content and the percentages of the empty stomach were not changed by the treatments.

Morphological Measurements of the Small Intestinal Mucosa

Table 8 presents the effect of dietary Thr to Lys ratio and of genotype for MUC4, on the intestinal morphology and on the mucin quantification in the intestinal scrapings of ETEC-challenged weaning pigs.
The morphometry of villous and crypt of jejunum was in general not affected by the diet, except that a trend of interaction Thr x MUC4 was seen for crypt depth (p = 0.091), however no statistically significant difference was seen within the different genotypes, when varying the diet. The mucin secreting Goblet cells tended to be more numerous in each villous with the 65% diet than with the 70% (p = 0.092).
In the colon an interaction Thr x MUC4 was seen for gland depth (p = 0.046). However no significant difference was seen when the test for multiple contrasts was applied. The number of Goblet cells tended to be higher in $MUC4^{+/+}$ than in $MUC4^{-/-}$ (P = 0.104), while the counts were not affected by the diet.
The diet did not affect the quantification of mucins in the scrapings of jejunum and of colon. The values of mucin concentration in jejunum tended to be higher in $MUC^{+/+}$ pigs, than in the other genotype (P = 0.096).

Discussion

The improvement of feed intake in the first week (before the challenge) indicated that the adoption of the 70% Thr to Lys ratio in the feed is safer, when the consumption of feed is influenced by the stress of the weaning. The effect of different SID or True Ileal Digestible Thr dietary supply on the performance of pigs has been recently object of several papers, but only few were related to weaning pigs (Lenehan et al., 2004; Fernandez and Stratez, 2009; Wang et al., 2010), and none reported the results for different weeks after the weaning. The effect on feed intake could be related simply to the known reduced preference for a diet that is deficient in one or more essential amino acids. This has been also evidenced for Thr in rats (Fuerté et al., 2000). However the range of variation of Thr to Lys ratio in our trial was quite narrow, compared to the strong imbalances used in aversion trials. Conversely, the first week after weaning is a critical phase were feed intake is depressed because of the convergence of several stresses related to the practice of weaning (Lallès et al., 2004). Thus the mechanism of Thr effect could be related to each one of the stressors (different diet, different physical structure of the diet, psycho-social stress, new bacteria) and a trail properly designed should be used to go more deeply in this study. The requirement of Thr in the piglet increase passing from parenteral to enteral nutrition (Bertolo et al., 1998), thus, for example, in the presense of the post-weaning anorexia, more Thr available could have been contributed to lighten negative feedbacks stimulated by Thr insufficiency. Nevertheless it should be considered that the higher Thr to Lys ratio was obtained with free Thr, that could be sensed more rapidly by the gut and by the brain, than the Thr present in feed proteins. Finally the presence of more free L-Thr, that is sensed as sweet in the pig (Tinti et al., 2000), could have had an appetizer effect.

Another reason of the best performance in the first week with more Thr could be related to an improved health of the piglets, and this could be associated with the interesting reduction of E. coli counts in feces of the 70% group. This may be a results of a specific effect of more threonine availability to the gut microflora. In fact it has been observed in in vitro coltures obtained from intestinal inocula, that addition of threonine to the medium inhibited E. coli growth (Dai et al., 2010). It is interesting to mention here that Fernandez and Stratez (2009) observed an improvement of the consistency of feces in pigs fed more Thr (69% Vs. 59% SID Thr : Lys) for 28 days post-weaning.

Thr is a relevant amino acid component of immune globulins (Tenenhouse and Deutsch, 1966), thus it could also have been expected that more Thr allowed a higher raise of the immune response that is in general observed immediately after the weaning, with the contact of many various new bacteria antigens on antigen-presenting cells in the gut of the young pig. However the total IgA and IgM contents on the pre-challenge blood samples were not changed by the diet, and it this can indicate that the Thr available with the low SID Thr to Lys ratio was sufficient for the production of IgA and IgM in the first week post weaning. Conversely the increase of IgA in ETEC-susceptible pigs fed the higher Thr to Lys ratio agrees with the increasing effect of Thr on IgG in blood of pigs injected with either Bovine Serum Albumin or Swine Fever attenuated vaccine (Li et al., 1999) and with IgG response to increased intake of true ileal digestible threonine (Wang et al., 2006). Thus it could be assumed that the requirement of Thr to mount the relevant K88-specific immune response is proportionally higher than the requirement for adapting the immune response to the post-weaning condition.

As regards the effect of ETEC challenge, the substantial absence of a raise of K88-specific IgA in $MUC4^{-/-}$ pigs, compared with the rapid response of $MUC4^{+/+}$ pigs, confirms indirectly the effectiveness of the challenge, but also the practical relevance of the presence of the receptors for a rapid immunization (Snoeck et al., 2008). Furthermore this observation pairs of course with the observed decrease of growth, the sharp raise of ETEC faecal shedding, and with the increase of diarrhea observed in $MUC4^{+/+}$ pigs, compared with $MUC4^{-/-}$ pigs. Finally also IgM in jejunal secretum raised in ETEC-susceptible pigs, but not in the blood serum This could be explained by the additional receptor-indipendent priming of the systemic immune system by the oral challenge with ETEC (Van den Broeck et al., 2002). Overall, these last effects of the challenge were more or less not contrasted by a higher Thr supply, notwithstanding the increased efficiency of IgA immune response in $MUC^{+/+}$ pig supplemented with more Thr. This on the whole was evidenced also by the absence of Thr effect in the post-challenge days. We were not able to find comparable research in the current literature, however, in mini-pigs, ileitis induced by trinitrobenzene sulfonic acid increased portal-drained viscera utilization of threonine from arterial but not luminal supply (Rémond et al., 2009). Thus it can be presumed that, at least in short period, the acute increased demand of threonine can be sustained by the mobilization of endogenous proteins, without apparent effect on the performance.

The effect of additional threonine on the number of mucin secreting Goblet cells and on the quantity of mucins collected from the jejunum scrapings was on the whole not consistent. Faure et al (2007) evidenced in rats that more Thr is required for acute phases proteins, intestinal proteins and mucins. However, in early-weaned pigs, a Thr deficient diet did not affect the total numbers of goblet cells per villous and per crypt in the ileum, and per crypt in the proximal colon (Hamard et al., 2007). If we consider the dietary range of Thr in our experiment, our data agree with the results of Wang et al. (2010) who observed that both low and high dietary threonine levels can reduce mucins presence or gene expression in the small intestine.

The release of mucins from Goblet cells and the Goblet cell numbers can be generally stimulated by resident intestinal flora (Mantle et al., 1989), but also by bacterial infection (Cohen et al., 1983). We were surprised that the challenge caused no variation of the presence of Goblet cells in the intestine of ETEC-susceptible pigs. However the quantity of crude mucin in jejeunum scrapings was mildly increased. It can be speculated that in our case the distance of the sampling (5 or 6 days post-challenge) was not sufficient to evidence morphological variations induced by the encounter with ETEC, but just to upset mucin secretion. Indeed the challenge did not affect also villous height and the other related morphological parameters in jejunum. In a similar trial were the Tryptophan requirement was assessed in ETEC-challenged piglets, the susceptible subjects had higher villi in the proximal jejunum (Trevisi et al., 2009), but their height was not affected by ETEC-susceptibility in distal jejunum were we also sampled in the present trial. Villus height was also not affect by the diet. Conversely the number of Goblet cells tended to be reduced with the higher supply of Thr. It is interesting to note that a 30% reduction of dietary Thr did not affect villous height in pair fed (Hamard et al., 2007) along the intestine, except for ileum. This indicates that the architecture of the intestinal mucosa is quite preserved, in a shortage of Thr.

An interesting observation was that Thr prevented the raise of body temperature observed at 10 h after the ETEC challenge. However we were not able to find in literature any reference that can link a possible effect of a different availability of Thr to the mechanism of control of body temperature or of inflammatory mediators.

In conclusion the overall favorable trend of improved growth in the whole trial suggest that the 70% Thr to Lys ratio is advisable for the piglet diet in the first two weeks, whatever is the genotype for ETEC susceptibility. This trend may result from the combination of different partial effect, like an initial better feed intake, an improved immune response and a better control of gut microbiota, but not from a reduction of colibacillosis.

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Table 3 . Effect of dietary Thr to Lys ratio and of genotype for MUC4, the marker for E. coli K88 susceptibility, in weaning pigs

	Thr to Lys ratio					Significance, *p*		
	65%		70%		SEM			
	$MUC4^{-/-}$	$MUC4^{+/+}$	$MUC4^{-/-}$	$MUC4^{+/+}$		Thr	MUC4	Thr*MUC4
BW, g								
d 0	7922	7709	7993	7662	278	0.968	0.344	0.828
d 7 (Challenge)	8276	8149	8554	8158	263	0.575	0.337	0.609
Final (d 13-14)[1]	9581	7695	10054	8097	345	0.224	<0.0001	0.923
ADFI, g								
d 0 to 7[2]	173	185	193	190	5	0.016	0.413	0.109
d 7 to Final[1]	297	100	325	105	21	0.427	<0.0001	0.588
d 0 to Final[1]	461	288	504	298	23	0.243	<0.0001	0.469
ADG, g								
d 0 to 7	51	63	80	71	12	0.116	0.899	0.380
d 7 to Final[1]	219	-85	253	5	38	0.125	<0.0001	0.500
d 0 to Final[1]	252	-26	308	84	46	0.087	<0.0001	0.583
G:F								
d 0 to 7	0.14	0.27	0.37	4.31	1.43	0.347	0.144	0.689
d 7 to Final[1]	0.55	-0.89	0.57	-0.22	0.25	0.087	<0.0001	0.259
d 0 to Final[1]	0.43	-0.08	0.57	0.22	0.11	0.056	0.0006	0.472

[1]On 33 pigs: 7 and 8 $MUC4^{+/+}$ died after challenge in groups 65% and 70% respectively.

[2]Within $MUC4^{-/-}$, 65% vs. 70%, $p = 0.031$; within 65%, $MUC4^{-/-}$ vs. $MUC4^{+/+}$, $p = 0.102$.

Table 4 . Effect of dietary Thr to Lys ratio and of genotype for MUC4, on rectal temperature on the day of ETEC challenge of the weaning pigs.

	Thr to Lys ratio					Significance, *p*		
	65%		70%		SEM			
	$MUC4^{-/-}$	$MUC4^{+/+}$	$MUC4^{-/-}$	$MUC4^{+/+}$		Thr	MUC4	Thr*MUC4
Before Challenge	39.17	39.03	39.03	39.13	0.10	0.829	0.806	0.239
After 5 h[1]	39.02	38.90	39.04	39.00	0.11	0.147	0.742	0.314
After 10 h[1]	39.11	39.34	39.02	38.84				
						Time	Time*Thr	Time*Muc4
After 5 h[1]	38.96 b		39.02		0.08	0.270	0.026	0.526
After 10 h[1]	39.24 a,A		38.92 B					

[1]With statistical analysis for repeated measures. Values before challenge were used as covariate (p = 0.007).

a,b; A,B: interaction Time*Thr, values paired for MUC4 with different letter differ for $p < 0.05$ and $p < 0.01$, after Tukey test.

Table 5 . Effect of dietary Thr to Lys ratio and of genotype for MUC4, on diarrhea score for the five days post challenge and on bacteria counts in feces of the ETEC-challenged weaning pigs.

	SID Thr to Lys ratio					Significance, *p*		
	65%		70%		SEM			
	$MUC4^{-/-}$	$MUC4^{+/+}$	$MUC4^{-/-}$	$MUC4^{+/+}$		Thr	MUC4	Thr*MUC4
Average faecal score [1]	2.22	3.77	2.51	3.50	0.21	0.959	<0.0001	0.194
Days with diarrhoea[2]	1.19	2.90	1.64	3.00	0.32	0.381	0.0001	0.556
Total E. coli, Log10 CFU/g								
d 7 (before Challenge)[3]	6.74	6.72	5.59	6.42	0.32	0.024	0.242	0.180
d 10	6.26	8.25	6.18	8.69	0.39	0.733	<0.0001	0.427
Total E. coli K88, Log10 CFU/g								
d 10[4]	0.45	8.46	0.59	8.55	0.50	0.814	<0.0001	0.967

[1]Faecal scores were recorded daily by visual appraisal of each subject using a 5-point scoring system (1 to 5); 1 = hard, 5 = watery faeces

[2]Counts of days with faecal score > 2.

[3]Within $MUC4^{-/-}$, 65% vs. 70%, $P = 0.014$; within 70%, $MUC4^{-/-}$ vs. $MUC4^{+/+}$, $p = 0.093$. .

[4]All the pigs were negative for E. coli K88 on day 7.

Table 6. Effect of dietary Thr to Lys ratio and of genotype for MUC4, on K88-specific IgA and on total IgM activity in blood serum collected before the challenge and at the end of the ETEC-challenged weaning pigs.

	SID hr to Lys ratio					Significance, *p*		
	65%		70%		SEM			
	$MUC4^{-/-}$	$MUC4^{+/+}$	$MUC4^{-/-}$	$MUC4^{+/+}$		Thr	MUC4	Thr*MUC4
K88-specific IgA activity (AU/ml)								
- Blood serum								
d 7 (before Challenge)	0.177	0.179	0.210	0.201	0.026	0.304	0.902	0.850
Final[1,2]	0.287	1.153	0.132	3.169	0.493	0.074	0.0006	0.048
- Jejunum secretum[3]	0.057	0.493	0.037	1.946	0.150	0.168	0.0002	0.102
K88-specific IgM activity (AU/ml)								
- Blood serum								
d 7 (before Challenge)	4.96	2.93	4.03	4.12	0.78	0.863	0.227	0.170
Final[4]	7.33	7.38	5.35	10.39	1.78	0.774	0.176	0.190
- Jejunum secretum	0.17	2.84	0.08	3.82	0.55	0.431	<0.0001	0.365
Total IgA activity								
- Blood serum (mg/ml)								
d 7 (before Challenge)	0.313	0.292	0.313	0.328	0.019	0.325	0.877	0.328
Final	0.295	0.267	0.296	0.311	0.028	0.427	0.802	0.462
- Jejunum secretum (µg/ml)	4.90	5.90	5.43	4.52	0.74	0.575	0.958	0.235
Total IgM activity								
- Blood serum (mg/ml)								
d 7 (before Challenge)	2.32	2.32	2.53	2.35	0.24	0.593	0.689	0.717
Final	2.87	2.63	2.50	2.35	0.34	0.349	0.577	0.894
- Jejunum secretum (µg/ml)	25.5	34.6	37.3	22.1	5.7	0.960	0.587	0.054

[1]Corrisponding values on d 7 were used as covariate, $p = 0.018$.
[2]Within $MUC4^{+/+}$, 65% vs. 70%, $p = 0.089$.
[3]Within $MUC4^{+/+}$, 65% vs. 70%, $p = 0.097$.
[4]Corrisponding values on d 7 were used as covariate, $p < 0.001$.

Table 7 . Effect of dietary Thr to Lys ratio and of genotype for MUC4, on the relative weights of the stomach, the intestine and their contents (on final body weight) of ETEC-challenged weaning pigs.

	SID Thr to Lys ratio					Significance, *p*		
	65%		70%		SEM			
	$MUC4^{-/-}$	$MUC4^{+/+}$	$MUC4^{-/-}$	$MUC4^{+/+}$		Thr	MUC4	Thr*MUC4
Stomach, % body weight								
Contents	2.87	1.47	3.10	1.81	0.44	0.523	0.005	0.908
Empty	0.76	0.76	0.77	0.83	0.03	0.242	0.447	0.380
Intestine, % body weight								
Contents	2.56	2.17	2.82	2.20	0.49	0.780	0.299	0.826
Empty	4.82	4.79	4.61	5.78	0.31	0.214	0.073	0.075

[1]Within 70%, $MUC4^{-/-}$ vs. $MUC4^{+/+}$, $p = 0.082$.

Table 8 . Effect of dietary Thr to Lys ratio and of genotype for MUC4, on the intestinal morphology and on the mucin quantification in the intestinal scrapings of ETEC-challenged weaning pigs.

	SID Thr to Lys ratio					Significance, *p*		
	65%		70%		SEM			
	$MUC4^{-/-}$	$MUC4^{+/+}$	$MUC4^{-/-}$	$MUC4^{+/+}$		Thr	MUC4	Thr*MUC4
Jejunum								
Villous height, µm	352	414	401	368	39	0.970	0.722	0.258
Villous width, µm	98	90	126	103	16	0.214	0.354	0.660
Crypt depth, µm	228	208	200	272	25	0.481	0.298	0.091
Crypt width, µm	44	41	57	47	7	0.207	0.357	0.674
M index[1]	7.81	8.78	9.16	8.55	0.70	0.200	0.579	0.960
Goblet cells, n /villous	8.64	10.25	7.78	6.80	1.22	0.092	0.798	0.324
Colon								
Gland depth, µm	343	396	362	325	20	0.211	0.688	0.046
Goblet cells, n/gland	42.8	49.9	44.4	46.2	2.7	0.710	0.104	0.366
Mucin quantification								
Jejunum, % dm	38.5	39.3	37.1	42.4	1.8	0.628	0.096	0.245
Colon, % dm	31.5	33.5	32.7	32.1	1.3	0.928	0.634	0.374

[1]M index = (villous surface + unit bottom − villous bottom)/unit bottom where villous surface = $\pi \cdot$ (villous length $\cdot$ villous width), unit bottom = $\pi \cdot$ (villous width/2 + crypt width/2)^2 and villous bottom = π (villous width/2)^2.

Chapter 2

Supplementary tryptophan downregulates the expression of genes induced by the gut microbiota in healthy weaned pigs susceptible to Enterotoxigenic *Escherichia coli* F4

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Supplementary L-tryptophan (L-Trp) limits the decrease of feed intake and growth in Enterotoxigenic *Escherichia coli* F4 (ETEC) susceptible pigs upon oral challenge with this pathogen. Susceptibility to ETEC is genetically controlled and related to the presence of receptors for the F4 fimbriae (F4R). We aimed to assess if dietary Trp affects genes involved in the intestinal barrier of healthy pigs carrying or not the F4R. Thirty-six littermate weaning pigs were selected to have potentially eighteen ETEC_susceptible and eighteen ETEC-non-susceptible subjects, based on a MUC4 gene polymorphism. For 21 days they were fed a diet with 0.17 or 0.22 ileal digestible Trp:Lys ratio. Using the test of ETEC adhesion to the intestinal villi, the pigs were divided into F4R negative (no bacteria adhering, $F4R^-$), F4R positive ($F4R^+$), and F4R mildly positive ($F4R^{m+}$). A preliminary test (GeneChip® Porcine Genome Array) highlighted the differentially expressed genes in the jejunum of 3 $F4R^-$ and 3 $F4R^+$ pigs. The expression of the most interesting genes was assessed on the whole sample. In $F4R^+$ pigs, Trp reduced the mRNA of four genes involved in the intestinal barrier and/or induced by several bacteria-associated molecular patterns, like lipopolysaccharide (LPS) (REG3G, Regenerating islet-derived 3 gamma; SFTPD, Surfactant pulmonary-associated protein D; CFB, Complement factor B; LBP, LPS-binding protein) (P<0.05). In pigs fed the low-Trp diet, Reg3g, SFTPD and LPB mRNA increased with F4R presence (P<0.05). Interleukine-8 tended to be less expressed with the higher Trp level whatever the F4R presence (P=0.09). No DNA from ETEC was detected in the jejunum contents. Trp favourably interacts to reduce the bacterial induction of some genes involved in the intestinal barrier in ETEC susceptible pigs, but the causative mechanism has yet to be established.

Key words: *Escherichia coli* F4 Immunity Intestinal receptors Tryptophan Weaning pigs

1. Introduction

Epithelial surface glycoconjugates are recognized as receptors for microbial pathogens but these molecules can also contribute to shaping the commensal microbiota (Bauer et al., 2006). Thus genetically or environmentally-driven shifts in glycan expression can also change the triggering of the innate immune system (Hooper, 2004) with consequences for metabolism and nutrient requirements. The innate immune system is activated by the formation of complexes between surface patterns of bacteria and membrane-bound or cytoplasmic primitive pattern recognition receptors that are proteins expressed primarily by cells of the innate immune system of the host. Typically one of the bacterial surface molecules, lipopolysaccharide (LPS) exposed on the outer cell wall of Gram-negative bacteria, contributes to the activation of the immune system (Wright et al., 1990). Recognition of the bacteria is necessary to initiate the host immune response, but proper compensatory tuning is also required to moderate or prevent the impact of the infection. The catabolism of tryptophan (Trp) through the kynurenine pathway is an important mechanism in regulating immunological and neurological responses (Wang et al., 2006). Several cell types, including cells of the immune system, have the pool of enzymes that break down Trp. Inflammatory signals like Tumor necrosis factor induced by LPS, regulate Trp catabolism by acting on key enzymes (Mellor and Munn, 2004). Thus, tissue inflammation can cause depletion of Trp. However most of the knowledge on the regulation of the catabolic pathway of Trp has been obtained in cellular systems. A possible significance of variations in dietary Trp available for immune regulation in the whole organism is suggested by these results but in vivo data are lacking. This also raises the hypothesis that intestinal infections could increase the requirement of Trp. Supplementary L-Trp limited the decrease of feed intake and growth in enterotoxigenic *Eescherichia coli* F4 (ETEC) susceptible pigs upon oral challenge with this pathogen (Trevisi et al., 2009). The ETEC infection in young pigs depends on the presence of the receptor for F4 fimbriae (F4R) of ETEC, on porcine enterocytes. The nature of the gene controlling this receptor has not been fully identified, but it has been established that its presence is inherited as a monogenetic trait. The dominant allele, that causes the susceptibility, is also associated with an intron mutation of Mucin 4 (MUC4) gene (Jørgensen et al, 2003). However it has not in general been considered if the presence of the receptors for ETEC can affect the host response to dietary factors in the absence of ETEC bacteria.

Our aim was to assess if genes involved in the intestinal barrier are changed by dietary Trp in healthy pigs that present F4R or not, but that are not ETEC-challenged.

2. Materials and methods

All procedures complied with the Italian law pertaining to experimental animals and were approved by the Ethics-Scientific Committee for Experiments on Animals of the University of Bologna.

2.1. Animals and experimental treatments

The polymorphism of Mucin 4 gene was used as a marker for susceptibility to ETEC (Jørgensen et al, 2003), to identify groups of littermates with or without this susceptibility, that can not be estimated in vivo. Sows and boars were screened for this polymorphism, following the protocol of Jensen et al. (2006), and then the litters from heterozygous parents were tested, to obtain homozygous piglets for the allele marker for the absence or for the presence of F4R (2 $MUC4^{-/-}$ and 2 $MUC4^{+/+}$ pigs within each litter). Following this screening method, thirty-six piglets were selected, weaned at 24 $\pm$ 2 days (6.51$\pm$1.51 kg live weight),

assigned to two different diets within each genotype per litter, and housed in weaning rooms with controlled temperature (30°C at start to 26°C at the end) and ventilation. Continuous access to feed and water was allowed throughout the trial. Pigs were individually penned in cages (125 cm x 50 cm), except the two first days when they were kept in groups of three, to improve their adaptation and feed intake.

For all the trial the piglets were fed the same standard pre-starter diet formulated as shown in Table 1 (supplying 18.3% crude protein, 10.52 MJ/kg net energy, 1.23% total Lysine, without growth promoters), but with two different Trp:Lys ratios (0.17 or 0.22, standardized ileal digestible basis (SID), obtained without or with 0.5 g/kg L-Trp addition). The Trp:Lys ratio in the first diet

was the one suggested by NRC (1998) for this category of pigs, however the content of the main nutrients (Crude protein, Ca, P, Lys) was mildly lower than suggested by NRC (1998), but typical for an Italian diet when no growth promoter or antimicrobial is added and a slightly lower growth is expected, as compared with American standards. Analyzed amino acid values of the two mixtures are presented in Table 2. SID was used because it best describes amino acid digestibility corrected for basal endogenous losses for the formulation of mixed diets (Mosenthin et al., 2007). The nutrient values were estimated by the EvaPig® software (Noblet et al., 2008 using information from the INRA-AFZ tables of feedstuff composition [Sauvant et al., 2004]).

Growth performance was measured and the results were reported by Trevisi et al. (2010a). Within each treatment, 50% of the pigs were slaughtered on day 20 of the trial; the remaining 50% were slaughtered on day 22. On each day of slaughter pigs were given access to feed for one hour and then had feed withdrawn for the last hour before killing. The pigs were anaesthetized with sodium thiopenthal (10 mg/kg body weight) (Zoletil 100, Virbac, Milano, Italy) and euthanized by an intracardiac injection of Tanax® (0.5 mL/kg BW) (Intervet Italia, Peschiera Borromeo, Italy).

2.2. Sampling and analyses

For each subject, the abdominal cavity was opened and samples of distal jejunum were collected. Firstly a sample of jejunal juice was collected in a sterilized vial and stored at –30°C. Afterwards a segment of jejunum tissue was collected and kept on ice, the mucosa was scraped using a microscope slide, and the sample was stored at -80°C, for further molecular biology analyses. Another segment of distal jejunum was collected for the *in vitro* ETEC villous adhesion assay. The intestinal villi and the *in vitro* tests were described by Bosi et al. (2004). Pigs with values ≥ 6 bacteria adhering per 250 µm length of villus were considered to be F4R positive ($F4R^+$, F4R negative with no bacteria adhering ($F4R^-$), and F4R mildly positive with values <6 and > 0 ($F4R^{m+}$).

After this classification, 3 subjects with no clear data on ETEC adhesion were excluded: they were negative for the gene marker MUC4, but were mildly positive on the in vitro adhesion test. All the others corresponded to the prediction by the marker gene: all $MUC4^{-/-}$ were $F4R^-$, and $MUC4^{+/+}$ were $F4R^{m+}$ or $F4R^+$.

On the intestinal tissue sample collected from each subject, the mRNA for Microarray and real time analyses was isolated according to the Qiagen RNeasy® Midi Kit protocol (Qiagen, Hilden, Germany). The purity and concentration of the total RNA extracted were checked using the Nanodrop ND 1000 spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE, USA) at 260 and 280 nm and the RNA integrity was controlled by agarose gel electrophoresis analysis. In addition, the RNA isolated from the

piglets selected for the Microarray analyses passed the evaluation by Agilent Bioanalyzer 2100. One microgram of RNA was reverse transcribed from each sample using the ImProm-II Reverse Transcription System (Promega, Madison, WI, USA).

The oligonucleotide-Microarray preliminary test was done to detect the most differentially expressed genes in jejunum tissue from 3 $F4R^-$ and 3 $F4R^+$ pigs fed the two experimental diets. The pigs were selected from two litters were the higher number of adhering bacteria at the *in vitro* ETEC villous adhesion assay was seen for $F4R^+$ subjects. The Affymetrix porcine genome microarray chip (Affymetrics, Liège- Belgium) was used. The row data derived from the chip were firstly normalized with the GC-RMA algorithm in the Affymetrix package of Bioconductor. The presence of artifacts and the consistency of normalisation across arrays were checked by *affy* and *affyPLM* packages available on Bioconductor, and a cluster representation of mRNA sequences was obtained by *affy*.

Among the genes differentially expressed between $F4R^-$ and $F4^+$ pigs, some genes with higher absolute values of expression were selected: REG3G (Regenerating islet-derived 3 gamma); SFTPD (Surfactant pulmonary-associated protein D; CFB (Complement factor B); LBP (LPS-binding protein); IL-8 (Interleukine-8); GPX2 (Glutathione peroxidase). IDO (indoleamine 2,3-dioxygenase) and TLR-4 (Toll-like receptors-4) were added to this list, resepctively because their relevance for the metabolism of Trp and for the detection of lipopolysaccharide (LPS) from Gram-negative bacteria. Two other genes, for SCPEP1 (serine carboxypeptidase 1) and for PSMB3 (beta type 3 proteasome subunit), were also added because they were affected by the SID Trp:Lys ratio. For all the genes, primers were designed based on a specific porcine nucleic acid sequence (Gen-Bank database) using Primer 3 version 0.4.0 (http://frodo.wi.mit.edu/primer3/). The sequence of the primers and the length of the fragments amplified (base pairs) are listed in Table 3.

Firstly a cDNA porcine target fragment was amplified for each gene, that served as the standard of a known nucleotide copy numbers. This product was purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). The cDNA quality and concentration were evaluated by the Nanodrop ND 1000 spectrophotometer. Then the fragment was serially diluted in 1:10 steps and a standard curve was created to perform the absolute quantitative analysis. The quantification reactions were performed in a Light Cycler instrument (Roche, Mannheim, Germany). The amplification was carried out in a 10 µL volume containing 2 µL of cDNA, 8 pmol of each primer, and 5 µL of SYBR® Premix Ex Taq™ II (Perfect Real Time) (Takara Bio Inc, Shiga, Japan). The fast protocol was 40 cycles at 95°C for 5 seconds and a temperature between 60°C and 66°C for 20 - 26 seconds on the bases of the specific conditions of primers amplification. The detection of the fluorescent product was set at the last step of each cycle. Melting curve analysis checked the specificity of each amplification; additional specificity analyses including product size verification was performed by 1.2% agarose gel electrophoresis. The data were expressed as gene transcript copies per microgram of RNA.

Because the aim of the present research was to assess the relevance of F4R in the absence of the triggering effect of ETEC, the presence of DNA from this strain of *E. coli* in the intestinal contents was assessed by PCR according to the method reported by Alexa et al. (2001), except for the DNA extracted from jejunal digesta. This was determined by the method described by Trevisi et al. (2010b).

2.3. Statistical analysis

Model fitting on normalized Microarray data and hypothesis testing were done by the *limma* package from Bioconductor. Two contrasts were fitted: $F4R^-$ vs. $F4R^+$; 0.17 vs. 0.22 SID Trp:Lys ratio. The empirical Bayes moderated *t* test was used to assess differential expression, the corresponding P-values were adjusted to control the false discovery rate, and the genes were declared differentially expressed when adjusted P-values <0.10 (Golkar et al., 2007), and were considered sufficiently noteworthy to justify further assessment.

The data on gene expression were analyzed by analysis of variance (GLM procedure of SAS; SAS Institute inc., 2007), based on the class variables SID Trp: Lys ratio, F4R presence, their interaction and litter. When the *p*-values for the interaction were ≤ 0.1, the single SID Trp:Lys ratio x F4R presence interaction values were considered, and the following linear contrasts were tested: Trp effect within $F4R^-$; Trp effect within $F4R^{m+}$; Trp effect within $F4R^+$; Linear effect of F4R in low Trp, Linear effect of F4R in high Trp.

Significance was declared if $P < 0.05$, and a trend was considered when $0.05 < P < 0.10$.

3. Results

No pig tested positive for DNA from ETEC in jejunum contents.

3.1. Microarray preliminary test

The cluster representation of mRNA sequences preliminary tested by Affymetrix Microchips on the six pigs is presented in Figure 1. It is evident from the figure that there was a cluster association related to the presence of F4R. Forty-five and five sequences were respectively more and less expressed in $F4R^+$ pigs compared with $F4R^-$ pigs. Most of the genes that could be attributed to these sequences and showed greater expression were related to the inflammatory response. Within this set of genes, the following subset was selected for further analysis because of their higher absolute values of expression and because of their affinity: REG3G (Regenerating islet-derived 3 gamma); SFTPD (Surfactant pulmonary-associated protein D; CFB (Complement factor B); LBP (LPS-binding protein); IL-8 (Interleukine-8); GPX2 (Glutathione peroxidase 2). With high Trp diets, four mRNa sequences showed greater expression, but were not attributable to any known gene; two other sequences showed lower expression compared with low Trp diets and were attributed to the genes for serine carboxypeptidase 1 (SCPEP1) and for beta type 3 proteasome subunit (PSMB3).

3.2. Expression of selected genes in the jejunum tissue

The effect of Trp and F4R presence on the expression of some genes in the jejunum tissue of all the piglets in the trial is shown in Table 4.

Interactions between Trp and F4R presence were observed for REG3G, SFTPD, CFB, LBP and TLR4. For these genes a linear effect of F4R presence in the low Trp group was seen (P-values = 0.023; 0.023; 0.059; 0.046; 0.056, respectively). In subjects with a higher F4R presence, supplementation of feed with Trp decreased the expression of all these genes (with P = 0.021; 0.006; 0.007; 0.011; 0.061, respectively). A 0.22 SID

Trp:Lys ratio in the diet tended to reduce IL-8 mRNA (P=0.090), as compared with the lower SID Trp:Lys ratio, while the gene expression of this cytokine was not changed by the degree of F4R presence. GPX2 was not changed by the diet or the F4R presence. The same was observed for indoleamine 2,3-dioxygenase (IDO) that was added to the list of genes that emerged from the preliminary Microarray due to its relevance to the catabolism of Trp. For SCPEP1 and PSMB3 no statistically significant effect was seen (Data not in table).

It was decided to test the gene expression for MUC4 too due to its possible association with the traits that were studied. However MUC4 mRNA in jejunal samples was detectable in only about 44% of the subjects. The frequency of detectable samples calculated by a chi square test did not differ for the MUC4 polymorphism. It is worth noting however that all the genes that resulted from the pre-screening process and were tested by Real-Time RT-qPCR, were significantly increased (P<0.01) in the pigs in which detectable MUC4 mRNA was found compared to the other pigs that did not show detectable levels of this gene (Figure 2).

4. Discussion

Our pigs in general recovered well from the post-weaning lag and were healthy throughout the trial. This was indirectly confirmed by the absence of DNA from ETEC, these bacteria being the main cause of pig mortality and scour to diarrhoea during the post-weaning period (Nagy and Fekete, 1999). Nevertheless, with the 0.17 SID Trp:Lys ratio, the expression of several genes related to the innate immune response increased with the presence of F4R in the distal jejunum. In the intestine Reg3g, SFTPD and LBP are produced mainly by the secretory lineages present in the crypt (Soerensen et al., 2005; George et al., 2008; Hansen et al., 2009). Reg3g and SFTPD are C-type lectins with antimicrobial activity (Brandl et al., 2008) or aggregating properties (Hogenkamp et al., 2007; Lehotzky et al., 2010) and they are often increased in bacterially-induced infections. Reg3g is stimulated on the first contact with symbiotic bacteria in germ-free mice (Cash et al., 2006), and also by the supplementation with a Gram+ bacteria like *Lactobacillus plantarum* (Gross et al., 2008). However it has already been observed that the homologue gene REG3A (also called Pancreatitis-associated protein) mRNA increases in porcine small intestinal loops infected with *E. coli* K88 (Niewold et al., 2005) and *Salmonella enterica* serovar typhimurium (Niewold et al., 2007), as compared with non-infected loops. SFTPD is a pattern recognition molecule with a variety of potential ligands, with different saccharide motifs, depending on the animal species (Crouch et al., 2006). LBP is an acute phase protein that contributes to the binding of LPS to cells presenting surface pattern recognition receptors, like TLR4 (Hansen et al., 2009). Factor B is a protein that plays a central role in the “alternative pathway” of complement activation (Zipfel and Skerka, 2009). It is also stimulated by various bacterial endotoxins, polysaccharides and cell walls, including LPS. The activation of all these genes leads to the hypothesis that bacteria-associated molecular patterns stimulate the mucosa of pigs with the alleles marker of ETEC susceptibility, notwithstanding the apparent health of all of them. In particular, an increase in the expression of LBP can lead to the suspect that more LPS was triggering the mucosa in pigs with high presence of F4R. Indeed LPS is broadly present in a large array of bacteria, like also in phylum *Bacteroidetes,* that includes friendly commensals and pathogens (Wexler, 2007), and that is the second phylum for the presence in the gut of growing pigs (Guo et al., 2008) .

This can be broadly confirmed by the tendency for TLR4 mRNA to rise in these pigs, because this surface pattern recognition receptor is stimulated by LPS (Abreu, 2010). Interestingly the inactivation of the gene MyD88, an adapter protein involved in the Toll-like receptor signalling pathway, blocks the expression of REG3G in mice (Vaishnava et al., 2008). In addition, ileal transcription of REG3B markedly declined in mice deficient in TLR2 (Dessein et al., 2009). Thus it can be supposed that in pigs fed a standard weaning diet, the high presence of F4R is associated with the development of an intestinal microbiota that maintain an elevated sensitivity in intestinal epithelial tissues to various chemical moeties found in bacteria, including LPS.

It has been estimated that about 95% of absorbed Trp is metabolized in the kynurenine pathway (Peters, 1991). Several metabolites formed in this process (NAD, kynuramines, kynurenic acid, quinolinic acid, picolinic acid) have an important impact on the complex regulatory effect of Trp on the immune system. In particular, the key enzyme IDO that degrades Trp acts in an anti-inflammatory way by generating other anti-inflammatory metabolites and inhibiting the replication of surrounding immune cells due to the shortage of Trp. Our observation that Trp reduced the gene expression of the inflammatory cytokine IL-8 is consistent with the ability of Trp to alleviate dextran sodium sulfate-induced colitis in pigs (Kim et al., 2010). This can be explained by the induction of apoptosis of activated immune cells due to the increased expression of apoptosis initiators caspase-8 and Bax by Trp (Kim et al., 2010). Indeed we did not find an effect of dietary Trp on the expression of the IDO gene but we cannot exclude an increase in its activity that could not be measured.

Nevertheless it is also well known that several bioactive molecules that are able to inhibit the production of inflammatory cytokines (serotonin, N-acetylserotonin, melatonin, anilinonaphthalene sulfonate) are metabolites of Trp (Wu, 2009). Thus a contribution from these Trp metabolites cannot be excluded.

In $F4R^+$ pigs, the higher SID Trp:Lys ratio in the diet prevented an increase in the expression of genes involved in the innate immune response (REG3G, SFTPD and LBP) in the jejunal tissue. In the literature we were unable to find any study that investigated direct links between the dietary supply of Trp and the expression of these genes. However, the increase in TLR4 mRNA found in $F4R^+$ pigs tended to be prevented by the higher SID Trp:Lys ratio. Since bacterial colonization starts in early life, responsiveness to LPS (Lotz et al., 2006) and TLR4 expression (Abreu et al., 2001) in intestinal epithelial cells is moderated to limit pro-inflammatory responses in the presence of commensal microbiota and allow toleration of endotoxins (An et al., 2010). Our observations could indicate that in subjects showing more F4R, this control was partially disrupted and Trp could help restore greater tolerance. This finally could explain the reduced stimulation to produce REG3G, SFTPD and LBP mRNA in $F4R^+$ pigs when more Trp was fed.

It is also tempting to hypothesize that the higher Trp level in the feed could have contributed to the formation of a different microbiota, or that other bacteria could be able to utilize the receptors of ETEC. In an associated paper (Messori et al, in preparation) we look at the impact of Trp supplementation and the presence of ETEC receptors on the intestinal bacterial population. Pigs with higher susceptibility to ETEC were characterized by a less varied bacterial population as assessed by the 16S rRNA gene-targeted denaturing gradient gel electrophoresis (DGGE) fingerprinting analysis. A loss of complexity of the microbiota can indicate a loss of the homeostasis (Falk et al., 1998) in the genetic susceptible pigs, may contribute to activate genes involved in the local innate defence, and more Trp could have helped in these subjects to restore an immune tolerance.

For the trial we selected littermate pigs differing in a XbaI polymorphism at the MUC4 gene (Jensen et al., 2006). MUC4 is a transmembrane O-glycosylprotein detectable in both goblet cells and enterocytes. The

g.243A>G mutation is in intron 17 (Jørgensen et al., 2003) thus not in the transcript region however it is interesting to observe that its oligosaccharide structure could be the basis of recognition by E. coli K88. Also, the MUC4 gene is associated with the unknown gene that controls susceptibility to E. coli K88. In our trial however we did not observe a link between the number of pigs presenting detectable MUC4 gene expression in jejunum tissue with the experimental variables (diet and genotype). Interestingly Sargeant et al (2010) observed that providing zinc oxide at pharmaceutical doses (3.1 g ZnO/kg diet) that contribute to the protection of piglets against colibacillosis, decreased the gene expression of MUC4. We also observed that pigs with detectable MUC4 mRNA had high levels of expression of genes involved in the innate response to gut bacteria. Recent studies in germ-free and conventional mice suggest that a well-established microbial community participates in the regulation of the gut mucosal layer (Comelli et al., 2009). Thus high levels of MUC4 mRNA in the piglet intestinal mucosa can be considered indicative of poor regulation of the gut microbiota.

5. Conclusion

In conclusion, several genes involved in the intestinal barrier are changed by dietary Trp in healthy pigs that are positive for the presence of the intestinal receptors for ETEC. This indicates the interest for developing feeding requirements designed for different genotypes of weaning pigs. The mechanism by which the tryptophan reduces the stimulatory effect of several bacteria-associated molecular patterns on the genes involved in the response of the intestinal barrier in ETEC susceptible pigs has yet to be established and may require studies on cell/tissue colture systems.

Acknowledgements

Ajinomoto Eurolysine financially supported this work.

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Table 1

Composition of the basal diet

Ingredient	%	Calculated composition	% or otherwise
Barley	25	Crude protein	18.26
Corn extruded	24.4	Crude fat	5.90
Barley extruded	8.15	Crude fiber	3.03
Wheat soft	5	Ash	5.73
Soybean debittered	5	NDF	9.83
Soybean meal 48% CP	14	Ca	0.792
		P tot	0.639
Spray-dried milk whey (10% CP)	10	P available	0.370
Soybean oil	1	SID Lys	1.11
Lard	2.5	SID Lys, g/Mcal NE	2.09
Calcium sulphate	1	DE, Mcal/kg	14.64
Monocalcium phosphate	1	NE, Mcal/kg	10.52
Mineral and vitamin premix[a]	1.05		
L-Lysine HCl (diluted 50%)	0.9		
DL-Methionine (diluted 30%)	0.65		
L-Threonine (diluted 50%)	0.35		
	100		

[a] Provided per kilogram diet: 11,000 IU of vitamin A; 1,100 IU of vitamin D_3; 2 mg of vitamin K_3; 45 mg of vitamin E; 4 mg of thiamine; 8 mg of riboflavin; 4 mg of pyridoxine; 0.04 mg of vitamin B_{12}; 55 mg of niacin; 0.15 mg of biotin; 30 mg of d-pantothenic acid; 1 mg of folic acid; 150 mg of Fe; 100 mg of Zn; 150 mg of Cu; 70 mg of Mn; 1 mg of I; 0.3 mg of Se.

Table 2. Analyzed amino acid contents of the diets (as-fed basis, g/100 g)

Item	Basal diet	High-Trp diet
Lys	1.23	1.24
Trp	0.22	0.27
Met	0.44	0.43
Cys	0.29	0.28
Thr	0.77	0.76
His	0.42	0.40
Ile	0.70	0.69
Leu	1.34	1.32
Phe	0.84	0.83
Tyr	0.57	0.57
Val	0.79	0.79

Table 3. Primers used for the real-time PCR analyses

Gene		Primer sequences	Size (base pair)	Accession number
REG3G	Forward	ACCCAAAACCTGGATGGATG		
	Reverse	AGGGAGGACACGAAGGATG	102	NM_001144847.1
SFTPD	Forward	CCCCTGGTCTATGCCAAC		
	Reverse	GCTCAGAACTCGCAGATCAC	149	NM_214110.1
CFB	Forward	ACATCAAGAATGGGGAAAGG		
	Reverse	AAGTGTTGGGGTCAGCATAG	147	EU282359.1
LBP	Forward	AGGAACACAGCCGAATGG		
	Reverse	GAAGGTGCGGAAGGAGTTG	161	NM_001128435.1
IL 8	Forward	CAAGCAAAAACCCATTCTCC		
	Reverse	TTTCTCTGGCAACCCTATGTC	123	AB057440
GPX2	Forward	GACATCAAGCGCCTCCTC		
	Reverse	AGACCAGAAAGGCAAGGTTC	183	DQ898282.2
IDO	Forward	ATGCCTCCCTCCCACAAG		
	Reverse	GTCCTCCTGCCCTTTGCTC	203	XM_001926701.1
TLR-4	Forward	AGGACCTGCCTGTGCTGAGT		
	Reverse	TCGTCCTGGCTGGAGTAGAT	211	AY535422.1
MUC 4	Forward	GTGCCTTGGGTGAGAGGTTA		
	Reverse	CACTCTGCCGTTCTTTCC	367	Jensen et al. 2006
SCPEP1	Forward	GCCATTCAGCAAGGGACCATCCA		
	Reverse	CCAGGAGACCACCGAATCAAGGG	90	XM_003131627
PSMB3	Forward	TCGGGCATGGGCGTCATCGT		
	Reverse	GGGAACCGCTAGTCCATTCGGG	86	NM_001144902
E. coli K88ac	Forward	TTTGCTACGCCAGTAACTG		
	Reverse	TTTCCCTGTAAGAACCTGC	436	Alexa et al. 2001

Table 4. Effect of Trp and F4R presence on the expression of some genes (gene copies/mg RNA) in the jejunum tissue of piglets.

		Genes							
		Reg3g	Sftpd	Cfb	LBP	IL-8	GPX2	IDO	TLR4
0.17 SID Trp:Lys	$F4R^-$	1623500	49582	291836	291836	47249	106335	916	4793
	$F4R^{m+}$	2967915	34260	291930	291930	53582	60832	437	5211
	$F4R^+$	8830267	123241	598117	598117	80585	130617	115	10657
0.22 SID Trp:Lys	$F4R^-$	2637501	58359	205989	205989	36859	98406	2512	6521
	$F4R^{m+}$	1161600	54866	277587	277587	38686	89222	386	6468
	$F4R^+$	197799	14717	124238	124238	23928	51383	345	3859
SEM[1]		1984646	20245	97237	97237	18677	28052	1308	1957
Statistical significance, P									
Trp		0.068	0.129	0.026	0.026	0.090	0.408	0.591	0.441
F4R		0.478	0.557	0.560	0.560	0.874	0.36	0.420	0.732
Interaction[2]		0.091	0.018	0.058	0.096	0.491	0.123	0.751	0.118
Linear effect of F4R in low Trp		0.023	0.023	0.059	0.046	-	-	-	0.056
Trp within $F4R^+$		0.021	0.006	0.007	0.011	-	-	-	0.061

[1]SEM: pooled standard error of means

[2]Five contrasts were tested: Trp within each level of F4R, linear effect of F4R in each Trp level. Only contrasts where P≤0.10 are presented.

Fig. 1. Cluster representation of the mRNA sequences tested by Affymetrix Microchips on six pigs differing for F4R presence and for the dietary level of Trp.

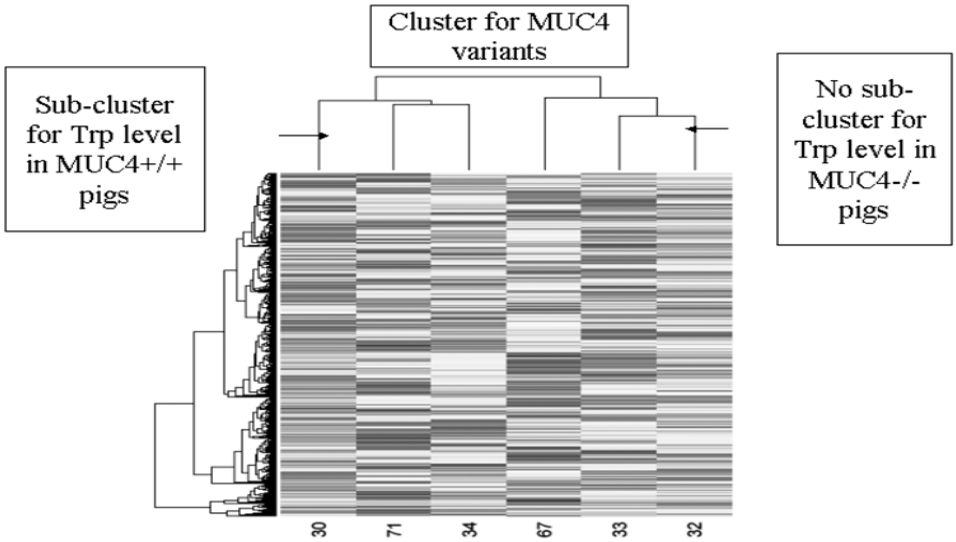
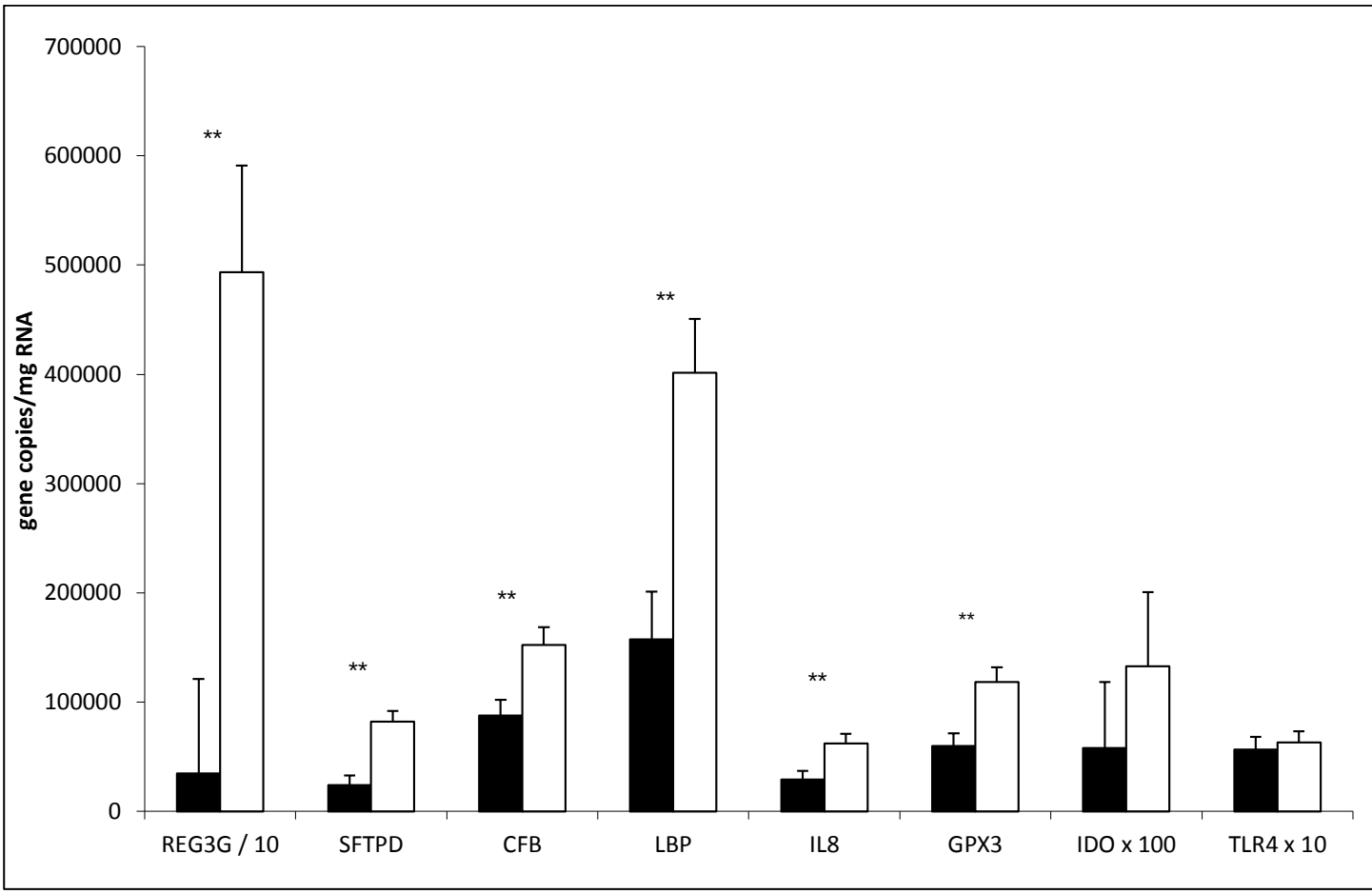


Fig. 2. Variations of some genes depending on the presence or not of detectable MUC4 mRNA. Values for each gene were scaled by different multiples of 10 for the presentation in the histograms. Results are expressed as means with their standard errors (n 19 in the Detected group and n 15 in the Not detected group). **: the values of the indicated gene significantly differed between the groups (P<0.01). Detectable □ or not ■ detectable MUC4 mRNA.



Chapter 3

Effect of susceptibility to enterotoxigenic *Escherichia coli* F4 and of dietary Tryptophan on gut microbiota variability observed in healthy young pigs

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Abstract

Weaned pigs susceptible to enterotoxigenic *Escherichia coli* F4 (ETEC) require more Tryptophan (Trp) to maximize their performance, either if they are challenged or not with ETEC. We aimed to assess if the susceptibility to ETEC and the dietary Trp affect the bacterial variability in the jejunal contents of healthy pigs. Thirty-six littermate weaned pigs were selected to obtain a set potentially formed of 50% ETEC-susceptible and 50% non-susceptible pigs, based on a *Mucin 4* gene polymorphism. Pigs were fed a diet with 0.17 (Trp^-) or 0.22 (Trp^+) standardized ileal digestible (SID) Trp:Lysine (Lys) ratio for 21 days. After slaughter, pigs were classified into non-susceptible, mildly susceptible, and susceptible, by testing ETEC adhesion to their intestinal villi. Bacteria variability was assessed by the 16S rRNA gene-targeted denaturing gradient gel electrophoresis (DGGE) fingerprinting analysis and expressed by the Shannon index. Susceptible pigs had a reduced bacteria variability and this effect was more evident with the Trp^- diet (p = 0.003). The quantification of Enterobacteria DNA was not affected by the experimental factors. One DGGE band, which referred to *Clostridium bartlettii*, was not evidenced in susceptible pigs, and less DNA from this microbe was quantified by RT-PCR in the jejunum contents from susceptible pigs fed the Trp^+ diet (p = 0.025) compared to those fed the Trp^- diet. *Beta-galactoside α-2,3-sialyltransferase 1* gene expression was higher in jejunal tissue of ETEC-susceptible pigs (p = 0.019). Bacteria variability is reduced in healthy weaned pigs susceptible to intestinal ETEC adhesion and an increase of the dietary Trp partially reverse this effect.

Keywords:

Escherichia coli F4, Gut microbiota, Pig, Tryptophan

1. Introduction

The pigs intestinal tract harbours a dense and varied microflora (Pryde et al., 1999): bacterial population is composed by about 400 different species and has been estimated around 10^{10}-10^{11} CFU/g intestinal content, that is to say that they outnumber the animal cells by a factor of 10 (Savage, 1977). This community co-exists in a delicate symbiosis with the host and it plays an important role on host's health, nutrition and physiology and on gut development (Cummings & MacFarlane, 1997). Multiple disease states may arise, at least in part, consequently to shifts in microbial communities.

Post-weaning colibacillosis derives from proliferation of enterotoxigenic *Escherichia coli* (ETEC), one of the majors sources of revenue loss for the swine industry because of increased mortality, growth stasis and treatment-associated cost in the post-weaning period (Fairbrother et al., 2005). The ETEC serotype the most involved in this colibacillosis is O149:F4 (Fairbrother et al., 2005).

Sellwood et al. (1995) identified two porcine phenotypes, on the basis of the ability of developing diarrhoea, dividing the pigs in susceptible and non-susceptible to ETEC F4-diarrhoea. Susceptible pigs, unlike resistant pigs, express the receptors for the F4 fimbriae which determine the intestinal adhesion of this bacterial strain. This character resulted to be inherited as an autosomal recessive trait (Bijlsma and Bouw, 1987) and, even if the DNA sequence responsible for the production of such receptor has not been identified, it has been linked to a polymorphism on *Mucine 4 (MUC4)* gene. On this basis, a DNA marker-based test has been developed on Muc4 gene allowing to genotype for ETEC F4-susceptibility (Jørgensen et al., 2004). However, it has not been assessed yet if the ETEC F4-susceptibility can affect the intestinal microbiota when pigs are healthy and apparently negative for ETEC presence.

Tryptophan (Trp) catabolism is linked to immune-regulatory processes. Mellor and Munn (2003) and Le Floc'h et al. (2004) showed that, in pigs, the level of dietary Trp was able to influence the inflammatory response. Tryptophan, in fact, is linked to the indoleamine 2,3-dioxygenase (IDO) pathway and there are evidences showing that IDO is able to mediate anti-microbial effects (Heseler et al., 2008) and to control lymphocytes proliferation (Mellor and Munn, 1999). Thus, it would be interesting to know whether Trp can interact in the maturation of intestinal microbiota of weaned pigs differing for the ETEC susceptibility.

The sialyl-transferase family is a group of enzymes involved in the process of glycosylation and in the formation of oligosaccharides, basal component of many glycoproteins used for mediating and regulating a variety of physiological cell-cell interactions and also exploited by a number of pathogens as adhesion ligands (Kyogashima et al., 1988). Two glycoproteins from brush borders of porcine intestinal cells were even identified as specific receptors for F4 by Grange et al. (1988). Marked changes in sialic acid linkages occur also during the life cycle of immune cells (Varki, 2008). Among sialyl-transferases, beta-galactoside alpha-2,3-sialyltransferase 1 (ST3GAL1) catalyzes the transfer of sialic acid from CMP-N-acetylneuraminic acid to galactose containing substrates. From the preliminary inspection by microarray analysis of mucosa samples in a sub-set of piglets from this experiment (Trevisi et al., submitted), emerged the differential impact of the *MUC4* genotype on the *ST3GAL1* gene expression.

The aim of this study was to assess if the susceptibility to ETEC and the different dietary Trp:Lys ratios affected the bacterial variability in the jejunal contents in healthy piglets. Additionally, the effect of these factors on the expression of *ST3GAL1* gene was evaluated.

2. Materials and Methods

2.1 Animals, experimental design, and diets

Sows and boars were screened for the *MUC4* polymorphism, following the protocol of Jensen et al. (2006), and heterozygous parents were selected to obtain litters which were presumed to include both ETEC-susceptible and not susceptible littermates.

Thereby thirty-six piglets were obtained from nine litters, by selecting two piglets homozygous for the resistant allele ($MUC^{-/-}$) and two homozygous for the sensitive allele ($MUC^{+/+}$) within each litter. The piglets were weaned at 24 ± 2 d (6.51 ± 1.51 kg live weight), housed in weaning rooms with controlled temperature and ventilation and assigned to two different diets differing by there standardized ileal digestible Trp to lysine ratio (SID Trp:Lys) within each genotype per litter. The piglets were individually penned, except the first two days when they were kept in groups of three, to improve their adaptation and feed intake.

The piglets were *ad libitum* fed the post-weaning diet, as reported by Trevisi et al. (submitted). Briefly, the basal diet (Trp^-) was composed by 18.3% of crude protein, 10.52 MJ/kg of net energy, 1.23% of total Lys and without antimicrobials. The basal diet was supplemented with a 0.5 g/kg L-Trp addition to increase the SID Trp:Lys ratio from 0.17 (Trp^- diet) to 0.22 (Trp^+ diet). The SID Trp:Lys ratio in the basal diet was adopted to be close to the values suggested by NRC (1998). The SID values were estimated by the EvaPig® database (Noblet et al., 2008).

Per each group, half of the piglets was slaughtered on d 20 and the remaining on d 22 post-weaning. The last meal was accessible for one hour and until one hour before slaughtering. The pigs were then anaesthetized with sodium thiopenthal (10 mg/kg body weight) (Zoletil 100, Virbac, Milano, Italy) and euthanized by an intracardiac injection of Tanax® (0.5 mL/kg BW) (Intervet Italia, Peschiera Borromeo, Italy).

All procedures complied with the Italian law pertaining to experimental animals and were approved by the Ethic-Scientific Committee for Experiments on Animals of the University of Bologna.

2.2 Susceptibility to ETEC intestinal adhesion

Piglets were classified for their susceptibility to ETEC intestinal adhesion (SEIA).

For each subject, after the opening of the abdominal cavity, the following samples of distal jejunum were collected: a) jejunal content, put in a sterilized vial and stored at –30°C; b) a segment of the tissue, stored at -80°C; c) a second segment, prepared as described by Bosi et al. (2004), to perform the *in vitro* ETEC villous adhesion assay. Pigs were considered highly susceptible with values ≥ 6 ETEC adhering per 250 µm length of villous, non-susceptible with no adhesion, and mildly susceptible with values <6 and > 0 ETEC adhering per 250 µm length of villous (Bosi et al., 2004).

2.3 Denaturating Gradient Gel Electrophoresis analysis and dominant bands sequencing

DNA was extracted from jejunal content samples as described by Trevisi et al. (2011). To analyse the total bacterial population of the jejunum, 16S rRNA gene-targeted denaturing gradient gel electrophoresis (DGGE) fingerprinting analysis was performed, following the protocol described by Trevisi et al. (2011). Each analysis was performed in duplicate. The proportional abundance of DGGE bands in each lane of each sample was calculated as Shannon's diversity index.

Visible dominant bands were excised from the gels with a scalpel and re-amplified using the same primers pair but without the GC clamp.

For the Universal bacteria fragments sequencing 3–5 µL of PCR product was treated with 2 µL of ExoSAP-IT® (USB Corporation, Cleveland, Ohio, USA) following the manufacturer's protocol. Cycle sequencing of the PCR products was obtained with the Big Dye v3.1 kit (Applied Biosystems, Foster City, CA, USA) and sequencing reactions were loaded on an ABI3100 Avant sequencer (Applied Biosystems). All sequences were visually inspected, edited, assembled, and aligned using the BioEdit v. 7.0.5.2 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and the CodonCode Aligner (http://www.codoncode.com/aligner) software. The sequences were compared using the BLASTN tool specific for bacterial genomes at the NCBI website (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi).

2.4 Bacteria quantification

The presence of specific DNA from *Escherichia coli* F4ac and F41 in jejunal content was detected by PCR analyses. As reference, the DNA extracted from a pure culture of each strain was used. The bacterial strains used in this trial were provided by the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia (Reggio Emilia, Italy). The E. coli O149:F4ac pure culture was isolated from pigs with colibacillosis, grown in Luria broth and further prepared as described by Bosi et al. (2004a).

The Escherichia coli F41 pure culture was received from National Institute of Public Health and the Environment (Bilthoven, Netherlands), it was grown in Brain Hearth Infusion Agar at 37°C for 24 hours in aerobic condition and further prepared as described by Bosi et al. (2004a).

The DNA was extracted following a standard protocol, as described by Trevisi et al. (2011).

To detect *E. coli* F4ac we used primers and amplification conditions described by Alexa et al. (2001). For *E. coli* F41, the primers were designed on the Genbank accession X14354.1 (http://www.ncbi.nlm.nih.gov/genbank/), using Primer 3 version 0.4.0 (http://frodo.wi.mit.edu/primer3/). The sequence of the primer is listed in Table 1. The PCR program included a 95°C denaturation for 5 min followed by 36 cycles at 95°C for 30 s, 58°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 5 min. The resulting product (197 bp) was resolved on 1.5% Tris borate/EDTA (TBE)-agarose gel stained with gel red reagent (Fisher Molecular Biology, USA).

Furthermore, to quantify the specific DNA from Enterobacteria and *Clostridium (Cl.) bartlettii* a semi-quantitative Real-time PCR was performed using the crossing point analyses.

For Enterobacteria the primers described by Castillo et al. (2006) were used. For *Cl. bartlettii* the primers were designed on the GenBank sequence NR_027573.1, within the same portion obtained from the sequencing of the DGGE band previously described (Table 1).

The quantification reactions were performed in a Light Cycler instrument (Roche, Mannheim, Germany). The amplification was carried out in a 10 µL volume containing 2 µL of cDNA, 8 pmol of each primer, and 5 µL of SYBR® Premix Ex Taq™ II (Perfect Real Time; Takara Bio Inc., Japan). The fast protocol included 40 cycles at 95°C for 5 s and 60°C for 20 s or 61°C for 24 s for Enterobacteria and for *Cl. bartlettii*, respectively The fluorescence detection was set at the last step of each cycle. The specificity of each amplification was determined by melting curve analysis and electrophoresis on 1.2% agarose gels. All amplifications were repeated twice and data were expressed as crossing point cycles.

2.5 Real-time quantification of ST3GAL1expression

The mRNA was isolated from the jejunal tissue sample collected from each subject, according to Qiagen RNeasy® Midi Kit protocol (Qiagen, Hilden, Germany). Purity and concentration of the total RNA extracted were checked spectrometrically by the Nanodrop ND 1000 (Nanodrop Technologies Inc., Wilmington, DE, USA) and RNA integrity was controlled by agarose gel electrophoresis analysis.

One microgram of RNA was reverse-transcribed from each sample using the ImProm-II Reverse Transcription System (Promega, Madison, WI, USA). A Real-time PCR quantification of the *ST3Gal1* gene expression was performed. The primers were designed using Primer 3 version 0.4.0 on the GenBank sequence NM 001004047.1 (*Sus scrofa* ST3 beta-galactoside alpha-2,3-sialyltransferase 1) (Table 1).

Firstly a PCR reaction was performed to amplify a fragment which served as the homologous DNA standard of a known copy number. This product was purified using the QIAquick PCR Purification Kit (Qiagen) and concentration was estimated by the Nanodrop ND 1000 spectrophotometer (Nanodrop Technologies Inc.). Then the fragment was serially diluted in 1:10 steps and a standard curve was created to perform an absolute quantitative analysis using internal primers. The quantification reactions were performed in a Light Cycler instrument (Roche, Mannheim, Germany). The amplification was carried out as described in the section 2.4. The protocol included 40 cycles at 95°C for 5 s and 61°C for 20 s. All amplifications were repeated twice and data were expressed as gene transcript copies /µl cDNA.

2.6 Statistical analysis

Data were analysed by analysis of variance, considering the diet (Trp), the SEIA, their interaction and the litter. When the *p*-values for the interactions were ≤ 0.10, the single interaction values were considered and the following linear contrasts were tested: Trp within no SEIA; Trp within mild SEIA; Trp within high SEIA; Linear effect of SEIA in Trp^- group, Linear effect of SEIA in Trp^+ group.

3. Results

3.1 DGGE analysis

All the samples of jejunal content were analysed by DGGE fingerprinting analysis and bands were counted for each sample. The band number of each group (dividing pigs by diet and genotype) was averaged and it is reported in Table 2. Additionally, to obtain more information about the microbiota variability, the Shannon's diversity index for bacteria was calculated on the basis of the number of bands detected by 16S rRNA gene-targeted DGGE.

The values of Shannon's diversity index and the band number did not differ for the SID Trp:Lys ratio, while both parameters were affected by SEIA (Table 2). The lowest values were observed for susceptible pigs. However, a trend for an interaction between the two factors was observed for the Shannon's diversity index (p = 0.096) and the band number (p = 0.097). The Shannon's diversity index and the band number increased linearly with the SEIA in pigs fed the Trp^- diet (p = 0.003 and p = 0.016, respectively). In not susceptible pigs the Trp^- diet tended to increase the Shannon's diversity index (p = 0.094).

3.2 Band isolation and sequencing

Figure 1 shows the result of DGGE comparing digesta samples from susceptible and mildly susceptible piglets. A differential band (band 1), present only in the mildly susceptible samples, and all the others dominants bands (bands 2 and 3) were isolated.

Band 1 resulted to be very similar to *Cl. bartlettii* (identity 99%), a Gram positive anaerobic bacteria belonging to the *Clostridium* genus, that has been previously isolated even in human faeces (Song et al., 2004).

All the others sequenced bands (bands 2 and 3), both present in samples with high and low microbial variability resulted to be strictly related to *Weissella paramesenteroides* (96% and 97%), a Gram positive bacteria included in the *Leuconostocaceae* family (order of *Lactobacillales*).

3.3 *Quantification of Enterobacteriaceae, ETEC F4ac and F41*

No sample was positive to the PCR screening, thus the presence of ETEC F4ac and F41 in the content of jejunum of all the experimental animals at the moment of the slaughtering was excluded.

Higher values of crossing point indicating lower concentrations of DNA, the gene copy number of total Enterobacteria in the intestinal samples was reduced in susceptible pigs, as compared to mild susceptible

or not susceptible pigs (p = 0.027), and it was not affected by Trp:Lys ratio (Table 2). No interaction between the two variables was observed.

3.4 Quantification of Cl. Bartlettii

A preliminary PCR screening was made and presence of specific DNA from *Cl. bartlettii* was found in samples of intestinal content of all the pigs. The results of Real-time PCR performed on jejunal samples to quantify the *Cl. bartlettii* are presented in Figure 2. *Cl. bartlettii* specific DNA was more abundant in non susceptible and mildly susceptible piglets than in susceptible ones (p = 0.052), while its concentration was not affected by the diet (Table 2). However, an interaction between the two experimental factors was observed: the quantity of *Cl. bartlettii* specific DNA decreased linearly with SEIA in the Trp^+ group (p = 0.002), but not in the Trp^- group. The 0.22 SID Trp:Lys ratio tended to increase the quantity of *Cl. bartlettii* specific DNA in mildly susceptible pigs (p = 0.062) and to decrease the value in susceptible pigs (p = 0.025), compared with the 0.17 SID Trp:Lys ratio.

3.5 Quantification of ST3GAL1gene expression

A Real-time quantitative PCR assay was performed on cDNA obtained from intestinal mucosa to quantify the expression of *ST3GAL1* gene. Diet effect and interaction between diet and susceptibility were not statistically significant and the values for the different degrees of SEIA are presented in Figure 2. The gene copies number was higher in susceptible than in not and mildly susceptible piglets (p = 0.019).

4. Discussion

The importance of different gut receptor phenotypes in the gastrointestinal bacterial disease pathogenesis, and those regarding the patterns of host glycosylation in particular, gained evidences in the last years (Moran et al., 2011). Nevertheless, the evidences that divergent phenotypes for the adhesion of one microbe can affect the performances in healthy pigs are scarce. Thus we were surprised to observe that the Trp requirement of healthy piglets in the first week after weaning was affected by a polymorphism for *MUC4*, marker of SEIA (Trevisi et al., 2010). We observed also that the intestinal gene expression of several genes related to the innate immunity and responsive to the bacteria products, were increased in susceptible healthy pigs, and that this increase was reversed by a Trp supplementation (Trevisi et al., submitted).

These observations raised the hypothesis that intestinal receptor for ETEC could be used also by other bacteria, or that the presence of this receptor could differentially affect the interplay of the host with the microbiota. Here we confirmed that the individual genetic susceptibility to ETEC contributes to shape the variability of microbiota: the bacterial variability decreased with the susceptibility to ETEC intestinal adhesion, particularly when pigs were fed the Trp^- diet. The pigs in the trial were in general fully healthy

and rapidly regained growth after the transient immediate post-weaning anorexia. Furthermore no DNA from ETEC was detected in jejunum content of the pigs although we can not exclude a transient mild and asymptomatic presence of ETEC in the first days of trial. Nevertheless, the Trp^{+} diet abrogated the reduction of feed intake observed in susceptible pigs during the first week post-weaning (Trevisi et al., 2010). Thus a more constant availability of feed in the gut could have partially favoured the establishment of a more balanced microbiota.

The exact nature of the receptors for F4 fimbriae is not known yet, but some researches indicate that they are placed on pig epithelial cells. Francis et al. (1988) isolated from brush borders of porcine intestinal cells two intestinal mucin-type glycoproteins (IMTGP), which bind F4ab and F4ac. Furthermore an essential component in recognition of IMTGP receptors by F4ac adhesions was the presence of β-linked galactose (Grange et al., 1988). We are not aware about the existence of commensal bacteria that specifically use galactose motifs on the brush borders of porcine intestinal cells to improve their settling in the gut, but such event can not be excluded. In fact, a previous research showed that some bacteria, proposed as probiotics, can use the adhesion to mucins sugars to improve their ability to colonize the intestine (Laparra and Sanz, 2009). However, we did not find by DGGE any band selectively present in susceptible pigs. On the contrary, an increased presence of *Cl. Bartlettii* seemed to characterise the pigs less susceptible to ETEC or fed with low Trp amounts. *Cl. bartlettii* was firstly isolated from human feces (Song et al., 2004), it is phylogenetically close to *Cl. difficile* (Bordeleau et al., 2011), with which it shares a high proportion of orthologues. In fact, 70% of the genes encoding for the spore proteome of *Cl. difficile* have orthologous in the *Cl. bartlettii* genome (Lawley et al., 2009).

It is not known if *Cl. bartlettii* is able to adhere to enterocytes, however the presence of a flagellum in *Cl. difficile* permits its specifical adhesion to cells and also the invasion (Tasteyre et al., 2001). Thus it is possible that a variation of the sugar motif on the enterocyte brush borders could favor or not *Cl. bartlettii.*

It is quite difficult to explain why different SID Trp:Lys ratios can interact with the high presence of F4 receptors to affect intestinal microbiota composition. It has been observed in *in vitro* cultures obtained from intestinal inocula that the addition of some amino acids to the medium can inhibit the growth of some selected bacteria, but this was not the case of Trp (Dai et al., 2010). However, in these *in vitro* tests, the interaction with enterocytes differing for surface properties was not tested.

Tryptophan is the substrate of the immunoregulatory enzyme IDO, expressed in dendritic cells in response to inflammatory stimuli. This enzyme catalyzes the first reaction regulating the product of severel metabolites, generally called kynurenines, with an important physiological mechanism capable of controlling both inflammation and immunity (Mellor and Munn, 2003; Le Floc'h et al., 2004). Thus it is reasonable to suppose that more Trp could allow a more effective response upon activation of the immune system. However, we were not able to find results suggesting a direct control of the gut microflora by dietary Trp.

The effect of a high degree of susceptibility to ETEC on intestinal *ST3GAL1* gene expression is quite intriguing, because of the hypothesized principal role of galactose on the brush borders of ETEC susceptible pigs, and because ST3GALl1 adds a molecule of sialic acid to a galatose sequence. However, no scientific evidence of an involvement of ST3GAL1 on the expression of the receptors for ETEC F4 has been reported yet. *ST3GAL1* gene was reported to be located close to the chromosomal region were the putative gene for the porcine receptor for ETEC F41 can be located (Yang et al., 2009), but anyhow we did not find DNA from this microbe in the gut of our pigs. Conversely, it is known that ST3GAL1 regulates CD8+ T lymphocyte homeostasis by modulating O-glycan biosynthesis (Priatel et al., 2000), that an increased expression of

sialylated cell surface structures is accompanied by a differentiation of the professional antigen-presenting dendritic cells and that sugar surface motifs can also vary depending on the involved enzyme (ST3GAL1 or ST6GAL1) (Videira et al., 2008). Thus the effect that we observed on *ST3GAL1* gene expression could be more realistically related to the tuning of the immune function induced by the variation of the composition of microbiota with the increasing susceptibility to ETEC and with the variation of dietary SID Trp:Lys ratio.

5. Conclusions

Collectively, these data indicate that susceptibility to intestinal adhesion of ETEC F4 has a role also in healthy pigs, by reducing microbial richness in the gut, particularly when a diet just covers the minimal Trp requirement. An increase in the Trp dietary content allows to partially offset this effect.

Our data also show that the relevance of genetically transmitted characters of the small intestinal surface should be more carefully considered in the studies to get a better understanding of the interaction between gut microbiota, diet and the young pig.

Acknowledgements

Ajinomoto Eurolysine S.A.S. financially supported this work.

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Table 1. The Primer sequence.

Gene	NCBI access number	Primer		Reference
Escherichia coli K88ac		For	TTTGCTACGCCAGTAACTG	
		Rev	TTTCCCTGTAAGAACCTG	Alexa et al. (2001)
Enterobacteria		For	ATGGCTGTCGTCAGCTCGT	
		Rev	CCTACTTCTTTTGCAACCCACTC	Castillo et al . (2006)
Total bacteria		For-GC	AACGCGAAGAACCT TACCGG TGTGTACAAGACCC - GC clamp	
		Rev	CGGTGTGTACAAGACCC	Nübel et al. (1996)
Escherichia coli F41		For	GCAGCGAAGATGAGTGATGG	
	X14354.1	Rev	CCAAATGACAAAGGAACAGAAAG	This study
Clostridium bartlettii		For	CCTAATCGCATCTTTCCCTTC	
	NR_027573.1	Rev	CGTCATCCCCACCTTCCTC	This study
*ST3GAL1**		For	AGGGTCTCCGCCTGGTTC	
	NM_001004047.1	Rev	AAGTTATTGGGCTGCTTCTCC	This study

**Beta-galactosidealpha-2,3-sialyltransferase*

Table 2. Effect of Trp and Susceptibility to ETEC intestinal adhesion (SEIA) on the Enterobacteria counts and microbiota variability by DGGE on the jejunum contents of piglets.

SID Trp:Lys ratio[1]	SEIA	Shannon index of bacterial variability	DGGE (N of Bands)	Enterobacteria (crossing point)	*Cl. bartletti* (crossing point)
	not susceptible	1.76	7.80	24.5	28.4
0.17	mildly susceptible	1.55	6.30	24.9	29.7
	susceptible	0.99	3.33	25.5	28.2
	not susceptible	1.43	5.50	24.3	26.4
0.22	mildly susceptible	1.85	8.57	24.3	26.4
	susceptible	1.10	3.83	25.7	33.9
SEM		0.16	1.17	0.439	1.40
Statistical significance, *p*					
SID Trp:Lys		0.863	0.875	0.590	0.937
SEIA		0.004	0.023	0.027	0.052
Interaction[2]		0.096	0.097	0.568	0.014
Linear effect of SEIA in 0.17 SID Trp:Lys ratio		0.003	0.016	-	-
Linear effect of SEIA in 0.22 SID Trp:Lys ratio		-	-	-	0.0017
Effect of the SID Trp:Lys ratio in					
- not susceptible		0.094	-	-	-
- mildly susceptible		-	-	-	0.062
- susceptible		-	-	-	0.025

[1] Standardized ileal digestible tryptophan to lysine ratio

[2] Only contrasts where $p \leq 0.10$ are presented.

Figure 1. Denaturating Gradient Gel Electrophoresis fingerprints of total bacteria obtained from jejunal samples of susceptible and mildly susceptible pigs.Each lane represents one animal, M: marker line. In the squares there are the excided bands that were reamplified and successfully sequenced. Vertical lines divide animal groups.

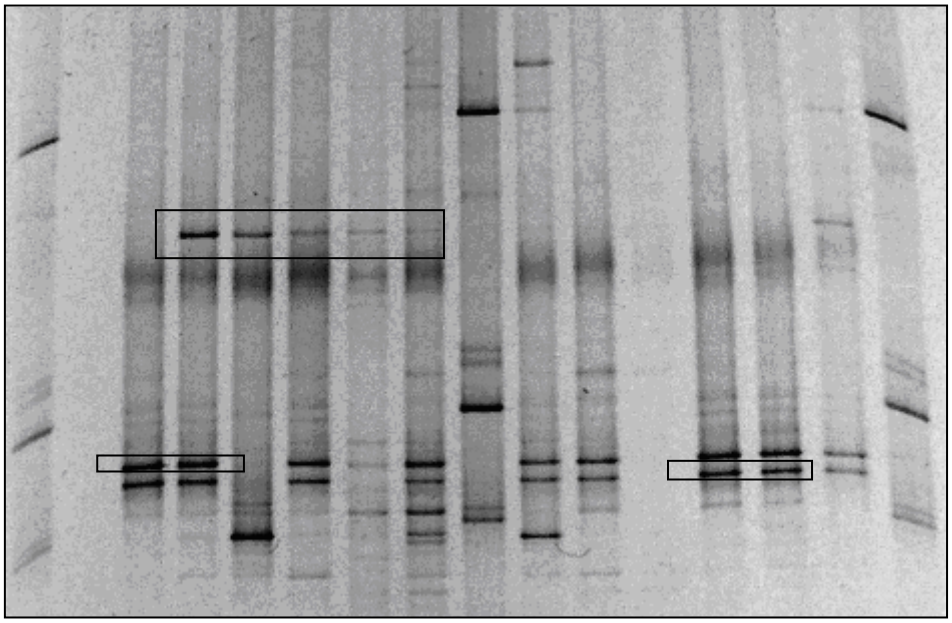
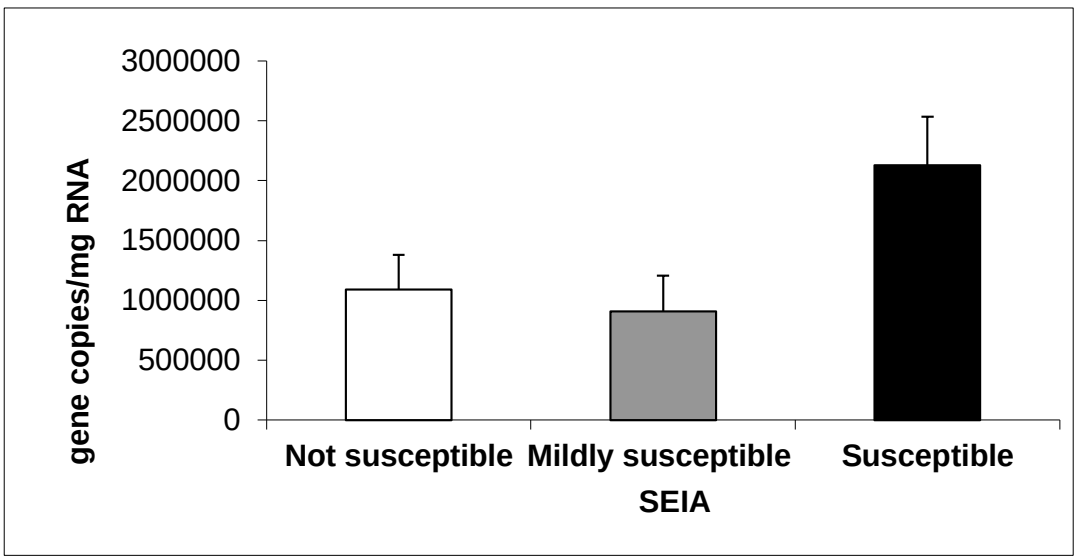


Figure 2. Effect of Susceptibility to ETEC intestinal adhesion (SEIA) on the gene expression of *ST3GAL1* in the jejunum tissue of piglets (Means ± SEM). Susceptible pigs differed from not and mildly susceptible pigs (p = 0.019).



Chapter 4

Distribution and developmentally regulated gene expression of polymeric immunoglobulin receptor in the gastric mucosa of pre and post weaning pigs

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Abstract

The mucosal immune system in gastrointestinal tract is critical during pigs growth, especially in the period from birth to after weaning. The polymeric immunoglobulin receptor (pIgR) mediates the transport of secretory pIgs from the lamina propria to the lumen, being a key element of both adaptive and innate mucosal immune system. Despite the emerging role of the stomach as a first barrier against pathogen colonization of the intestinal tract in piglets, the gastric mucosal immune system has been poorly characterized to date and no studies have been devoted to the characterization of pIgR in pigs. We aimed to investigate the pIgR time course expression and protein distribution in three functionally different sites of the stomach of pre and post-weaning pigs.

Oxyntic, oxyntic-cardiac and pyloric mucosa samples were collected from 84 pigs, slaughtered pre-weaning at 14, 21 and 28 days of age (23, 23 and 19 pigs, respectively) and at 42 days (14 days post-weaning, 23 pigs). mRNA expression levels were evaluated by RT-qPCR, and pIgR protein and mRNA were localized by immunohistochemistry and *in situ* hybridization techniques.

Results indicated that pIgR is expressed in the gastric mucosa of growing pigs, and its transcript levels are developmentally regulated with regional differences among the three sites of the stomach. The pIgR protein and mRNA were localized in the gastric glands located in the lamina propria, indicating that the pIgR is actively synthesized in gastric mucosa and it could play a crucial role in gastric mucosal immune defence of growing pigs.

Keywords: polymeric immunoglobulin receptor; gastric mucosa; pigs; weaning

Introduction

The mucosal surfaces of the gastrointestinal tract play a primary role in the interaction between the host and the endogenous or invading microorganisms in mammals. The mucosal immune system represents the first defence against potential pathogens and it requires a thigh regulation and control. In fact, the mucosal immunity must provide an active response against infectious agents and at the same time it must express tolerance to the 'harmless' antigens and to commensal bacteria [1, 2].

In the periods after birth and after weaning, the young pigs are exposed to the maximum amount of novel antigens. The development of appropriate immune responses to pathogens and to harmless dietary and commensal antigens is therefore critical during the gastrointestinal mucosa maturation in young pigs [3]. An inadequate response to pathogens or inappropriate active reaction against harmless antigens can impair the piglets development and affect the productive performance of the animals [1].

A key element of the mucosal adaptive immune system is the production and active transport of polymeric immunoglobulins (pIgs), produced by plasma cells in the lamina propria and actively transcytosed through the epithelial cells by the polymeric immunoglobulin receptor (pIgR) [4]. The pIgR is a type-I transmembrane glycoprotein of about 100 kDa, synthesized by epithelial cells lining mucous membranes and exocrine glands. The pIgR binds the polymeric immunoglobulins A (pIgA), and to a lesser extent immunoglobulins M (pIgM), at the basolateral surface of epithelial cells and mediate their transcytoses to the apical surface, where the complex pIgs-pIgR is proteolitically cleaved to generate secretory immunoglobulins (SIgs). Consequently, the SIgs released into the lumen are composed of the pIgs and the extracellular portion of pIgR (defined as secretory component, SC) [4,5]. In absence of specific immunoglobulin production, SC is released in its free form into the lumen, representing an important component of the innate anti-microbial defence [6]. It was demonstrated that pIgR is critical for the maintenance of the mucosal homeostasis and food tolerance in mice [7]. The pIgR expression was observed at high levels in the intestine and at low levels in lung, kidney, pancreas and endometrium in humans [8]. Low pIgR mRNA expression was reported in liver and stomach of mice [9]. Due to its importance in the regulation of both innate and adaptive immune response at mucosa surfaces, pIgR could play a key role in the development of mucosal immune system in the gastrointestinal tract of young piglets.

To date, the gastric mucosal immune system in pigs has been poorly characterized, in comparison with the extensively investigated intestinal mucosal immunity. In fact, the mucosal immune responses in the stomach have been considered of minor importance in relation to gut host diseases, possibly due to the inhospitable microbial environment and the shorter time of exposure to the feed compared with intestine. However, the possible role of the gastric immune system as a first barrier against pathogen colonization of the intestinal tract in piglets is emerging. The occurrence and the distribution of lymphoid follicles in functionally different parts of the stomach in conventional piglets have been recently reported [10], suggesting the existence of a basic machinery for the adaptive immune response in stomach. To the best of our knowledge, no studies have been devoted to the characterization of pIgR during the development of the gastric mucosa in the period pre and post weaning in pigs.

The aims of the present study were therefore: a) to determine whether pIgR was expressed in the stomach of piglets; b) to investigate the time course mRNA expression in three functionally different sites of the gastric mucosa of pre- and post-weaning piglets, c) to investigate the protein distribution in the three sites of the gastric mucosa; d) to confirm the active synthesis of the pIgR in the gastric mucosa by mRNA localization.

Here we report that pIgR is expressed in the gastric mucosa of growing piglets, and its transcript levels are developmentally regulated with regional differences among the three considered sites of the stomach. The pIgR protein and mRNA were localized in the gastric glands located in the lamina propria, indicating that the pIgR is actively synthesized in gastric mucosa and it could play a crucial role in gastric mucosal immune defence.

Materials and methods

All experimental procedures were carried out in accordance with the guidelines of the French National Institute for Research in Agriculture (INRA) for the care and use of laboratory animals.

Experimental design, animals and feeding

In this trial, we used eighty-four piglets obtained from twenty-three sows reared at the farm of INRA Saint-Gilles. Eleven sows were treated orally with the antibiotic amoxicillin from day –10 pre-farrowing to day 21 post-farrowing, while the remaining twelve sows were untreated.

The piglets were randomly slaughtered, balanced per sow and per treatment, at 14, 21 and 28 days of age (pre-weaning), or at 42 day of age (14 d post-weaning, weaned at 28 d of age) (23, 23, 19 and 19 pigs per each age, respectively). For the post-weaning groups, every litter was kept separated from the others and all the piglets received a standard weaning diet without any addition of antibiotics or growth promotants.

Pig slaughter and tissue sampling

At slaughter, the piglets were anesthetized by electric shock, and immediately killed by exsanguination 4 h after the last meal. For each pig, a midline abdominal incision was made and the stomach was gently removed. The stomach was opened along the greater curvature, emptied of its contents, and rinsed with double-distilled water.

Tissue samples were collected in three functionally different sites of the stomach: in correspondence to the transition from cardiac to oxyntic mucosa in the lesser curvature**,** in the proper gastric gland region

(oxyntic mucosa, in the body) and in the pyloric gland region (in the antrum). These three gastric sites are referred to as Cd, Ox, and Py respectively in the present report.

For RNA extraction and expression analysis, samples from these three gastric sites were collected, snap frozen in liquid nitrogen and stored at –80°C until analysis.

For immunohistochemistry and *in situ* hybridization analyses, whole thickness tissue specimens of ~1 cm^2 were removed, pinned tightly to balsa wood and fixed in 10% buffered formalin for 24 h. The samples were then removed from the fixative, dehydrated in a graded series of ethanol and embedded in paraffin.

Real-time quantitative PCR

Total RNA was isolated from tissue samples according to Takara Fast Pure™ kit (Takara Bio Inc) protocol. For each sample, 1 µg of RNA was reverse transcribed using the ImProm-II Reverse Transcription System (Promega, Italy).

The *pIgR*-specific mRNA abundance was determined by real-time quantitative PCR (RT-qPCR), performed in a LightCycler® Real-Time PCR Systems (Roche, Germany).

The reactions, performed in duplicates, were carried out in a 10 µL volume containing about 100 ng of cDNA, 0.5 µM of each primer, and 5 µL of SYBR Premix Ex Taq II (Perfect Real Time) (Takara Bio Inc). Reactions consisted of an initial denaturation step at 95°C for 30 s and 40 cycles of 95°C for 5 s and the annealing/extension temperature for 20 s. The information related to the primers sequences and annealing/extension temperatures are indicated in Table 2. Threshold cycles were converted to mRNA molecules/µL using a standard curve, obtained for each gene as described previously by Trevisi et al. (2008) [11]. The specificity of each amplification was checked by melting curve analysis at the end of the reaction. To ensure that the primers used for *pIgR* expression analysis were specific for the intended target, the PCR products were sequenced (BMR, Italy) and confirmed to correspond with the expected porcine *pIgR* gene sequences. The *pIgR* expression data were normalized by geometric mean of the expression of the two housekeeping genes *Hydroxymethylbilane Synthase* (*HMBS2*) and *Ribosomal Protein L4* (*RPL4*). Primers and amplification conditions for the two housekeeping genes are reported in Table 2. All the individual data were analysed by analysis of variance using the GLM procedure of SAS (SAS Inst., inc., Cary, NC) with a factor design, including the antibiotic treatment of sows, the age of piglets, their interaction, and sows within treatment. Age was considered as a repeated measure inside each sow.

Immunohistochemistry

For immunohistochemistry, the avidin-biotin-peroxidase complex (ABC) method was used as described elsewhere [12]. Briefly, paraffin sections were deparaffinized and rehydrated; the slides were heated in sodium citrate buffer (pH 6.0) in a microwave to unmask the antigenic sites. Endogenous peroxidase was blocked with 1% aqueous hydrogen peroxide solution for 30 min at room temperature (RT), and subsequently incubated for 30 min in PBS containing 10% normal goat serum, 1% normal swine serum and 10% bovine serum albumin to prevent nonspecific binding of the antibodies. The sections were then incubated overnight at 4°C with the polyclonal rabbit antiserum anti-PIGR (Sigma-Aldrich, HPA012012), diluted 1:600. After washing, the sections were incubated at RT for 1 h with the biotin-conjugated

secondary antibody goat anti-rabbit IgG, diluted 1:500 (Vector, Vector Laboratories) and then treated with ABC complex (Vector elite kit, Vector Laboratories). The immune reactions were visualized applying a 3,3'-diaminobenzidine chromogen solution (Vector DAB kit, Vector Laboratories, Italy).

In situ hybridization

An LNA™ -enhanced DNA oligonucleotide probe, containing 30% of LNA™ nucleotide and digoxigenin-labelled at the 5' end, was purchase from Exiqon Inc. (The Netherlands). The probe (TTCTCTGGGAAGTTGGTGAGGT) was designed on the porcine pIgR mRNA reference sequences [GenBank:NM_214159] using the online probe designer tool on the Exigon website (http://www.exiqon.com). Paraffin 10 µm-sections mounted on poly-L-lysine-coated slides were deparaffinized, rehydrated and post-fixed in 4% paraformaldehyde in DEPC-treated PBS 10 min at RT. The sections were acetylated in 0.1 M triethanolamine buffer at pH 8.0, containing 0.25% (v/v) acetic anhydride for 10 min and incubated with proteinase K (5 µg/mL in PBS) for 15 min at RT. After washing with PBS, slides were incubated 1 hour at 37 °C with the hybridization buffer containing 40% formammide, 5× saline sodium citrate (SSC), 2.5× Denhardt's solution, 250 µg/mL yeast RNA, 500 µg/mL salmon sperm DNA, 2% blocking reagent (Roche Diagnostic). The sections were then incubated for 20 h at 55 °C in the hybridization buffer containing 80 nM of the denatured LNA probe and 0.1% Tween. The sections were soaked in 5× SSC at 60°C, quickly washed in 1×SSC at RT, incubated 10 min in 1×SSC at 55°C, 1 hour in 0.2×SSC at 55°C and finally washed in TBS buffer (Tris-HCl 0.1M ; NaCl 0.15M). After incubation in blocking buffer (1% blocking reagent and 0.1% Tween in TBS) for 30 min, the sections were incubated overnight at 4 °C with sheep alkaline phosphatase-conjugated anti-digoxigenin polyclonal antibody (Roche Diagnostics, cat. 11093274910) diluted 1:500 in blocking buffer. Staining reactions were carried out using NBT/BCIP Ready-to-Use Tablets (Roche) following manufacturers' instruction. Sections were stained for 24 h at RT, washed 3 times × 10 min with TBS and once with sterile water, and finally mounted using an aqueous medium. Negative controls were performed following the same procedure but omitting the probe in the hybridization step or the anti-digoxigenin antibody.

Results

***PIgR* mRNA expression at different piglet ages and in three sites of gastric mucosa**

Specific *pIgR* mRNA expression was detected in the gastric mucosa of piglets at different ages by RT-qPCR. For *pIgR* mRNA expression values, the effect of the interaction between the antibiotic supplementation to the sows with the piglets age was not statistically significant. Therefore, these two effects can be discussed separately. In the current paper we described the effect of the piglets age on *pIgR* mRNA expression in different sites of gastric mucosa. The presentation of the effect of the antibiotic supplementation to the sows will be addressed in a - subsequent study.

The normalized *pIgR* mRNA expression values at four different ages and in three different gastric sites are reported in Figure 1. On average, mRNA expression in Oxy was 10-fold lower than the expression in Cd and

in Py. In the Cd, the pIgR expression increased linearly during suckling (*P*=0.019) and it increased post-weaning compared with pre-weaning (*P*=0.001). Also in the Py, the *pIgR* expression increased at post-weaning age (*P*=0.049). In the Ox, the *pIgR* expression increased linearly during suckling (*P*=0.036) and, differently from the other two sites, it showed a marked decrease after weaning.

PIgR protein localization in different sites of gastric mucosa

The immunohistochemical localization of the pIgR protein in sections of the gastric mucosa was performed in three sites of the stomach from piglets at 42 days of age (Fig. 2). The detection of pIgR protein in the three sites confirmed the presence of pIgR in stomach of piglets, as indicated by the detection of *pIgR* gene transcript by RT-qPCR. The pIgR protein was located in the bottom part of the gastric glands. The pIgR was more abundantly localized in the Cd compared with the other sites, in accordance with the RT-qPCR results, and it was distributed in more than half thickness of the tubular mucous-producing glands (Fig. 2 A, 2D). In Ox, the protein was found in the bottom of the tightly packed glands (Fig. 2 B, 2E). In the Py the label was less defined and it showed a spotted distribution pattern (Fig. 2C, 2F).

***in situ* localization of *pIgR* mRNA**

The localization of mRNA was achieved by *in situ* hybridization technique, to further confirm the active synthesis of the pIgR protein at the gastric mucosal sites. The *pIgR* mRNA localization was performed in the region of transition between cardial and oxyntic mucosa (Cd), were the strongest protein signal was detected.

The mRNA was detected in correspondence of the gastric glands within the lamina propria (Fig. 3 A), mirroring the protein localization (Fig. 3 C) and confirming the active synthesis of pIgR in these cells.

Discussion

Stomach has been usually considered an almost aseptic organ because of its inhospitable acidic environment and not so much attention has been given on its emerging potential role in immunity. To gain insight on the role of the stomach in the defence against pathogens in young piglets, we hypothesised that the gastric mucosa is provided with the basic machinery of the innate and adaptive mucosal immune system. This hypothesis was supported by a previous research that described the distribution of the gastric-associated lymphoid tissue in conventional piglets [10].
Regulation of pIgR expression in mucous membranes involves complex interactions among host, microbial, and environmental-derived factors and the extensive literature on this topic recently has been reviewed [6,13].

The present study focused on the characterization of the pIgR occurrence and expression pattern in the gastric mucosa of piglets, since pIgR can be considered a key player in the mucosal immune system, participating in both adaptive immunity and innate immune defence. In fact, the trancytosis of pIgA by pIgR promotes the intracellular neutralization and transcellular excretion of antigens and pathogens and ensures continuous delivery of SIgA to the epithelial surface [6]. Upregulation of pIgR expression thus would increase the capacity of mucosal epithelial cells to transport dimeric IgA.On the other hand, the free SC, produced by transcytosis of pIgR in the absence of pIgA ligand, interacts directly with several bacteria or their products and it is an important component of innate anti-microbial defense [14].

In this study, the *pIgR* mRNA was detected for the first time in the stomach of piglets, in good agreement with the study by Asano and colleagues (2004)[9], in which the pIgR mRNA was detected by Northern blot in the stomach of mice.

Levels of pIgR transcription were found to be developmentally regulated along the mucosa maturation in growing piglets, suggesting that the progressive functional maturation of mucosa associated lymphoid tissue is associated with an increase in pIgR gene expression.

After birth, the gastrointestinal tract undergoes a complex process of growth and differentiation and several genes involved in this process are developmentally regulated along the course of postnatal development, mainly at the level of transcription, as proposed by Jenkins et al.(2003) [15]. This development process include the maturation of the mucosal adaptive immune system, which is immature in neonatal and suckling animals [16]. In the intestine, within days after birth the organized lymphoid tissue expand and antigen-presenting cells and T-cells begin to infiltrate the mucosa [17]. The maturation of an efficient mucosal immune system appears to be dependent on colonization by commensal and pathogenic microbial flora [3]. The maturation of the mucosa from after birth to post-weaning age is reflected by the raise of pIgR mRNA expression found in this study. Accordingly, pIgR expression was found to raise from pre to post weaning period in the small intestine of mice [14], while it is not expressed in the intestine of rats during early pre weaning postnatal life [18].

However, we observed regional differences among functionally different parts of the stomach in the pIgR expression profiles. In fact, in this study different sites of the stomach, known to provide different functions, were taken in consideration. The selected oxyntic mucosa (Ox), pyloric mucosa (Py) and cardiac to oxyntic transition mucosa (Cd) corresponded to the sites where a previous study found several submucosal lymphoid follicles, defined in that research as sites 1, 4-5 and 7 respectively [10].

In the pyloric and in the transition between cardiac and oxyntyic mucosa, the pIgR gene expression was higher than in the oxyntic mucosa, and it increased from pre to post weaning period, probably mirroring the functional development of mucosal immune system as well as the adaptation to the change of the diet.

On the other hand, the reduced mRNA expression of pIgR in the oxyntic mucosa of post-weaning pigs may be related to the typical functional raise of HCl secretion observed after the end of the suckling period in this region. Increased HCl production could lead to the reduction of the local bacteria-driven Igs secretion, in agreement with the decreased pIgR expression at 42 days after weaning.

The expression of pIgR at these gastric sites was confirmed by protein immunostaining. This is the first report of the pIgR localization in the gastric mucosa, where the pIgR protein was mainly found within the gastric glands in the lamina propria. Previous researches described the expression of pIgR in the epithelial and glandular cells in various organs (including kidney, lung, pancreas and rat but not human liver [4, 9]) and especially in the columnar epithelial cells of the intestine, where it exert its function of secretory Igs

transcytosis from lamina propria to the lumen [5, 6]. In the stomach of piglets, pIgR is likely involved in the release of secretory Igs, as well as of free form of the SC, within the coiled gastric glands that empty into the base of the foveolae (gastric pits, which represent invaginations of the surface epithelium). The active synthesis of pIgR protein in the glandular cells of gastric mucosa was confirmed in the present study by the *in situ* localization of the *pIgR* gene transcript within the same cells.

The ability of *pIgR* gene to react to the increased presence of pathogens in the stomach (typically *Helicobacter* spp.) has been already evidenced in mice [19]. However, the piglets used in this study were in general healthy and the impact of commensal for the development of immunity in the stomach is in general not considered. Our data show that the stomach of pig is able to react to the increasing contact with commensal bacteria and, presumably, with the increasing complexity of microbiota, that is observed in the suckling and in the post-weaning period.

Conclusion

We can conclude that pIgR is present in the stomach of the piglet during the mucosa maturation. The developmentally regulated expression of pIgR in the gastric mucosa found in this study suggests a linkage between physiological and immunological activity. The pIgR presence was confirmed by mRNA and protein localization, which confirmed a stronger protein presence in the cardiac-oxyntic transition mucosa, previously found rich of submucosal lymphoid follicles.

The presence and the developmentally regulated expression of pIgR in the gastric mucosa of pre and post weaning pigs suggest that also the stomach may play a primary role in the adaptive and innate mucosal immunity in growing pigs.

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Table 1 - Primers information and RT-qPCR conditions used in the trial

Gene	NCBI accession number	Oligo sequence (5'→3')		Amplicon length	Annealing T
PIGR[1]	NM_214159.1	Forward Reverse	AGCCAACCTCACCAACTTCC CTGCTAATGCCCAGACCAC	105 bp	62°C
HMBS2[2]	DQ845174	Forward Reverse	AGGATGGGCAACTCTACCTG GATGGTGGCCTGCATAGTCT	83 bp	62°C
RPL4[3]	DQ845176	Forward Reverse	CAAGAGTAACTACAACCTTC GAACTCTACGATGAATCTTC	122 bp	60°C

[1] Polymeric immunoglobulin receptor
[2] Hydroxymethylbilane synthase
[3] Ribosomal protein L4

Figure 1 - Expression of polymeric immunoglobulin receptor (*pIgR*) gene in three parts of the stomach from pre and post weaning piglets

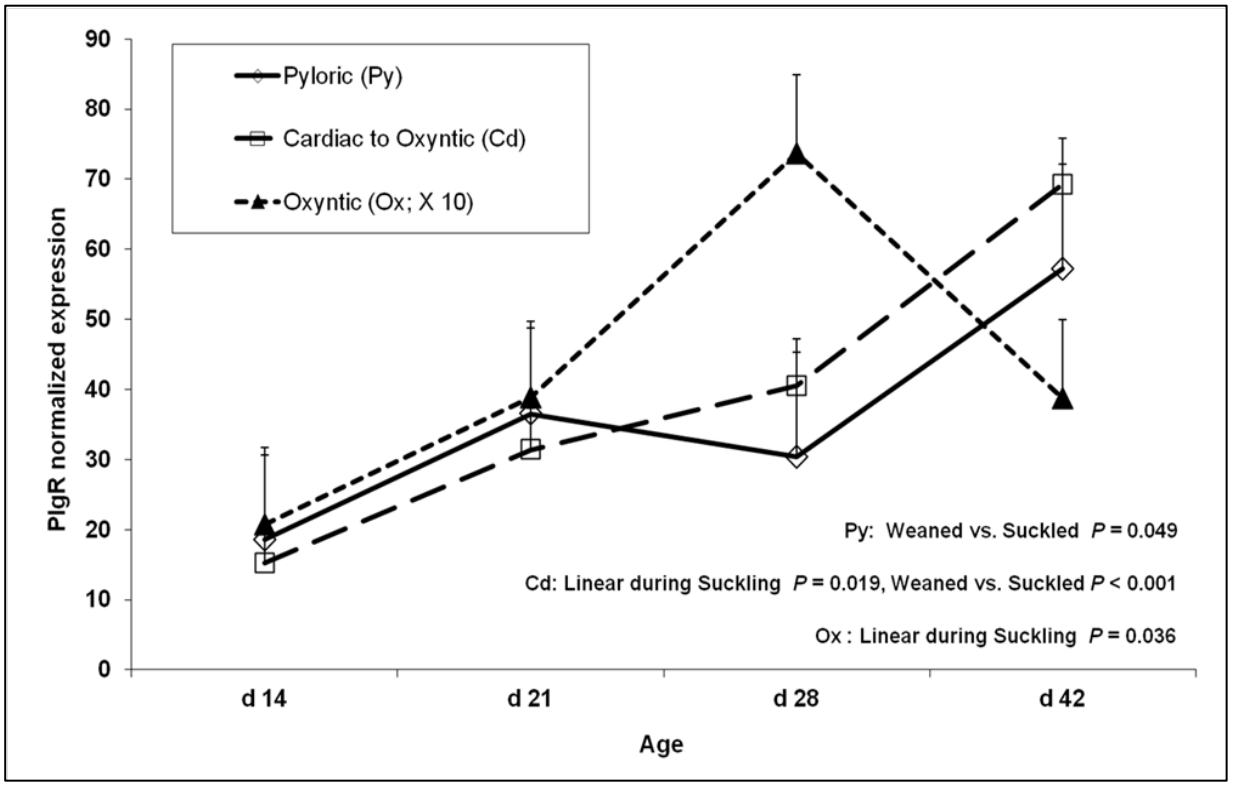


Figure 2 - Localization of polymeric immunoglobulin receptor (pIgR) protein in three sites of the gastric mucosa from 42 days-old piglet**.** PIgR protein immunostaining in the transition from cardiac to oxyntic mucosa (A, D), in the proper glandular region (B, E) and in the pyloric region (C, F). Bar indicate 200 µm (A-C; 50× magnification) or 100 µm (D-F; 100× magnification).

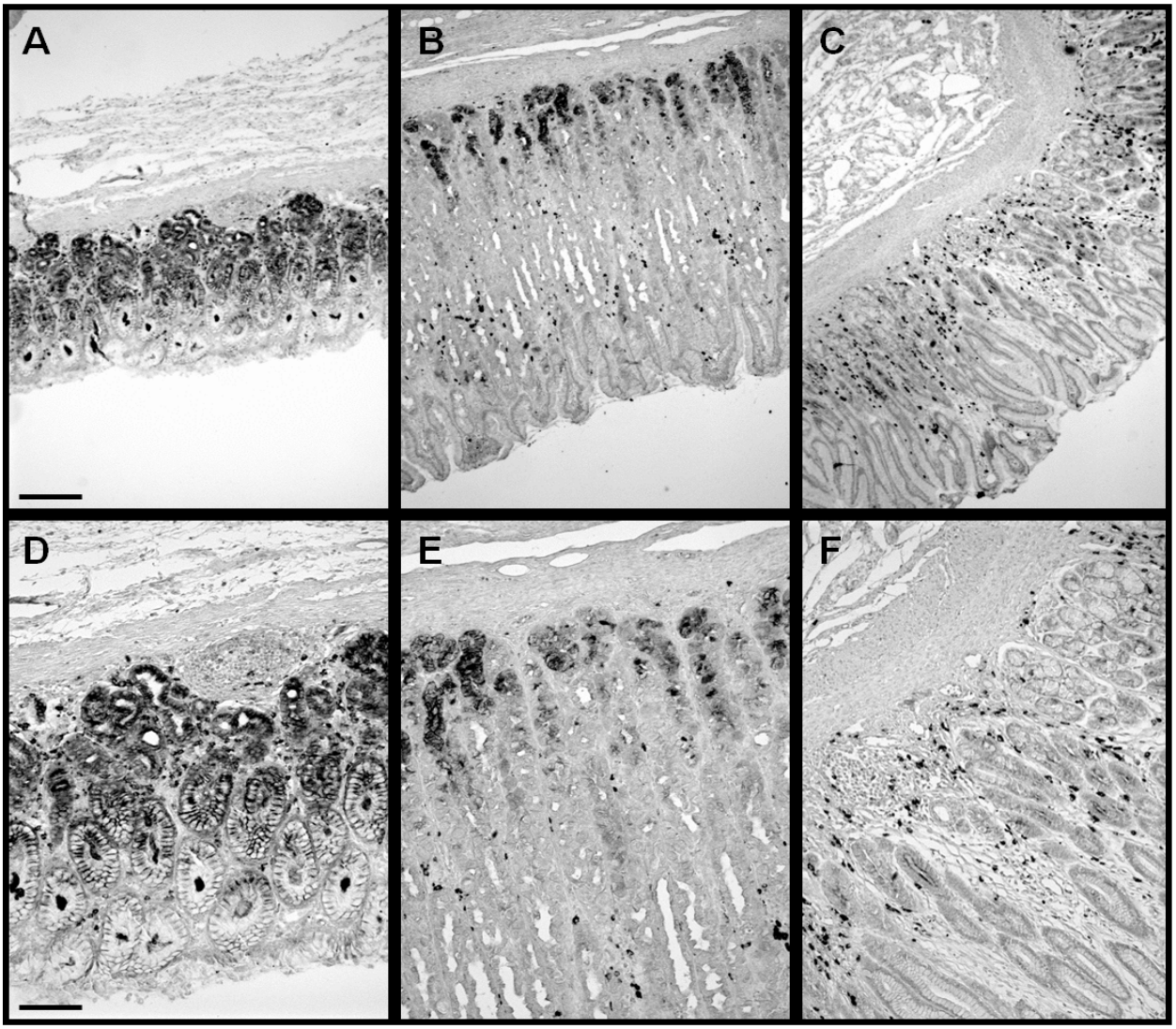
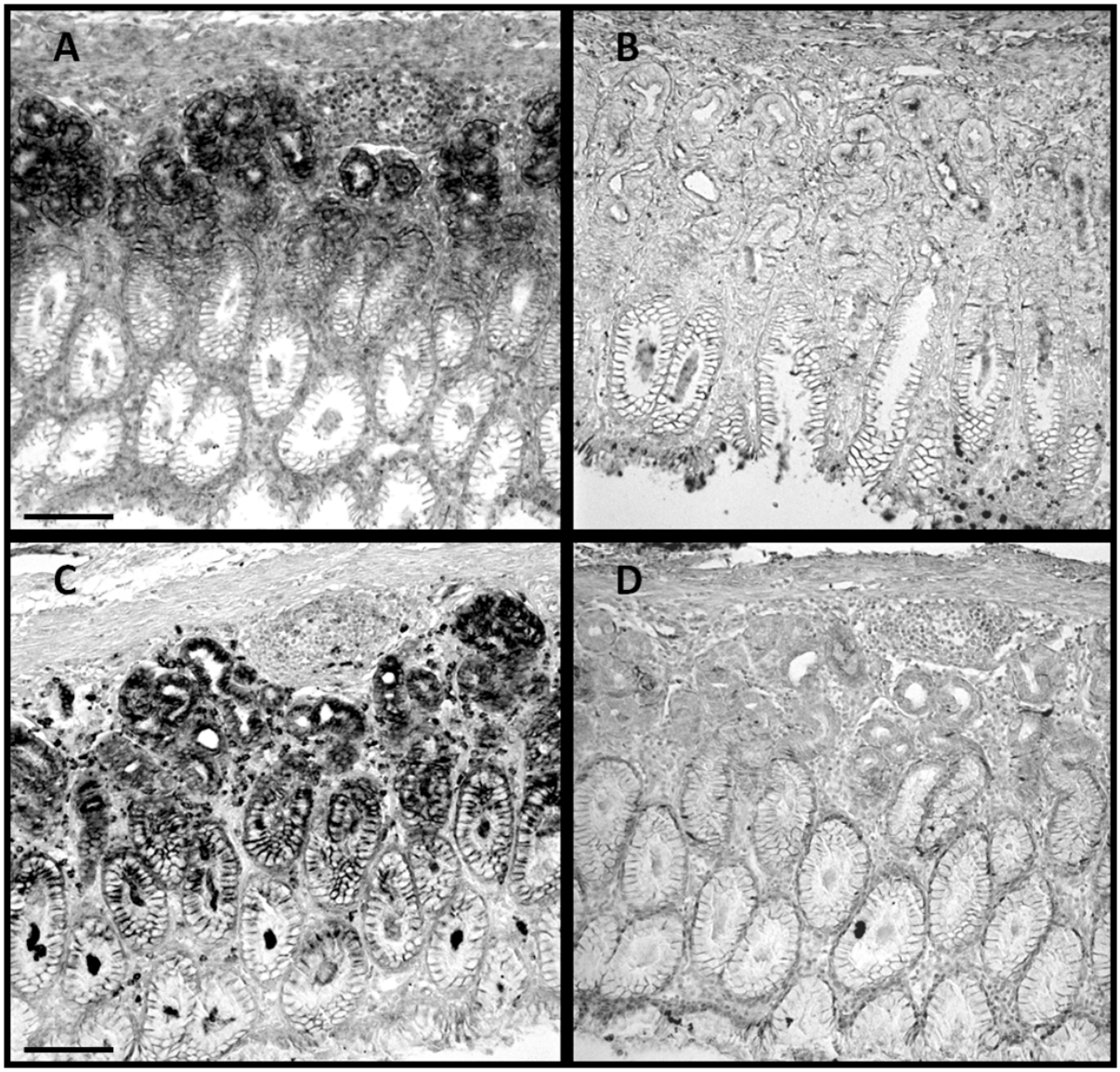


Figure 3 – Localization of polymeric immunoglobulin receptor (pIgR) messenger RNA and protein in the gastric mucosa from 42 days-old piglet**.** PIgR mRNA in situ localization (A) and corresponding protein immunostaining (C) in the transition from cardiac to oxyntic mucosa in 42 days-old piglets. The absence of signal in the respective negative controls, obtained with the omission of the probe (B) and of the primary anti-pIgR antibody (D), confirmed the specificity of the reactions. Bar indicate 100 μm (100× magnification).



Chapter 5

Effect of fasting and refeeding on the expression of the complex of genes involved in the gastric nutrient sensing and orexigenic control of pigs

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ABSTRACT

Knowledge on orexigenic signals in the pig stomach is poor. The gastric release of the active ghrelin (octanoyl-ghrelin) is under the complex control of 3 genes: prepro-ghrelin, proprotein convertase (PC1/3), for the posttranslational cleavage, ghrelin *O*-acyltransferase (GOAT), for acylation of pro-ghrelin.
Twelve pigs (12.0 kg LW) were adapted to a base diet for 1 week, were then divided into 3 groups of 4 pigs and slaughtered 3 days later. Each group had an individual feeding schedule: a. Control (C), fed twice a day for the whole trial, b. Refeeding (R), fasted on d 8 and then re-fed after 24 hours until the slaughter; c. Fasting (F), fasted on d 9 until the slaughter. Per each pig, total RNA was isolated from fundic and pyloric mucosa (Takara Fast Pure^{TM} kit protocol) and reverse transcribed (ImProm-II Reverse Transcription System). Specific primers were designed on the sequence of pig Pre/Pro Ghrelin, PC 1/3, and GOAT (GenBank), by Primer 3. The quantification was performed in a LightCycler instrument, using Takara SYBR Premix Ex Taq II.
In the fundic mucosa the prepro-ghrelin gene expression tended to be increased with the refeeding ($p=0.09$), fasting and refeeding increased PC1/3 ($p<0.01$), and GOAT was not affected. In the pylorus mucosa the GOAT mRNA was increased by refeeding ($p<0.05$), while prepro-ghrelin and PC1/3 were not affected. Compared with the other two genes, the expression of PC1/3 is higher in pyloric mucosa.
The pool of genes involved in the secretion of active ghrelin are active both in fundic and pyloric mucosa, while in general in other species only fundic mucosa has been evaluated; fasting and refeeding can affect the expression of genes that control octoanoyl-ghrelin production; a higher PC1/3 mRNA presence in pyloric mucosa is consistent with the observation that PC1/3 is required also for the processing of gastrin.

Keywords: ghrelin, fasting, pig, taste receptor, nutrient sensing

Introduction

In medical sciences research attention is now powerfully addressed to evidence the relevance of oral and digestive signals to explain ingestive behavior and control of body metabolism. Conversely the same topics did not receive the same attention in domestic species. Particularly the involvement of stomach is not sufficiently considered. The stomach, due to its aggressive secretion and activity is a relevant stop point along the digestive tract to sample newly released molecules and arrange the behavior of the lower tracts; furthermore the nervous connection mediated by afferent fibers support the role of the stomach as an energy balance regulator (Mazzoni et al., 2011).

Ghrelin is secreted mainly in the stomach and plays a role in food intake regulation: in humans and rodents, shortly before the meal, plasma ghrelin level rises and then declines when consumption stops.

X/A endocrine cells, mainly located in the oxyntic mucosa, produce the ghrelin precursor peptide (preproghrelin), that is then cleaved by proprotein convertase 1, and stored in secretory vesicles. With the addition of an octanoyl group to a serine residue by ghrelin O-acyltransferase, the most biologically active form (octanoyl-ghrelin) is obtained.

The local signals regulating ghrelin secretion have not still identified, however it can be presumed that they originate from the compartments in the stomach where they are more distributed. In spite of the finding that gastric ghrelin-producing cells have no direct contact to the stomach lumen, a regulation of ghrelin release by stimuli from the gastric lumen seems feasible: one candidate gene involved is the calcium-sensing receptor (CASR), that is already known to regulate gastrin secretion (Feng et al., 2010). The involvement of this receptor can also explain the relationship the stimulation of gastric acid secretion by L-amino acids (Busque et al., 2005).

The taste receptor type 1 member 3 (T1R3) is a G-protein coupled receptor and component of the heterodimeric complexes necessary for the amino acid (“umami”) sensing (with T1R1) or for the sweet sensing (with T1R2). T1R3 is expressed in brush cells and ghrelin-producing cells of murine stomach (Hass et al., 2010). As regards the pig, Widmayer et al. (2011) evidenced that the chemosensing machinery useful to process taste-related signals is present in the porcine stomach. Furthermore, after a preliminary inspection on samples from pyloric and oxyntic mucosa we were not able to find detectable mRNA for T1R2 gene, thus we presumed that the gastric area is more specialized for the amino acid sensing, and that more research should be addressed on T1R1, that is characteristic for this recognition.

Fasting is an important determinant of several digestive adaptation and can be considered also a tool to evidence primary regulatory adjustments in gastric functions.

Thus our aim was to assess the effect of fasting and refeeding on the expression of the complex of genes involved in the gastric nutrient sensing and orexigenic control of pigs.

Material and methods

The procedures were based on Italian laws on experimental animals and approved by the Ethic-Scientific Committee for Experiments on Animals of the University of Bologna.

Four littermate triplets of castrated male pigs (12.0 ± 0.3) kg averagely, Large White breed) were adapted to a base diet for 1 week, were then divided per litter into 3 groups of 4 pigs and slaughtered 3 days later. Each group had an individual feeding schedule: a. Control (C), fed a post-weaning standard diet twice a day for the whole trial, b. Refeeding (R), fasted on d 8 and then re-fed after 24 hours until the slaughter; c. Fasting (F), fasted on d 9 until the slaughter.

On the day of sacrifice, C and R pigs had their morning meal available for 1 hour and were then sacrificed after two hours. F pigs were sacrificed in the same batch, but continued to be fasted. The pigs were deeply anaesthetized with sodium thiopental (10 mg/kg body weight, Zoletil 100, Virbac) and killed by an intracardiac injection of Tanax ® (0.5 mL/kg BW; Intervet Italia). After slaughter, the gastrointestinal tract of each piglet was gently removed, rinsed with 0.01 M phosphate buffer saline (PBS) and sampled in the oxyntic (in the greater curvature) and pyloric (close to the pyloric sphincter) stomach. Tissue specimens were pinned flat on balsa wood and fixed in 10% buffered formalin for 24 h at room temperature (RT). The specimens were then dehydrated in a graded series of ethanol and embedded in paraffin. From each sample, serial (5 µm thick) sections were obtained and mounted on poly-L-lysine coated slides and then processed for immunohistochemistry. An additional sample was collected in the previous sites for molecular biology, snap-frozen in liquid nitrogen, and stored at -80°C until analysis.
Samples of duodenum, jejunum and ileum were formalin-fixed and paraffin wax-embedded samples were deparaffinized in xylene and stained with hematoxylin-eosin; the height of 10 villi was measured per sample.

Immunohistochemistry

Paraffin sections were processed for single and double labelling immunofluorescence using antibodies directed to ghrelin. Briefly, sections were deparaffinized through graded ethanols up to xylol and then rehydrated. For antigen unmasking, sections were heated in sodium citrate buffer (pH 6.0) in a microwave (2 cycles at 800 W, 5 min each) and, in order to prevent aspecific binding of antibodies, slides were incubated in 0.01 M PBS containing 15% normal horse serum for 1h at RT. Sections were placed in a humid chamber and incubated at 4°C overnight with rabbit anti ghrelin primary antibodies (cod. H-031-52 Phoenix Pharmaceuticals) diluted 1:2000 in PBS. After washing for 3 x 5 min in PBS, sections were incubated for 1 h at RT with donkey anti rabbit fluorescein isothiocyanate (FITC)-conjugated diluted 1:800 in PBS (Table 1). Finally, sections were washed in PBS and coverslipped with buffered glycerol, pH 8.6.

Specificity of Antibodies

The anti-ghrelin antibody specificity has been assessed by preadsorbing the primary antibody with an excess of homologous ghrelin peptide (code 031-52) purchased from Phoenix Phrarmaceuticals, Inc., Burlingame, CA, USA. Specificity of the secondary antibodies has been proved by omitting the primary antibodies. No staining has been observed in section incubated without primary antibodies or incubated with the mixture antibody plus the homologous peptides as illustrated above.

Gene quantification by real-time RT-PCR

Total RNA was isolated from each tissue samples and collected according to Takara Fast Pure™ kit (Takara Bio Inc) protocol. A sample of 1 µg of RNA was reverse transcribed using the ImProm-II Reverse Transcription System (Promega).

For all the genes, primers were designed based on a specific porcine nucleic acid sequence (Gen-Bank database) using Primer 3 version 0.4.0 (http://frodo.wi.mit.edu/primer3/). The sequence of the primers and the length of the fragments amplified (base pairs) are listed in Table 1.
The quantification reaction was performed in a LightCycler® Real-Time PCR Systems (Roche Applied Science) by a shuttle PCR (2 steps). The standard curve was obtained as described previously by Trevisi et al. (2008). The amplification was performed in a 10-µL volume containing 2 µL of cDNA, 5 pmol of each primer, and 5 µL of SYBR Premix Ex Taq II (Perfect Real Time) (Takara Bio Inc). After 40 cycles at 95°C for 5 s and 65°C for 21 s, the detection of the fluorescent product was set at the last step of each cycle. The specificity of each amplification was checked by melting curve analysis. The data were normalized to the HBMS2 gene.

Cell counting and statistical analysis

Cell counting was performed with a 40X objective lens using a Zeiss Axioplan microscope equipped with the appropriate filter cubes to discriminate between FITC, TRITC, Alexa 488 and Alexa 594 fluorescent. Images were cropped with a Polaroid DMC digital photocamera (Polaroid, Cambridge, Mass.,USA) and minimal alterations (minor adjustment to brightness and contrast) were performed using Corel Photo Paint and Corel Draw (Corel, Dublin, Ireland).
In the oxyntic and pyloric mucosa the number of total ghrelin immunoreactive (IR) cells were counts manually per 10-20 whole glands. Only entire glands located perpendicularly to the mucosa were counted in each slide.
Data were analyzed by analysis of variance using the GLM procedure of SAS (SAS Inst., inc., Cary, NC) with a factor design, including the feeding treatment and the litter. Contrasts between feeding treatments were tested with the test of Tukey. Significance was declared if $P<0.05$, and a trend was considered when $0.05<P<=0.10$.

Results

The effect of fasting and refeeding on the height of small intestinal villi of pigs is presented in Figure 1. The fasting decreased villus height of duodenum ($p < 0.01$) and of ileum ($p < 0.05$), as compared with control and refed pigs. Villus height of jejunum was not significantly affected, but visually presented the same pattern.
The effect of fasting and refeeding on the relative expression of genes involved in active ghrelin regulation, in oxyntic and pyloric mucosa of pigs is presented in Figure 2.

In general gene expression for preprogrelin was higher in the oxyntic mucosa, PC1/3 was more expressed in the pylorus, while GOAT gene expression was quite comparable in the two mucosae. PC1/3 mRNA relative quantification in oxyntic mucosa was higher in R than in C ($p = 0.037$). The gene expression for preproghrelin in oxyntic mucosa and for GOAT in pylorus, tended to be increased in R ($p = 0.060$ and $p = 0.072$), than in C.

No effect of the treatments was seen for the counts of ghrelin positive cells in the two mucosae.

The effect of fasting and refeeding on the relative expression of gastrin and of genes involved amino acid sensing, in oxyntic and pyloric mucosa of young pig is presented in Figure 4. Gastrin expression tended to be higher in R than in C and F ($p = 0.068$ and $p = 0.055$). CASR expression was lower in pyloric than in oxyntic mucosa, where values tended to be higher in R than in C ($p = 0.072$).

Discussion

The profound effect on small intestinal villi length of the imposed fasting period shows that a short-term feed privation can disturb seriously the pig gut. This was observed also by Lallès and David with a similar protocol, that also evidenced that fasting may cause the raise of several stress protein in the gastro-intestinal complex. These observations are also consistent with the effect of post-weaning anorexia (Lallès et al., 2004). In our experiment three meals seemed to be sufficient to restore partially the small intestinal villi, while this was not observed in the trial from Lallès and David.

However one day and half of fasting did never affect significantly the genes involved in the active ghrelin secretion and amino acid sensing. The effect of fasting on total ghrelin and acyl ghrelin in blood in other species has not been always consistent and also different across human, rat and mouse (Hassouna et al., 2010); these differences may be also related to the duration of fasting and the timing of blood samples. The absence of increase of the gastric expression of genes involved in ghrelin control after fasting, is consistent with observation in rat, that a base line of ghrelin secretion is sufficient to sustain GH secretion and maintain glucose tone (Zhao et al., 2010). It should also be considered that we used young pigs were the pressure for growth is relevant and thus, we can presume also to sustain GH secretion (Salfen et al., 2003).

Conversely the increased values in refed pigs, show that the refeeding activates a positive orexigenic loop when feed is again available.

The sharp increase of gene expression for PC1/3 in the fundic mucosa after fasting and refeeding may indicate that the cleavage of preprogrelin is the key step for the production of mature ghrelin (Walia et al., 2009).

However, the time fixed for the fasting treatments seems to have been not enough to affect the presence of cells involved in ghrelin production.

The observation done for the ghrelin-related genes are somewhat in agreement with the activation of gastrin and of CASR in the pylorus: thus a trend of increase in both gene expression was observed after the refeeding. This is consistent with the role of CASR in the control of gastrin secretion (Feng et al., 2010)

The tendency of increasing gastrin and CASR expression in refed pigs could be explained by the pressure to restore gastric digestive mechanisms after the fasting, as compared with pigs normally fed. According to research in CASR-null and wild-type mice possible dietary signals are peptone and some aromatic amino

acid (mainly phenylalanine) (Feng et al., 2010). The absence of effect on T1R1 gene expression could be explained by the sensitivity to different amino acids. In pig the umami taste has not extensively studied. Nevertheless it has been reported that L-amino acids are ligands for T1R1 (Roura and Tedò, 2009). Thus other explanation should be considered.

Conclusion

The pool of genes involved in the secretion of active ghrelin are active both in fundic and pyloric mucosa, while in general in other species only fundic mucosa has been assessed.
One day and a half of fasting affects the small intestinal morphometry, but do not change the expression of gene machinery controlling ghrelin production and of T1R1 and CASR.
Refeeding after one day of fasting can affect the expression of genes that control octanoyl-ghrelin production, gastric gene and, marginally, CASR gene.
A higher PC1/3 mRNA presence in pyloric mucosa is consistent with the observation that PC1/3 is required also for the processing of gastrin

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Table 1. Primers

Gene	Access number Ensebl/NCBI		primer	pb	Annealing
T1R1	ENSSSCT00000003753	for	ccgtggtattcttgacttgg	166	62°C
		rev	gagcccagcatgaggaag		
Preproghrelin	NM213807	for	gacagtggtgaggtggaagg	202	
		rev	gaacagaggtggctggtctc		
PC1/3	U20545	for	acaggggagacaaggaaagg	116	51°C
		rev	tgatggagatggtgtagatgc		
GOAT	XM_001924204	for	ctgggctcttcaaactcacc	125	60°C
		rev	gtctgcatcagggacaaaac		
CASR	AH006097	for	tcctcctcttctccctgctc	110	60°C
		rev	cacgaagctaatgccaaagg		
Gastrin	NM_001004036	for	gactctgcgcctatgtcctg	123	60°C
		rev	gctctttgcccctgttgg		

Figure 1. Effect of fasting and refeeding on the height of small intestinal villi of young pig.

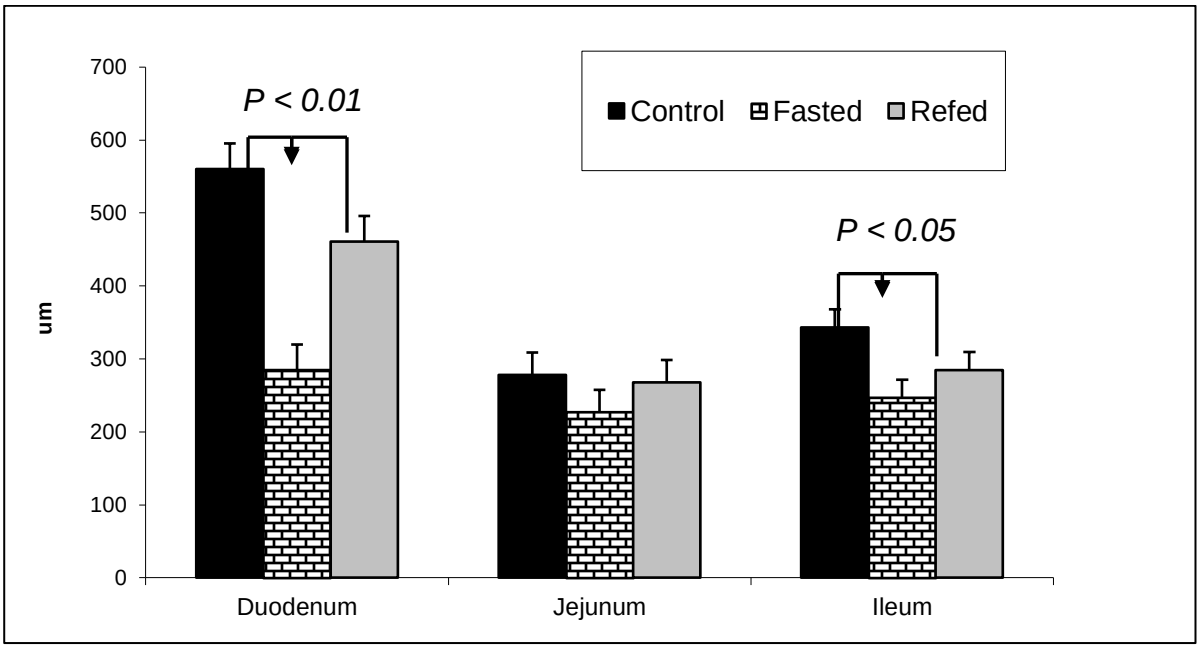


Figure 2. Effect of fasting and refeeding on expression of genes involved in active ghrelin regulation, in oxyntic and pyloric mucosa of young pig.

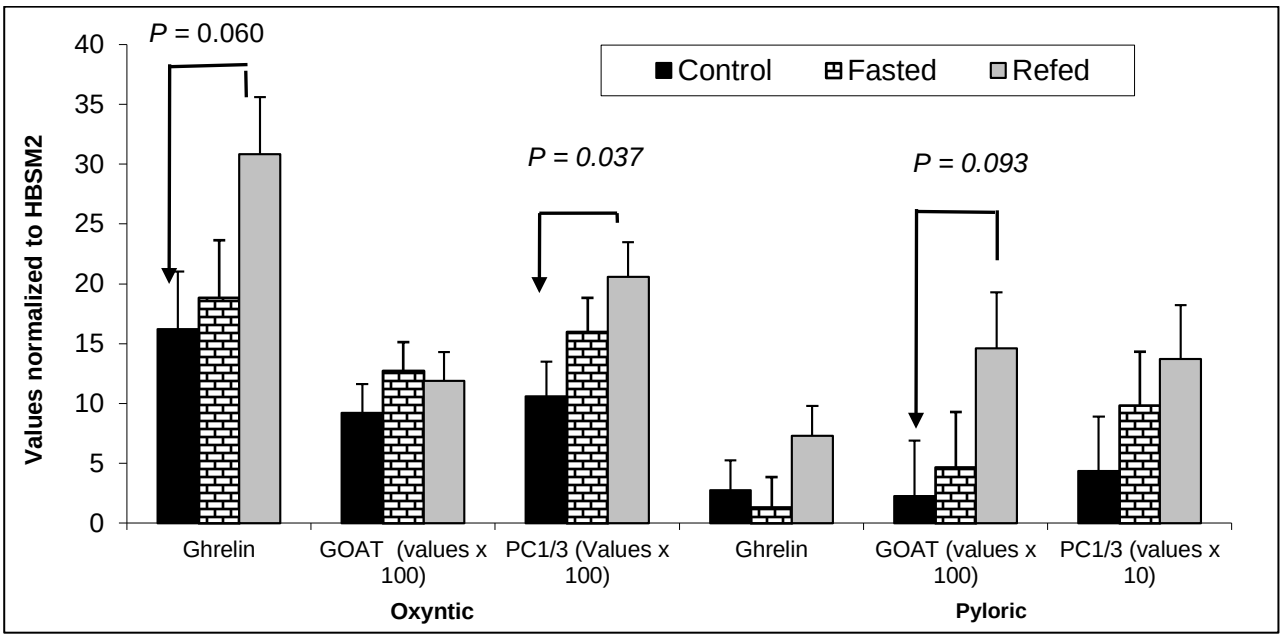


Figure 3. Effect of fasting and refeeding on counts of ghrelin^{+} cells in oxyntic and pyloric mucosa of young pig.

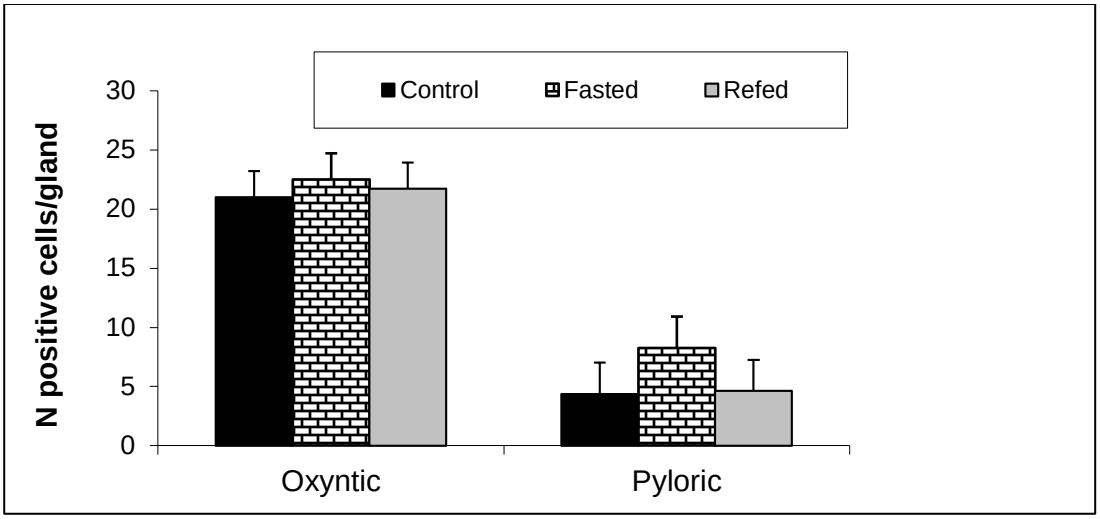
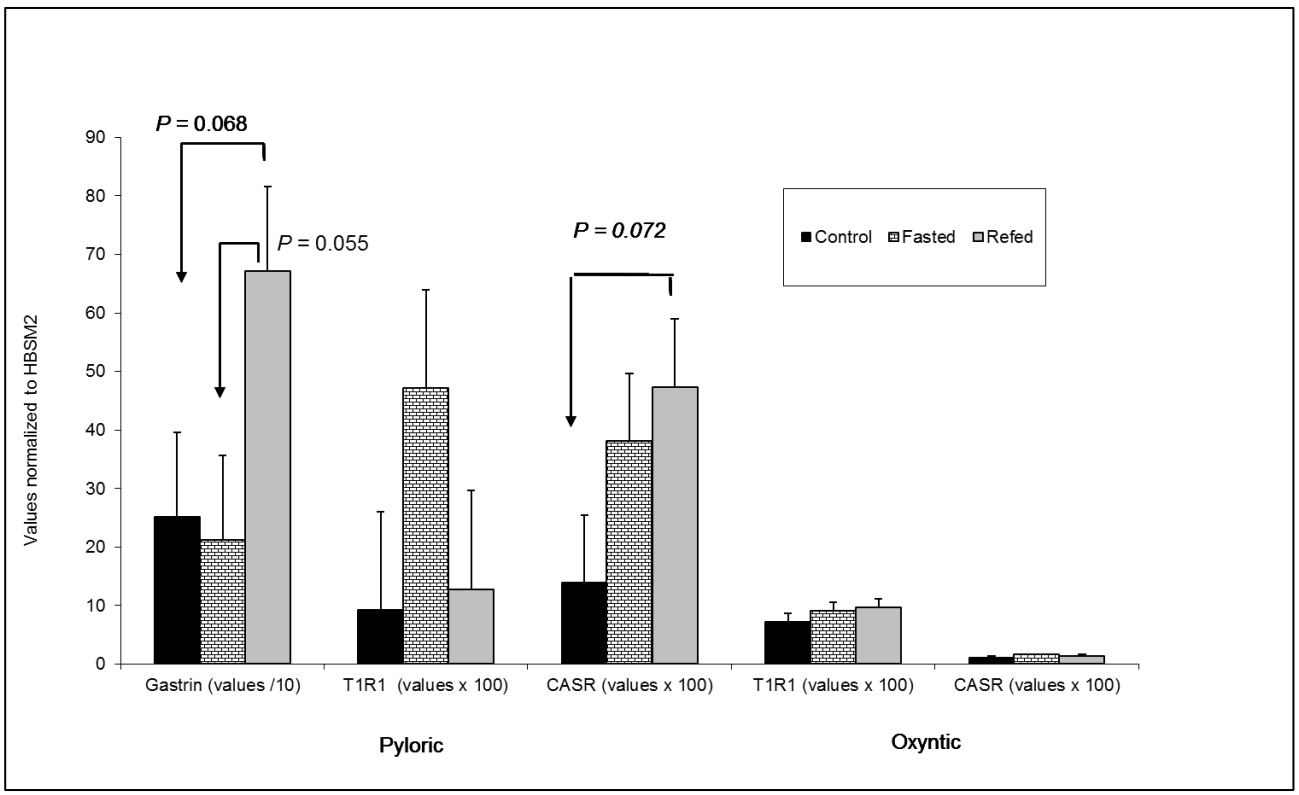


Figure 4. Effect of fasting and refeeding on expression of gastrin and genes involved amino acid sensing, in oxyntic and pyloric mucosa of young pig.



GENERAL DISCUSSION

Weaning is a complex step involving dietary, environmental, social and psychological stresses which interferes deeply with feed intake, GIT development and adaptation to the weaning diet. In the perinatal period the maturational program of the intestinal epithelium is influenced by a complex interplay of local, systemic and luminal factors. During this early period the newborn pig it is especially susceptible to gastrointestinal infections because of its immature immune system and the subjection to several stressors (new feed, stop of mother contact, animal mixing etc.). The piglet may succumb to infections if it fails to acquire an adequate supply of immunoglobulins, and colibacillosis is a major cause of illness and death in weaned pigs. The reduction in feed intake, growth rate, PWD and mortality that occurs following weaning is of major economic consequence to the pig industry. After the ban on the use of antibiotics as growth promoters in the European Union since January 1, 2006, there is the need to look for viable alternatives that could enhance the natural defence mechanisms of animals and reduce the massive use of antibiotics. In the last years more evidences emerged on the importance of the interplay among genome, microbiota, immunity in determining animal health. For these reason the aim of this work was to investigate the importance of the diet and ageing on food and gastrointestinal barrier control in piglets around weaning considering all these aspects within five different studies.

The study shown in chapter 1 aims to investigate different parameters influenced by a diet with high content of Threonine and ETEC susceptible genotypes respect colibacillosis induced by E.coli K88 challenge. Starting from background knowledge that threonine is a main component of mucins and immunoglobulins, it could be hypothesized that a diet with high content could influence positively the response of piglets in post weaning period to this disease and could have different effects respect genotyping. The results obtained confirm only partially this hypothesis because data show that a high threonine diet influence positively only some of them. Indeed increase of the feed intake and decrease of total E.coli count in feces only before the infection in the first week after weaning. Moreover, there was a tendency to increase of specific K88-IgA in ETEC susceptible piglets with more threonine in the blood and in the jejunum secretum. Conversely total IgA and IgM content in blood and jejum secretum were not changed. Interestingly more effects related to ETEC susceptible genotype were not influenced by threonine rich diet. Indeed body weight and feed intake, decreased in susceptible type while fecal shedding, E.coli counts in feces, K88-IgA specific in blood, K88-IgM and K88-IgA specific in jejunum secretum increased. Although there was no significant increase of total IgA, IgM and mucins as aspected at the beginning, the overall favorable trend of improved growth in the whole trial suggests that the 70% Thr to Lys ratio is advisable for the piglet diet in the first two weeks in post weaning period, whatever is the genotype for ETEC susceptibility.

The study in chapter 2 was designed to investigate the effects of a Tryptophan enriched diet and ETEC susceptible genotype without inducing infection towards to defense genes expression in piglets around weaning period. The background is that the catabolism of tryptophan is involved in immunological and neurological response including serotonin synthesis and the hypothesis is that a tryptophan enriched diet could interfere with defence gene expression and could be associated to susceptible genotype. The data

confirm that several defence genes are down-expressed with a 22 % Trp to Lys enrich diet in ETEC susceptible genotype, concluding that this diet is advisable for such piglet genotype in post weaning period to prevent or mitigate ETEC infection consequences even if the molecular mechanism should be investigate deeply.

The study in chapter 3 is in contiguity with the previous one in which was followed the same experimental design but it proposed to investigate the effects of a tryptophan enriched diet toward intestinal microbiota. The back ground is that the previously data obtained show that tryptophan has healthy effect on gene expression of ETEC susceptible genotypes and the hypothesis is the potential influence of the same diet on microbiota balance on the same genotypes. The data obtained show that ETEC susceptible genotypes has less variability in microbiota content and a 22% Trp to Lys enriched diet has sharp opposite effects; this in turn allows to partially offset the negative effects of ETEC susceptibility. This elucidates other beneficial effects of this enriched diet supporting the concluding advices suggested before for piglets of such genotype.

The chapter 4 shows a study focused on the presence and distribution of the polymeric immunoglobulin receptor in gastric mucosa during the development around weaning. The background is that there is very little attention and very few information about this important immunity receptor in particular in stomach pig around weaning and a recently research describes as the focused lymphoid tissue is distributed differently in different zone of the stomach in pig. The aim was to investigate the possible presence of pIgR in different zones of the stomach and its gene regulation during the maturation around weaning. The data obtained evidence the presence of pIgR in different zone of the stomach in particular where were found more lymphoid tissue and confirm that the expression is regulated by mucosa development and possibly physiology. This elucidation shows a realistic and active immunological function probably towards microbiota of the stomach despite its almost aseptic role generally considered because its acidic inhospitable environment.

Finally, in the chapter 5 is shown a pilot study focused on the effects of fasting and refeeding on the expression of genes involved in orexigenic signal and nutrient sensing in gastric piglet mucosa. The background is again that there is very little attention on the orexigenic signal and nutrient receptors in gastric pig mucosa, also because the pig genome have still to be sequenced. The hypothesis was that there could be an association of effects among orexigenic signal and nutrient sensing receptors in piglet gastric mucosa. The data show that the presence of orexygenic signal and nutrient sensing was both in fundic and pyloric mucosa but higher in the first one and in second one respectively. Moreover the trend of ghrelin, gastrin and calcium sensing receptor was higher in refed status as well and the similar trend in the same mucosa of the last two could relate them each other as was indicate in previously research but a real relation among them have to be investigate deeply when data from pig genome will be complete.

As consequences of these studies it could be stated that a higher diet content of threonine and/or tryptophan during the first week after weaning and all the post weaning period respectively could help the animal to stimulate in any case defence mechanism in different way even if same aspects and the real reason have to be elucidate deeply. Moreover new knowledge about the immunity role and the orexygenic signal linkage with nutrient sensing in the stomach could stimulate further studies focused on potential influences of microbiota and nutrient on gastric physiology response.

CONCLUSION

The overall aim of these studies was to better elucidate protective functions of the gastrointestinal tract in piglets around weaning period through more or less deeply knowledge of defence mechanism and how they are influenced by the feeding and the genotype.

All together these studies help to elucidate the impact of pig nutrition on genome, microbiota , immunity and physiological functions moreover highlights the close interplay relationship among them.

This underlines the need for a deep knowledge of all these individual parameters and their variability to setup better formulation and establish best health status avoiding expensive and dangerous medications.

This kind of research will drive farmer and animal industry to setup new nutrition strategies and medical scientist to elucidate harmful or healthy interplay related to nutrition also in human in the next future.