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RELEVANCE OF THE PIG STOMACH FOR THE DETECTION OF DIETARY FACTORS AND GUT MATURATION AND CONTROL

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ABSTRACT

The first chapter was dedicated to the study of the distribution of the expression of genes of several bitter and fat receptor in several gastrointestinal tracts, due to the scarcity of information about them in pigs, we performed a qualitative analysis by PCR reaction. A set of 7 genes for bitter taste (TAS2R1, TAS2R3, TAS2R7, TAS2R9, TAS2R10, TAS2R16, and TAS2R38) and for 3 genes for fat taste (GPR40, GPR43, and GPR120) was amplified with real-time PCR from mRNA extracted from 5 gastrointestinal segments of weaned pigs: oxyntic, pyloric , and cardiac to oxyntic transition mucosa, jejunum, and colon. Data indicate that colon is the preeminent tract where fat detection by GPR120 takes place. The presence of gene expression for several chemosensing receptors for bitter and fat taste in different compartments of the stomach confirms that this organ should be considered a player for the early detection of bolus composition.

In the second chapter another kind of sense receptor was considered in the GIT: the butyrate-sensing olfactory receptor (OR51E1). We investigated in young pigs the distribution of this receptor along the GIT, its relation with some endocrine markers, its variation with age, and after interventions affecting the gut environment and intestinal microbiota in piglets and in different tissues. Our results indicate that OR51E1 is strictly related to the normal GIT enteroendocrine activity.

In the third chapter we investigated the differential gene expression between oxyntic and pyloric mucosa in seven starter pigs. Total RNA expression was analyzed by whole genome Affymetrix Porcine Gene 1.1_ST array strips. The stomach is often considered a single compartment, although morphological differences among specific areas are well known. In fact the obtained data indicate that there is significant differential gene expression between OXY of the young pig and PYL and further functional studies are needed to confirm their physiological importance.

In the last chapter, thymol, that has been proposed as an oral alternative to antibiotics in the feed of pigs and broilers, was introduced directly into the stomach of 8 weaned pigs that were slaughtered 12 h later and sampled for gastric oxyntic and pyloric mucosa. The analysis of the whole transcript expression was performed using Affymetrix© Porcine Gene 1.1 ST array strips. In response to thymol treatment, 72 and 19 gene sets were significantly enriched in the oxyntic and pyloric mucosa, respectively. Several gene sets involved in mitosis and its regulation ranked near the top, primarily in the oxyntic mucosa; the gene set DIGESTION ranked first and ninth in the pyloric and oxyntic mucosa, respectively. Within this group, somatostatin (SST), SST receptors, peptide transporter 1 (SLC15A1) and calpain 9 (gastrointestinal tract-specific calpain) were the most strongly upregulated genes. Thymol reduced the enrichment of 120 and 59 gene sets in the oxyntic and pyloric mucosa, respectively. Several gene sets related to ion transport and channeling and

aqueous pores across membranes, including short transient receptor potential (TRP) channel 4, potassium voltage-gated channel members 1 and 2, and ryanodine receptors 2 and 3, were less enriched. The down regulation of these genes sensitive to thymol in vitro could depend on the thymol dose and contact with the gastric tissues that causes an adaptive response with their reduced activation. Conversely, the activation of the TRPA1 gene indicates the involvement of another TRP-regulating cellular calcium storage. In conclusion, the stimulation of gastric proliferative activity and the control of digestive activity by thymol can influence positively gastric maturation and function in the weaned pigs.

Chapter one

Introduction

1. INTRODUCTION

For long time the gut has been described as a simple organ with only digestive and immunity functions but now it is a fact that intestine may be regarded as a true sensory system (Furnees *et al.*, 1999), able to perceive the environment and the internal content and adapt independently without any interaction with the central nervous system. This starts a wide set of physiological responses including gut secretions and motility. The mechanisms by witch the gut "senses" nutrients and other non-nutritive chemical compounds is a recent and exciting objective of many studies both in human and animal science.

Taste perception influences food intake but most recent is the discovery that taste receptors are distributed throughout the gastrointestinal tract and not only limited to the mouth (Hofer *et al.*, 1996; Wu *et al.*, 2002). As a consequence gut works as a sensory organ responsible for conveying information to the brain and other organs in relation to ingested foods and enteric fermentation.

The gut chemosensory system is represented by a population of highly specialized cells (i.e. enteroendocrine cells) that are found singly scattered through the gut's epithelium in direct contact with luminal contents and also by diffuse chemosensory system (DCS) described for the first time from Sbarbati and Osculati, (2005) and composed of solitary chemosensory cells (CSS) (Sbarbati and Osculati, (2003)), with some analogies with taste cell but not aggregated in buds as in the oro-pharyngeal cavity.

In pig breeding, as known, feed intake, strictly related to taste, is one of the most limiting factors and may become restrictive in several conditions. For example, pigs at weaning go through a period of stress that depresses feed intake partially caused by a transition from the sow's milk to a dry feed. Furthermore all along the pig's life, dietary changes usually cause neophobia reactions resulting in temporary drops in feed intake, particularly if low palatability ingredients are used (i.e., rapeseed meal) (Roura, 2006).

The advancement of knowledge on the chemosensory system along the gastrointestinal tract of the pig can have different fields of application: stimulating appetite and feed intake, which could greatly benefit performance of piglets at weaning; improving of the health of animals by reduction of mucosal inflammation; the promotion of intestinal growth to weaning, which could lead to a better GI integrity and health. In addition, considering the similarities in terms of food intake, body size, lifespan and body proportion, the pig is found extremely relevant and coherent as a case study regarding the GI physiology and diseases in human.

1.1. GASTROINTESTINAL TRACT AND TASTE RECEPTOR

1.2 The sense of taste

Animals evaluate the nutritious value, toxicity, sodium content and acidity of food by the gustatory system. Taste permits the sensing of nutrients and harmful substances in order to guide selection of nutritious food and avoidance of toxin.

The palatability of a feed is positively correlated to the taste experienced by animals. The sensation associated with eating includes the senses of smell and taste. The sense of smell is often experienced prior to consumption. Therefore, aroma is important as an initial attractant to feed.

Groups of cells located on the tongue, commonly referred to as taste buds, are the major sensory organs responsible for taste and where taste receptors are expressed .The majority of taste buds are in the lingual epithelium, on papillae. Based on the morphological structure, four types of papillae have been described on the mammalian tongue (figure 1).

Fungiform papillae are mostly located on the dorsal surface in the anterior two-thirds of the tongue. **Foliate papillae** are on lateral margins towards the posterior part of the tongue, c**ircumvallate papillae**, the one containing the highest number of taste buds, are few (8 to 12 in humans) and arranged in a V-shaped row at the back of the tongue. **Filiform papillae** do not contain taste buds and are found all over the surface of the tongue. They are considered to have a mechanical function and to be not directly involved in taste sensation (Chandrashekar *et al.*, 2006).

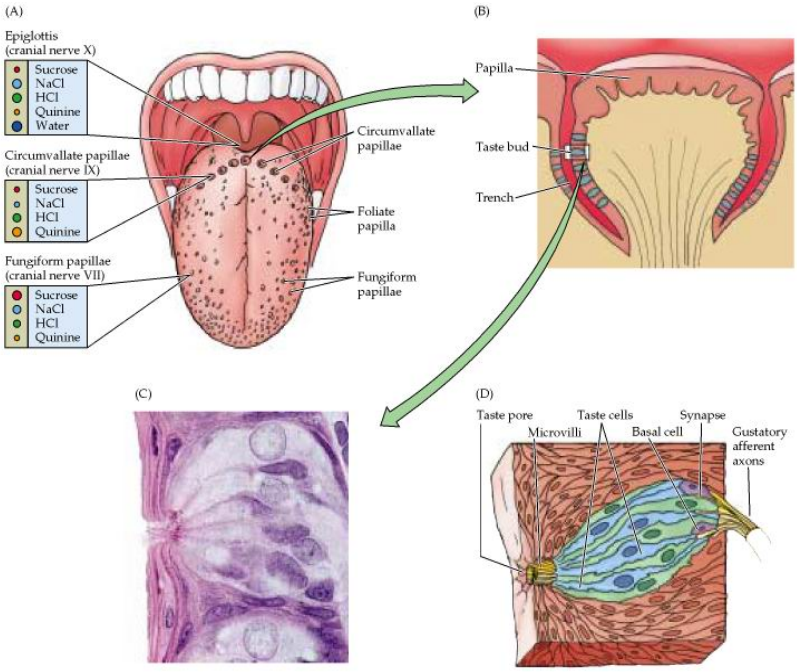

Figure 1. Taste buds and the peripheral innervation of the tongue. (A) Distribution of taste papillae on the dorsal surface of the tongue. (B) Diagram of a circumvallate papilla showing location of individual taste buds. (C) Light micrograph of a taste bud. (D) Diagram of a taste bud, showing various types of taste cells and the associated gustatory nerves. The apical surface of the receptor cells have microvilli that are oriented toward the taste pore (image from Purves *et al.*, 2001).

At ultra-structural level, we can classify different cell types in taste buds: basal cells, type I, II, and III taste cells, whose functions have not been fully established (Murray & Murray (1967); Roper (2006)) (figure 2).

Basal cells are undifferentiated cells precursors of other cellular elements.

Type I cells are characterized by the presence of large granules apical and are widely regarded as elements of support and secretion (Kataoka *et al.*, 2008; Yoshida *et al.*, 2009) and may also be involved in the perception the salty taste (Vandenbeuch *et al.*, 2008).

The cells of Type II are those that possess receptors and GPCRs are immunoreactive gustducina, have lanceolate shape, and since they do not possess granules or synapses appear clear to the microscope (Chandrashekar *et al.*, 2000; Chaudhari *et al.*, 2000; Nelson*et al.* 2001; Nelson *et al.* 2002). The cells of type III have apical and basal processes that create synapses with axons and also seem to perceive the taste acid (Frings, 2010).

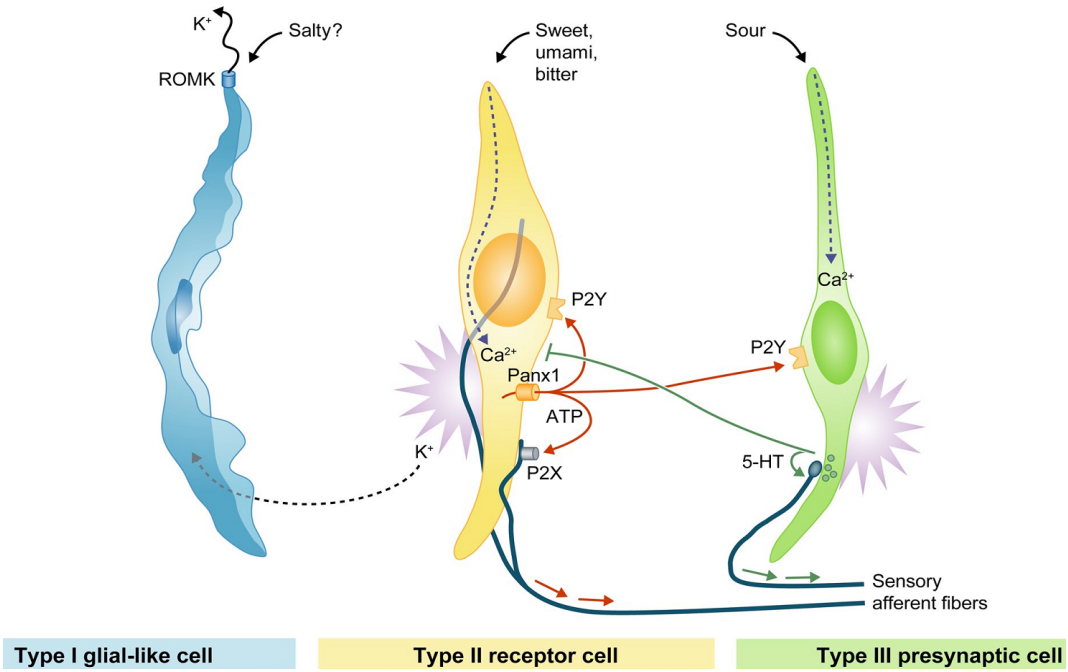

Figure 2. The three major classes of taste receptor cells. (image from Chaudhari and Roper, 2010)

Anatomical studies show that pigs have some of the highest number of taste buds (Chamorro *et al.*, 1993). Pigs have ~5000 fungiform taste buds (Chamorro *et al.*, 1993), compared with ~1600 in humans (Miller, 1986). The density of fungiform papillae is especially high along the rim of the pig's tongue, where they form a frill. Also, the posterior area of the pig's tongue has >10 000 taste buds in the vallate papillae, compared with 6000 in the human, and ~4800 in the foliate papillae, compared with 3000 in the human (Tuckerman, 1888).

Since studies in humans show a positive correlation between number of fungiform papillae and ability to taste (Miller and Reedy, 1990, Delwiche *et al.*, 2001; Tepper and Nurse, 1997), it is likely that pigs' sense of taste is not inferior to that of humans, but may in fact be superior.

1.3 Taste and olfactory receptor

Sweet, umami and bitter tastes are perceived when a ligand interacts with a taste receptor,

which are seven-transmembered heterodimertic guanine nucleotide-binding protein (g-protein) coupled receptors (Adler, Hoon *et al.* 2000). When a receptor member of the family of GPCRs is activated, the transduction of the signal starts by the activation of heterotrimeric G-protein associated with it.This causing a release of calcium ions from internal stores in the cell. This calcium release triggers other ions to end the cells, resulting in depolarization, and ultimately transmission of nerve impulse to the brain causing theperception of sweet, umami or bitter (reviewed by Margolskee 2002) (figure 3).

Sweet and umami are perceived through heterodimers, where bitter is perceived through a single receptor, although a single ligand may activate different bitter taste receptors.

In vertebrates, bitter chemicals are detected by a small family of receptors (T2Rs or TAS2R) and range in number from 3–49, depending on the species (Chandrashekar *et al.*, 2000; Matsunami *et al.*, 2000; Shi and Zhang, 2006). In pig, the consistency of T2Rs was recently investigated by genetic analysis resulting composed at least 15 porcine Tas2rs, of which 13 are orthologous to human sequences (Groenen *et al.*, 2012, da Silva *et al.*, 2014).

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Due to the their different receptive capacity were determined two classes of receptors for the bitter: the so-called “specialists "which detect one or a few chemicals bitter and "generalist" able to detect many of this (Behrens and Meyerhof, 2009).

In general, receptors that detect many ligands do so at the expense of sensitivity: mutations in the receptor that increase affinity for one agonist, decrease affinity for others (Born *et al.*, 2013).

T2Rs are expressed on taste cells and elsewhere in the body: 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. A wide variety of bitter compounds are known to activate human T2Rs in vitro (Bufe, Breslin *et al.* 2005; Bufe, Hofmann *et al.* 2002; Chandrashekar, Mueller *et al.* 2000; Kim, Jorgenson *et al.* 2003; Kuhn, Bufe *et al.* 2004; Pronin, Tang *et al.* 2004; Pronin, Xu *et al.* 2007). In general, each bitter responsive taste receptor cell expresses multiple types of bitter receptors (Mueller *et al.*, 2005), such that bitter chemicals cannot be readily distinguished by taste alone.

The perception of the dietary fat in human, has been attributed mainly to olfactory and postingestive mechanisms (Greenberg and Smith 1996). Over the recent years, however, evidence has been accumulating for a possible involvement of gustatory cues in fat perception of rodents and human (Takeda *et al.*, 2001; Fukuwatari *et al.*, 2003; Hiraoka *et al.* 2003; Chale-Rush *et al.* 2007).

In the gastrointestinal tract the presence of receptors that respond to nutrients or their immediate breakdown products, (fatty acids, sugars, amino acids and proteolytic products) serve mainly as chemosensors for food intake (Conigrave and Brown, 2006; Egan and Margolskee, 2008; Engelstoft *et al.*, 2008). Among the sensors identified so far are the membrane protein CD36 (Fukuwatari *et al.*, 1997; Laugerette *et al.*, 2005; Degrace-Passilly and Besnard, 2012), the G protein-coupled receptors GPR120 and GPR40 (Matsumura *et al.*, 2007; Cartoni *et al.*, 2010), and the calcium-activated cation channel TRPM5 (transient receptor potential cation channel M5) [Perez *et al.*, 2002; Liu *et al.*, 2011).

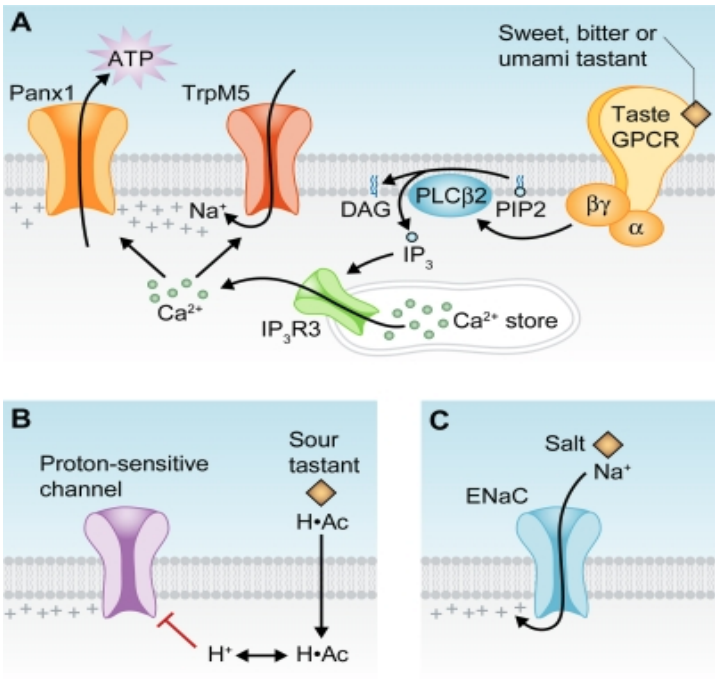

Figure 3. Mechanisms by which five taste qualities are transduced in taste cells. (A) In receptor (Type II) cells, sweet, bitter, and umami ligands bind taste GPCRs, and activate a phosphoinositide pathway that elevates cytoplasmic Ca^{2+} and depolarizes the membrane via a cation channel, TrpM5. Shown here is a dimer of T1R taste GPCRs (sweet, umami). (B) In presynaptic (Type III) cells, organic acids (HAc) permeate through the plasma membrane and acidify the cytoplasm where they dissociate to acidify the cytosol. Intracellular H^+ is believed to block a proton-sensitive K channel (as yet unidentified) and depolarize the membrane. (C) The salty taste of Na^+ is detected by direct permeation of Na^+ ions through membrane ion channels, including ENaC, to depolarize the membrane. The cell type underlying salty taste has not been definitively identified (image from Chaudhari and Roper, 2010)

1.4 OLFACTORY RECEPTORS

Olfactory receptors (ORs) were discovered in olfactory epithelium about twenty years ago (Buck and Axel, 1991) in rodent.

Now we know that the superfamily of ORs is formed by a very large number of G-protein coupled receptor proteins that detect volatile odorant molecules. Recent evidence shows that ORs are expressed in many tissues that are not considered classical chemosensory tissues; however, in the majority of these cases, the physiological functions of the ORs remain elusive (Kang e Koo, 2012;

Flegel *et al.*, 2013). Several of them are well expressed in the respiratory tract and in other tissues (Flegel *et al.*,2013); in the gastrointestinal tract (GIT), they are detected in enterochromaffin cells and can affect the secretion of serotonin (5-hydroxytryptamine; 5HT) in response to fragrant molecules (Braun *et al.*, 2007), and subsequent effects on gut motility (Foster *et al.*, 2014).

Genome sequencing has demostrated that the ORs are part of a large, multigene family: it is estimated that humans express #350 functional ORs, while rodents express more than 1000. The transcripts encoded proteins with key hallmarks of GPCRs, including seven putative membrane-spanning helices, three putative intracellular and three putative extracellular loops connecting these helices, and a conserved aspartate-arginine-tyrosine amino acid motif (a hallmark of GPCRs) within the second intracellular loop (Buck and Axel 1991; Mombaerts 2004).

The encoded proteins also contain hypervariable regions that likely contribute to selective binding of different odorants (Buck and Axel 1991; Bozza *et al.* 2002; Krautwurst *et al.* 1998; Abaffy *et al.* 2006; Shirokova *et al.* 2005).

1.5 GASTROINTESTINAL TRACT

The stomach is a muscular and dilated organ responsible for storage, initiating the breakdown of nutrients, and passing the digesta into the small intestine. It is a reaction chamber where chemicals are added to the food, mixed, 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.

In addition, the stomach is also an important endocrine organ and secretes various peptide hormones with different functions.

Anatomically, the stomach of the pigs, consists of a simple compartment that is divided into four functionally and structurally different regions. The pars oesophagea is a non-glandular extension of the esophagus into the proper stomach. The glandular cardia is very large and occupies approximately one third of the stomach luminal surface.

The fundus, or proper gastric region, is located between the cardia and pylorus.

All three regions contain secretory glands located in ‘gastric pits’ (fig. 4) .

The major surface of the stomach and lining of the pits are covered with surface mucous cells that produce thick, tenacious mucus to protect the epithelium against injury from acid and grinding activity.

The gastric pits of the fundic mucosa contain HCl-producing parietal cells that are clustered in the neck of the gland. Between these cells are mucous neck cells that produce mucus and proteases. As the only cells of the stomach lining, mucous neck cells divide and migrate either down into the gland or up into the pits and differentiate into any of the mature cell types. In the fundic region, pepsinogen-producing chief cells are located at the base of the fundic glands. In addition, the fundic mucosa also contain endocrine/paracrine somatostatin producing D cells, endocrine G cells producing gastrin, serotonin producing enterochromaffin (EC) cells, and histamine producing histamine-immunoreactive cells and mast cells.

The cardia and pyloric region (antrum in other species) contain different cells types than in the fundic region.

The cardiac glands have no chief cells, but have mucous cells that produce mucus, proteases and gastric lipase. The pyloric glands do contain mucous neck cells that produce mucus and proteases. Furthermore, the pyloric glands also contain G cells and D cells (producing gastrin and somatostatin respectively), but the mucous cells are the most representative.

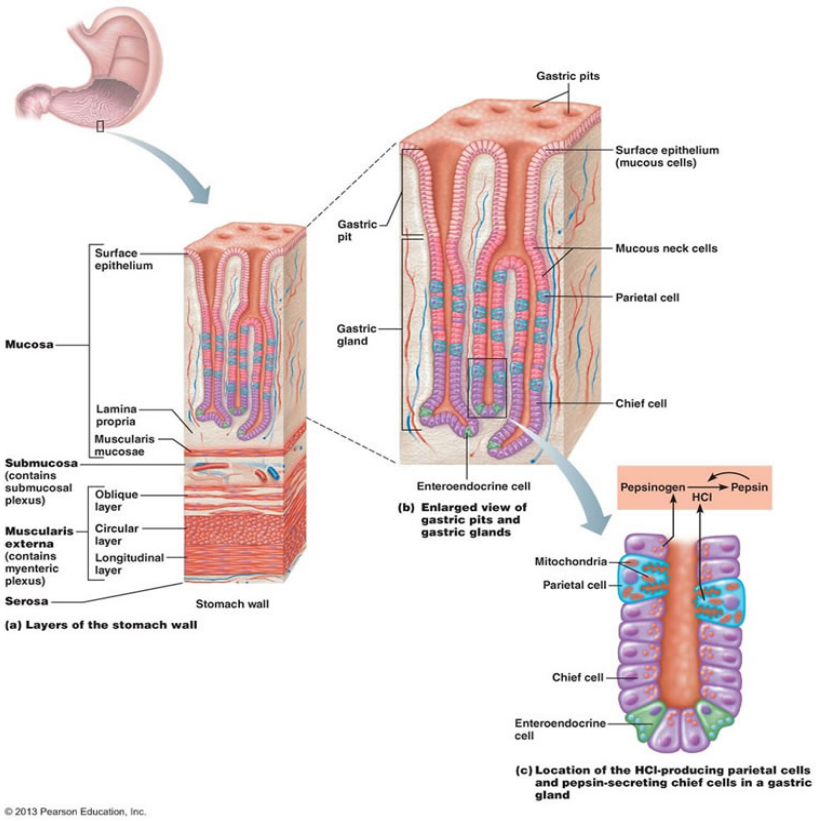

Figure 4. Microscopic anatomy of the stomach

INTESTINE

The small intestine

The small intestine comprises the duodenum (4-4.5%), jejunum (88-91%) and ileum (4-5%). Although there are distinctive morphological features, the duodenum, jejunum and ileum share a lot of common features.

The small intestine consists of four major layers; the *serosa*, the *muscularis*, the *submucosa* and the *mucosa* (Figure). The *serosa* contains connective tissue, large blood vessels and nerves.

The muscular layer contains two types of muscle fibres; an outer layer of longitudal muscles and an inner layer of circular muscles that are involved in gastrointestinal motility. The *submucosa* is a layer of connective tissue holding together the large blood and lymphatic vessels and neural complexes. The *mucosa* consists of three sublayers; the *muscularis mucosa*, the *lamina propria* and the *epithelium*. The muscularis mucosa produce transient intestinal folds. The lamina propria consists of blood vessels, free lymphocytes and lymph nodes called Peyers patches, and neurons held together by connective tissue. It supports the structure and nourishes the epithelial layer. The epithelial layer consists of a single layer of epithelial cells. They cover the whole luminal surface of the intestine, which is severely folded by the formation of finger-like projections called villi, and at the base of these crypts of Lieberkuhn, that are moat-like invaginations (figure 5).

There are three types of epithelial cells on the villus surface: absorptive cells, goblet cells and enteroendocrine cells. They all originate from stem cells located near the base of the crypts. The enterocytes migrate from the base to the top of the villi and during migration, the enterocytes maturate. The digestive function (enzyme activity) begins as the enterocytes migrates over the basal third of the villi. The absorptive function starts to develop as they reach the upper to mid-level and continues to increase until they reach the top of the villi, where they are shed into the lumen.

Hence, enterocytes at the surface of the villi are continuously renewed. Goblet cells are secreting viscous mucus, and are interspersed among the enterocytes. Goblet cells increase in number from the proximal jejunum to the distal ileum. Enteroendocrine cells are specialized endocrine cells of the gastrointestinal tract. They produce hormones such as secretin (S cells) and cholecystokinin (I cells).

The formation of villi increases the mucosal surface at least 5 times compared to a flat surface of equal size. Furthermore, the cell-surface of the enterocytes facing the lumen has an apical membrane forming microvilli (brush-border) that further enhances absorptive surface 14-40-fold. The microvilli have important digestive enzymes and other proteins attached. They extend into a jelly-like layer of glycoprotein known as the glycocalyx that covers the apical membrane. The remaining part of the enterocyte plasma membrane is called the basolateral membrane, referring to the base and side of the cell. The length of villi increases from the duodenum to the mid-jejunum and then decreases again towards the terminal ileum. This reflects the various functions of the different segments of the small intestine.

Crypts also vary in size and composition along the intestine. They are deepest in the proximal small intestine (duodenum and jejunum) and shorter distally in the ileum. Paneth cells are located

adjacent to stem cells at the base of the crypts. Their exact function is unknown but due to the presence of lysozymes and defensins they most likely contribute to maintenance of the gastrointestinal barrier.

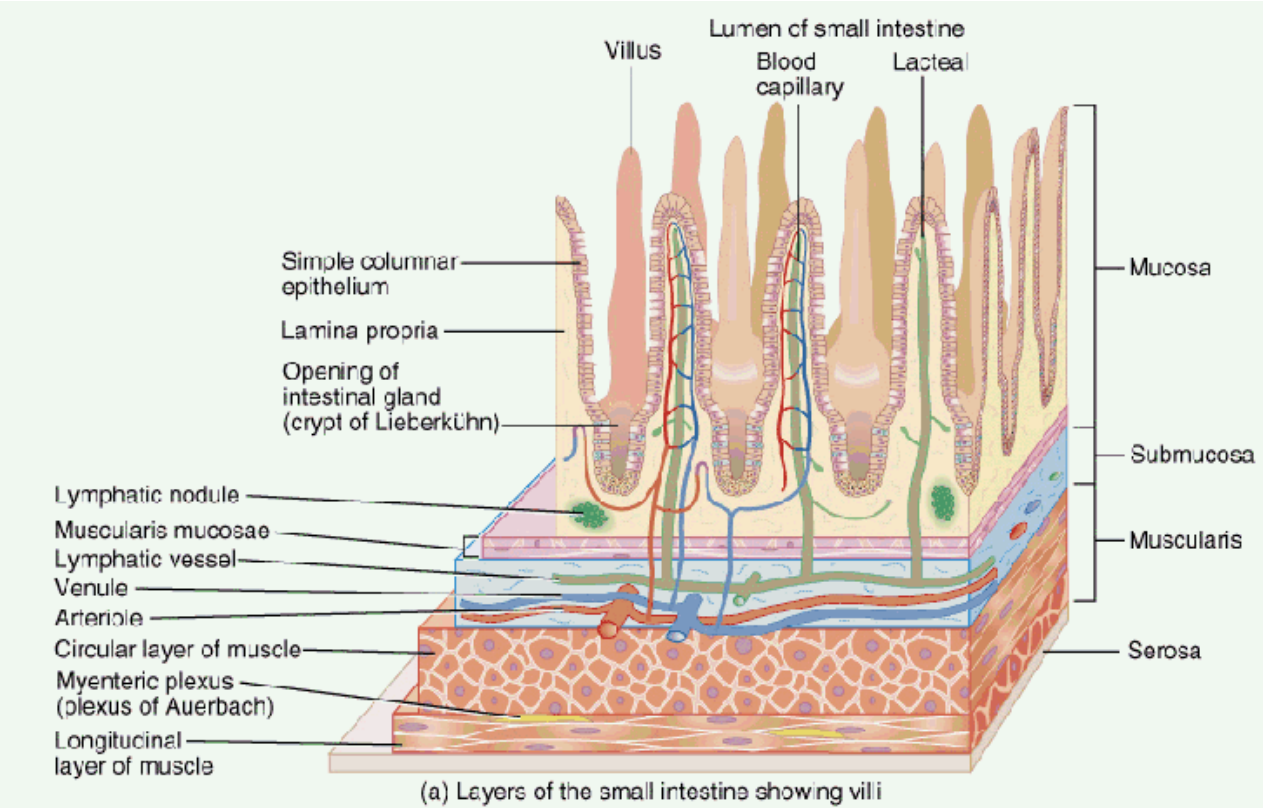

Figure 5. Diagram of the mucosa of the small intestine (online from udilab.bmed.mcgill.ca/HA/html/dig_29_E.html).

The large intestine

The pig has a relatively short caecum and a long colon, consisting of an ascending, transverse and descending colon. The caecum is located at the proximal end of the colon. The caecum, the ascending and transverse colon and the proximal portion of the descending colon are arranged the "spiral colon" namely in a series of centrifugal and centripetal coils. The rectum is embedded in fat and is dilated to form ampulla recti just before ending at the anus. The mucosa of the large intestine has no villi, but columnar epithelial cells with microvilli formed into straight tubular crypts. Numerous goblet cells secreting sulphated carbohydrate-protein complex intersperse the columnar cells to lubricate the colon. The rectum has a simple structure with columnar cells and only few goblet cells.

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Chapter two

Aims

The aims of this thesis were to:

1. Identification of fat and bitter receptors along the gastrointestinal tract of the piglet, through qualitative analysis of gene expression;
2. study receptor OR51E1 along the pig gastrointestinal tract, its association with cell endocrine markers, its variation with age and during different treatments that affect the intestinal environment;
3. Gene expression analysis in different parts of the piglet stomach;
4. Effect of thymol, a molecule with potential antibiotic effect, on gene expression in the mucosa of the stomach of the piglet.

Chapter three

Assessment of the presence of chemosensing receptors based on bitter and fat taste in the gastro-intestinal tract of young pig

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ABSTRACT: Knowledge on porcine bitter and fat taste receptors and on their expression in gastrointestinal tract of pigs is scarce. We searched for the presence of porcine homologous sequences for 13 human transcripts of bitter and fat taste receptors, in ENSEMBL and NCBI databases. For *taste 2 receptor (TAS2R) 8*, alignment was not observed; for *TAS2R13* and *TAS2R46* the porcine predicted sequence aligned with several other human bitter genes. For 7 genes for bitter taste (*TAS2R1*, *TAS2R3*, *TAS2R7*, *TAS2R9*, *TAS2R10*, *TAS2R16*, *TAS2R38*) and for 3 genes for fat taste (*GPR40*, *GPR43*, *GPR120*), a full homology for exons sequences was found and primers were designed by PRIMER3. These 7 genes were amplified with real-time PCR and verified on agarose gel, in 5 gastro-intestinal segments of weaned pigs: oxyntic (ST1), pyloric (ST2) and cardiac to oxyntic transition mucosa (ST3); jejunum (JEJ) and colon (COL). Suitability of mRNA was verified by amplifying *RPL4* and *HMBS2* genes. Each bitter taste gene was detectable on agarose gel in at least one subject of all the gastro-intestinal segment except for *TAS2R3* and *TAS2R38* that were never detected in ST1 and COL, respectively. The inspection of bitter taste genes amplification curve indicated that the expression was in general very low. *GPR43* and *GPR120* were present in all segments from all pigs. Expression was not detected for *GPR40*. Data also indicate that colon is the preeminent tract where fat detection by *GPR120* takes place ($P < 0.001$). The presence of gene expression for several chemosensing receptors for bitter and fat taste in different compartments of the stomach confirms that this organ should be considered a player for the early detection of bolus composition.
Key words: bitter, fat, pig, stomach, taste receptor

INTRODUCTION

Humans recognize sweet, umami, sour, salty, and bitter, and novel tastes have been described such as fat taste, and metallic (Chaudhari and Roper, 2010). Recent research indicates that the presence of chemosensing receptors for bitter and fat taste is not restricted to the mouth, but is extended to other organs and tissues. Particularly, their location along the whole digestive tract contributes to the control of secreting activity, regulation of several hormones, and afferent neuronal modulation. In pigs, the taste chemosensory system has been investigated for its impact on the feed preference and feed intake (Roura and Tedò, 2009). The diffuse gastro-intestinal chemosensory system consists of solitary chemosensory cells, that express molecules of the chemoreceptorial cascade activated by G protein-coupled receptors (**GPR**) and α-gustducin interaction (Iwatsuki and Torii, 2012). Taste 2 receptors (**TAS2R**) are the GPR identified as

receptor for bitter taste. Bitter taste is initiated by a large number of different and unrelated organic molecules recognized by a broad range of receptors of the TAS2R family: humans have 25 functional bitter taste receptors genes. Other GPRs have been identified in taste buds and likely contribute to the detection of nutrients. These include *GPR40*, *GPR43* and *GPR120* genes which are expressed in subsets of taste cells and detect fatty acids (Cartoni *et al.*, 2010). To date, we are not aware of any study that investigated *TAS2Rs* or *GPRs* genes in pigs.

The goal of this study was to assess the presence of porcine homologous sequences for the known human transcripts of bitter and fat taste receptors in different segments of the gastro-intestinal tract of the young pigs.

MATERIAL AND METHODS

We searched for the presence of porcine homologous sequences for 13 human transcripts of bitter and fat taste receptors, in ENSEMBL and NCBI databases. Genes for which a full homology for exon sequences was found in the pig were tested for their expression in the gastro-intestinal tract. The animal protocol was approved by the deputed Committee of our University.

The following 5 gastro-intestinal segments were obtained after euthanasia from 5 Large White castrated male pigs (42 d of age, 11.8 $\pm$ 0.33 kg average BW): oxyntic (**ST1**), pyloric (**ST2**) and cardiac to oxyntic transition mucosa (**ST3**); jejunum (at 75% of length, **JEJ**) and ascending colon (near the centrifugal turns, **COL**). Prior to the sacrifice the pigs were receiving a typical post-weaning diet, based on cereals (extruded in parts), cereal byproducts, milk whey, and soybean meal. The tissues were immediately snap frozen in liquid N and preserved at -80° C till analysis Total RNA was isolated according to Takara Fast PureTM kit (Takara Bio Inc, Otsu, Japan) protocol and reverse transcribed using the ImProm-II Reverse Transcription System (Promega, Madison, WI). The following primers were designed using Primer 3 (http://frodo.wi.mit.edu/primer3/) on pig sequences: *TAS2R1* (Forward: ATCCCTCACCCAATCTTCTC, Reverse: TCCATTTACGTTTGCTCTGG), *TAS2R3* (F:CCCATCCTACATTCCTCTGG, R:ATCCACCGATTCCACTGAGA), *TAS2R7* (F:ATGCAATTCAATGCCACAG, R:AGCTGGAGGTGGCTACAAGA), *TAS2R9* (F:CAAGCCATTCATTCATCCTG, R:TGTCTCCTTCTTTTGTGGAAA), *TAS2R10* (F:

TTTGCTCAATCTGGGTGTCA, R:CTGTGCTGGGGTCTCTGAAT), *TAS2R13* (F:CCAGGACCAAGGTCCATACA, R:TCCAATCGTCTCACAAAGCA), *TAS2R16* (F:ACCGTGACGGAGAGGCTTGA, R: ACGTGCTGGGACAATGAGGC), *TAS2R38* (F:AATTTTTGGGACGTGGTGAG, R:TCAGTGGGTCCTTCATCCTC, *GPR120* (F:CCAGTGTTGCTGGAGAAATC, R:TGATGCCTTGGTGATCTGTA), *GPR40* (F:GCATCAACACGCCAGTCAAT, R:TCCTGTTCTTCTTTCTGCCTC), *GPR43* (F:CTCATGGGTTTCGGCTTCTA, R:GCAGCAATCACTCCGTACAA).

Amplification was performed in a LightCycler Real-Time PCR Systems (Roche Applied Science, Mannheim, Germany) by a shuttle PCR (2 steps) 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). Amplifications consisted of 40 cycles at 95°C for 5 s and 60°C for 22 s. The specificity of each amplification was checked by melting curve analysis. For *GPR43* and *GPR120* genes an absolute quantification was performed, afterwards the data were normalized by geometric mean of the absolute expression of the two housekeeping genes *hydroxymethylbilane synthase* (***HMBS2***) and *ribosomal protein L4* (***RPL4***), obtained with the primer pairs F:AGGATGGGCAACTCTACCTG, R:GATGGTGGCCTGCATAGTCT and F:CAAGAGTAACTACAACCTTC, R:GAACTCTACGATGAATCTTC, respectively. The quantification of TAS2R genes was not possible due to low gene expression: thus the analysis was limited to determining the presence/absence of the specific amplicon. Then 10 µL of the PCR reaction were migrated in 1.5% TBE agarose gel to check the product size of the fragment.

Data of gene expression were analyzed by ANOVA using the MIXED procedure (SAS Inst., Inc., Cary, NC), testing point in the gastro-intestinal tract as repeated measure in each pig. Statistical significance was set to $P < 0.05$. The Tukey test for multiple contrasts was used.

RESULTS AND DISCUSSION

The in silico analysis showed that for *TAS2R8* alignment from human to pig was not present. For *TAS2R13* and *TAS2R46* the porcine predicted sequence aligned also with several other human bitter genes. For 7 genes for bitter taste (*TAS2R1*, *TAS2R3*, *TAS2R7*, *TAS2R9*, *TAS2R10*, *TAS2R16*, *TAS2R38*) and for 3 genes for fat taste (*GPR40*, *GPR43*, *GPR120*), a full homology for exons sequences was found. Each bitter taste gene was detectable on agarose gel in at least one subject of all the gastro-intestinal segments except for *TAS2R3* and *TAS2R38* that were never detected in ST1 and COL, respectively (Table 1). Conversely, *TAS2R1*, *TAS2R9* and *TAS2R10* were present in all tested ST3, and *TAS2R16* in all JEJ samples. The inspection of bitter taste genes amplification curve indicated that the expression was in general very low; hence, it was not possible to perform a quantitative analysis.

Putative receptors for bitter molecules are diffused also in the porcine gastro-intestinal tract, similar to observations in mouse and rat. Concerning the stomach, the identification in pig gastric tissues of some of the tools to translate the taste signals (Widmayer *et al.*, 2011), can be put in connection with the presence of transcripts for some TAS2Rs, as well as of other taste receptors. Particularly, TAS2R in the stomach may participate in the control of gastric emptying in the pig, as suggested in mice (Janssen *et al.*, 2011).

The *GPR43* and *GPR120* transcripts were detected in all the segments, while no expression was found for *GPR40*. For *GPR43*, differences were not observed among the gastro-intestinal segments, while *GPR120* had increased expression in the colon compared to each of the other segments ($P < 0.001$) (Table 2). Our observation of higher gene expression of GPR120 in the colon, as compared with small intestine, agrees with the major role of GPR120 in the colon in the incretin rise after stimulation with fatty acids in mice (Hirasawa *et al.*, 2004). Furthermore, this response of incretin was not seen for GPR40. Conversely the possible activation of GPR43 and GPR120 in different gastric areas has never been fully described.

In conclusion, chemosensors for bitter and fat tastants contribute to the diffuse chemosensory system in the gut and provide also the basis for further studies aimed at assessing the potential genetic variability in pigs and the screening of potential adjuvant or masking molecules.

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Table 1. Frequency of pigs with expression of *TAS2Rs* genes in 5 gastrointestinal points

	Point of measure[1]				
Gene	ST1	ST2	ST3	JEJ	COL
TAS2R1	4/5	4/5	4/4	3/5	2/5
TAS2R3	0/5	2/5	3/4	1/5	3/5
TAS2R7	3/5	4/5	3/4	2/5	2/5
TAS2R9	4/5	4/5	4/4	3/5	3/5
TAS2R10	4/5	4/5	4/4	4/5	1/5
TAS2R16	1/5	1/5	1/4	5/5	3/5
TAS2R38	2/5	1/5	2/4	1/5	0/5

[1] Oxyntic (ST1), pyloric (ST2) and cardiac to oxyntic transition mucosa (ST3); jejunum (JEJ) and colon (COL).

Table 2. Effect of the point of measure on the expression of *GPR43* and *GPR120* genes

	Point of measure[1]						
Item	ST1	ST2	ST3	JEJ	COL	SEM	*P*-value[2]
GPR43, normalized expression	0.025	0.036	0.038	0.060	0.063	0.022	NS
GRP120, normalized expression	0.017	0.009	0.012	0.004	0.081	0.058	< 0.001

[1] Oxyntic (ST1), pyloric (ST2) and cardiac to oxyntic transition mucosa (ST3); jejunum (JEJ) and colon (COL).

[2]COL vs. each of the other points.

Chapter four

The olfactory receptor OR51E1 is present along the gastrointestinal tract of pigs and is modulated by intestinal microbiota

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Abstract

The relevance of the butyrate-sensing olfactory receptor OR51E1 for gastrointestinal (GIT) functioning has not been considered so far. We investigated in young pigs the distribution of OR51E1 along the GIT, its relation with some endocrine markers, its variation with age and after interventions affecting the gut environment and intestinal microbiota. Immuno-reactive cells for OR51E1 and chromogranin A (CgA) were counted in cardial (CA), fundic (FU), pyloric (PL) duodenal (DU), jejunal (JE), ileal (IL), cecal (CE), colonic (CO) and rectal (RE) mucosae. OR51E1 co-localization with serotonin (5HT) and peptide YY (PYY) were evaluated in PL and CO respectively. FU and PL tissues were also sampled from 84 piglets reared from sows receiving either or not oral antibiotics (amoxicillin) around parturition, and sacrificed at days 14, 21, 28 (weaning) and 42 of age. JE samples were also obtained from 12 caesarean-derived piglets which were orally associated with simple (SA) or complex (CA) microbiota in the postnatal phase, and of which on days 26-37 of age jejunal loops were perfused for 8 h with enterotoxigenic *Escherichia coli* F4 (ETEC), *Lactobacillus amylovorus* or saline (CTRL). Tissue densities of OR51E1+ cells were in decreasing order: PL=DU>FU=CA>JE=IL=CE=CO=RE. OR51E1+ cells showed an enteroendocrine nature containing gastrointestinal hormones such as PYY or 5HT. *OR51E1* gene expression in PL and FU increased during and after the suckling period ($p<0.05$). It was marginally reduced in offspring from antibiotic-treated sows (tendency, $p=0.073$), *vs*. control. Jejunal *OR51E1* gene expression was reduced in piglets early associated with SA, compared with CA, and in ETEC-perfused loops vs. CTRL ($p<0.01$). Our results indicate that *OR51E1* is related to GIT enteroendocrine activity. Moreover age, pathogen challenge and dietary manipulations influencing the gastrointestinal luminal microenvironment significantly affect the *OR51E1* gene expression in GIT tissues presumably in association with the release of microbial metabolites.

Introduction

The superfamily of olfactory receptors (ORs) is formed by a very large number of G-protein coupled receptor proteins that detect volatile odorant molecules. They were initially discovered in the olfactory epithelium, but recent evidence shows that several of them are well expressed in the respiratory tract and in other tissues [1], where their function is still unclear. In the gastrointestinal

tract (GIT), they are detected in enterochromaffin cells and can affect the secretion of serotonin (5-hydroxytryptamine; 5HT) in response to fragrant molecules [2], with subsequent effects on gut motility [3]. During a comparative investigation of the transcriptome between the gastric fundic and the pyloric mucosae of pigs [4], it was evidenced that one gene- *OR51E1* (olfactory receptor, family 51, subfamily E, member 1, previously named GPR164) - was expressed more than other genes assigned to this category.

Deorphanization studies by cell-reporter systems evidenced that 3- and 4- methyl-valeric acids [5], nonanoic acid [6] and butyric acid [7] are agonists of this receptor. The sensitivity of OR51E1 to butyrate is of particular interest due to the multiple implications of this enteric bacterial metabolite in regulating GIT tissues in normal and pathological states [8].

Butyrate-sensing olfactory receptor OR51E1 has been identified only in GIT carcinomas [9], but its relevance for GIT physiology has not been considered so far.

In mammals, the maternal environment is a major determinant shaping the gut microbiota in early life [10,11]. Initial qualitative and quantitative colonization by environmental bacteria in the neonate and in the suckling mammal may play a role in the programmed maturation of the GIT. Both transiting and colonizing microbiota may contribute to the latter [12,13]. The effects could be exerted via multiple ways: influencing nutrient availability, synthesis of gut-active metabolites, interactions with host systems for detection of xenobiotics, involvement in the induction and activation of innate and acquired immune defences, via the differentiation and proliferation of GIT cells and development of the architecture of the intestinal mucosae. A part of these actions is explained by the intestinal release of the peptide YY (PYY) stimulated by luminal butyrate [14].

Microbial imprinting has been studied mostly in mice, and more frequently for the maturation of the immune system, and in poultry in a few studies [15,16]. The relevance of the maternal environment is also indirectly confirmed by the copious literature promoting breastfeeding in babies. Both inadequate early contact with favourable bacteria and their insufficient seeding are also implicated in the ontogeny of several metabolic and immune-related diseases in humans [17].

Here, we investigated the OR51E1 distribution along the GIT and its cellular association with some relevant endocrine markers, e.g. 5HT and PYY in young pigs. Furthermore, we hypothesized that OR51E1 tissue distribution may vary with changes in GIT microbiota or by microbial challenge. For investigating the former, we used piglets born from sows either or not treated with antibiotics during the gestation and lactation phase and piglets born by caesarean section and orally associated with different microbiota in the postnatal phase.

Materials and Methods

Study A: distribution of OR51E1 along the GIT

Ethics statement: The procedures carried out on the pigs were conducted in compliance with Italian laws on experimental animals and were approved by the Ethic-Scientific Committee for Experiments on Animals of the University of Bologna (Protocol submission to Italian Ministry of Health, number 34613-X/10).

Three Large White littermate pigs, one female and two males, were fed a typical post-weaning diet and housed individually in pens with a mesh floor in a temperature-controlled room and tap water freely available. At six weeks of age and a body weight (BW) of 13.4 ± 0.4 kg, the piglets were slaughtered and whole tissue samples for each of the following parts of the GIT were collected for immunohistochemistry analysis: gastric cardia (CA), fundus (FU), pylorus (PL); duodenum (DU), jejunum (JE), ileum (IL); cecum (CE), colon (CO) and rectum (RE).

Study B:*OR51E1* gastric gene expression in pigs born to sows either treated orally with an antibiotic around parturition or not

Ethics statement: The experiment was designed and executed in 2010 in compliance with French and European law (Decree No. 2001–464 29/05/01, 86/609/CEE) for the care and use of laboratory animals. At that time (2010) getting approval from an ethic committee was not mandatory. One of us (JPL) held the authorization certificate No. 006708 for experimentation on living animals delivered by the French Veterinary Services. INRA Saint-Gilles, including the on-site slaughterhouse has an institutional license (agreement No. A35-622) from the French Veterinary Services.

The study aimed at assessing if early disturbances in microbial colonization have an impact on *OR51E1* gene expression in the stomach. This was the part of the GIT showing in the highest number of OR51E1 staining cells along its different segments, in study A. Stomach tissue samples

were harvested from offspring born to control mothers (CTRL, n = 12) or mothers treated with the antibiotic amoxicillin around parturition (day -10 till day + 21) (ATBC, n = 11) in a larger study described in more detail by Arnal *et al.* [18, 19]. Broad spectrum antibiotic amoxicillin (Vetrimoxin PO containing 10% amoxicillin; CEVA Santé Animale, Loudéac, France) was provided daily to the sows (40 mg/kg BW) orally together with their morning meal (2 kg/day). They were fed the rest of their daily feed allowance without supplemental antibiotic. Amoxicillin was used as antibiotic because the experiment with pigs was primarily set up as a model for humans in the context of so called "DOHaD" (developmental origins of health and disease). Two experiments reporting long-term effects of neonatal antibiotic administration on gut physiology (barrier) had already been published in rodents using amoxicillin [20,21].

Litter size was adjusted within treatment groups at n = 12 piglets per litter at the end of farrowing. Four pigs per sow were randomly assigned to slaughter at the ages of 14, 21, 28 (age of weaning), and 42 days, respectively. Piglets were selected from all available litters to keep the mean BW and its variation in the sub-groups to be sacrificed as equal as possible. However, for two litters per each sow treatment, low numbers of offspring (but equalized piglet numbers during the suckling period, with fosters) did not allow slaughtering one pig per litter each time (given the fact that the general plan included also four additional pigs for the long-term part of the experiment, as already published [18,19]). Thus four pigs less were sampled for the slaughtering at 28 d and 42 d, and total eighty four offspring pigs are included in the present data set. This experimental design allowed testing the effect of treatment (ATBC vs. CTRL) within litters.

Sows and piglets were fed balanced diets formulated to cover all known nutritional requirements for gestating and lactating sows, and for starting (pre-starter and starter), respectively. Complete feed composition is available from Arnal *et al.* [18]. Sows were fed the gestating diet (3.5 kg/day) or the lactating diet (*ad libitum*) in two meals. Offspring had *ad libitum* access to their successive diets. At slaughter, the whole tissue samples of the stomach were obtained from FU and PL mucosae of each pig, and were immediately snap-frozen in liquid nitrogen for molecular analysis at a later stage.

Study C: Effects of early microbial association and intestinal loop treatments in caesarean-derived (CD) piglets

Ethics statement: The protocol of the study was approved by the Committee on the Ethics of Animal Experiments of Wageningen University and Research Centre in Lelystad, The Netherlands (Permission Number: 2012083.e).

Twelve piglets from sows [(Great York × Pie) × 'Dalland' cross] were obtained by caesarean delivery (day 0) and were divided over two microbiota association treatments housed in separate clean, non-sterile rooms and balanced for BW and litter of origin. Piglets were housed in two pens per room suited with an automatic feeding system for supply of a moist diet. Average birth weight was 1273 ± 138 g and 1275 ± 153 g for the two treatment groups, respectively. Each time at 1 h and 5 h after birth, each CD-piglet received 45 mL pasteurized (30 min at 60 °C) sow colostrum by oral gavage [22,23]. All piglets received a simplified starter microbiota consisting *of Lactobacillus amylovorus* (LAM) (3.6×10^7 CFU*), Clostridium glycolicum* (5.7×10^7 CFU), and *Parabacteroides* spp. (4.8×10^7 CFU) by oral inoculation (2 mL) on days 1, 2, and 3 after birth. These bacterial species are, among the phylogenetic groups, the most frequently identified in the intestine of piglets and were proposed by Laycock *et al.* [24] as intestinal colonization microbiota for gnotobiotic pigs. On days 3 and 4 of age, the piglets received either a complex microbiota by providing them with 2 mL of an inoculant consisting of 10% saline diluted feces of an adult sow (complex association, CA) or a placebo inoculant (10% saline) (simple association, SA). Piglets were fed *ad libitum* a milk replacer diet during a period of 5 d (days 0 - 4). It consisted of bovine skimmed milk powder, whey powder, vegetable oil, hydrolysed wheat protein, wheat starch, sucrose and a vitamin and mineral premix, and contained 230 g crude protein per kg milk replacer. A moist diet based on whey powder, maize, wheat, toasted full fat soybeans, oat flakes, sucrose, soybean meal, vegetable oil, coconut oil, wheat gluten, potato protein, rice protein, and brewer's yeast was fed during the remainder of the study.

On days 26 - 37 of age, intestinal tissue samples were obtained from intestinal loops prepared for applying the "*in vivo* small intestinal segment perfusion" (SISP) technique, as described by Nabuurs *et al.* [25,26]. In brief, the abdomen was opened along the *linea alba* and three small intestinal segments of 20 cm in length each were made in the mid-jejunum, starting at a distance of 200 cm distal to the ligament of Treitz. These segments retained their vascularization and were cannulated with a rubber tube at the proximal and distal ends to perfuse and collect fluid, respectively.

Three different isolated jejunal loops per pig were perfused with 8 mL fluid for 8h (saline with 0.1% glucose and 0.1% amino acids per h) containing the following solutions: enterotoxigenic *Escherichia coli* F4 (ETEC) as pathogenic strain (5.5×10^9 CFU per loop), or

LAM as beneficial strain (7.5 x 10^9 CFU per loop) or saline as control (CTRL). At the end of the SISP study, a sample of jejunal tissue per loop was collected and snap-frozen in liquid nitrogen for further molecular analysis.

Immunohistochemistry

Gastrointestinal samples were washed with PBS and fixed in 4% buffered formalin overnight. Standard procedure for paraffin embedding was followed and then 8 µm-thick of cross-sectional sections of the organ tissue were obtained from microtome cutting and mounted on microscope poly-L-lysine coated slides (Sigma-Aldrich, Milan, Italy). For immunohistochemical analysis, the sections were dewaxed, rehydrated and heated in citrate buffer pH 6 for 10 min at 700 w in microwave for antigen retrieval. Then, they were pre-incubated in PBS + 0.3% TritonX + 5% of donkey or goat normal serum for 1 h and incubated with the following primary antisera diluted in PBS + 5% serum overnight at +4°C: rabbit anti-human OR51E1 (code GTX100361; GeneTex Prodotti Gianni, Milano, Italy) 1:200, mouse anti- CgA (code MON 9014, Monosan Xtra, DBA Italia, Segrate, Italy) 1:600, mouse anti-5HT (code Ab16007, Abcam, Cambridge, UK) 1:200, guinea pig anti-pig PYY (code PAB17185; ABNOVA, Taipei, Taiwan) 1:1000. The porcine epitope for OR51E1 antibody shares 96% of homology compared to humans by sequence blast while the anti-GgA antibody was previously successfully tested on porcine CgA [27]. Then, the tissue sections were incubated with the following secondary antibodies for 1 h at room temperature: donkey anti-rabbit Alexa 488 (code ab150073; Abcam) 1:500, donkey anti-mouse Alexa 594 (code A21203; Molecular Probes, Eugene, OR, USA) 1:600, goat anti-guinea pig TRITC (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) 1:100. The slides were finally mounted via VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA) for microscope visualization. Preparations were examined on a Nikon Eclipse Ni microscope (Nikon Instruments, Sesto Fiorentino, Italy) equipped with the appropriate filter cubes to distinguish the fluorochromes employed. The images were recorded with a Nikon DS-Qi1Nc digital camera (Nikon Instruments) and NIS software (BR 4.20.01; Nikon Instruments).

In order to analyse a large and comparable area for each slide, the mosaic software procedure was used to capture a large composite microscope image, and only the mucosal area correctly cut transversally were selected as regions of interest for each slide. Three slides per

sample and a minimum of 3 mm² per region of interest in each slide were analysed. Immunoreactive cells were visually evaluated for the reactivity and manually counted.

RNA Isolation and gene quantification by real-time RT-qPCR

Total RNA was isolated from the intestinal tissue samples collected in trials B and C according to FastPure™ RNA kit (TaKaRa Bio Inc., Shiga, Japan). All other procedures were in agreement with the manufacturer's protocol. RNA purity and integrity were evaluated by Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA) just before real-time quantitative PCR (RT-qPCR) analysis.

The expression of *OR51E1* gene was quantified by RT-qPCR. One microgram of total RNA was reverse-transcribed using the ImProm-II Reverse Transcription System (Promega, Milan, Italy). 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 RT-qPCR reactions were performed in a LightCycler® Real-Time PCR Systems (Roche Applied Science, Bazel, Switzerland) by a shuttle PCR (2 steps) following the procedure described by Trevisi *et al.* [28]. The expression of data was normalized by geometric mean of expression data for two housekeeping genes: hydroxymethylbilane synthase (*HMBS*) and ribosomal protein L4 (*RPL4*) genes for gastric tissue samples (study B), and *HMBS* and TATA box binding protein (*TBP*) genes for jejunal tissue samples (study C), following Nygard *et al.* [29]. Primers and amplification conditions for *OR51E1* and housekeeping genes are reported in Table 1.

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

Gene[a]	NCBI accession number	Oligo sequence (5'→3')		Amplicon length	Annealing tempera-ture
OR51E1	XM_005656617.1	Forward Reverse	CGCGTCAACATCATCTATGGC CGCACACATGGGAGACACAC	160pb	59°C
HMBS	DQ845174	Forward Reverse	AGGATGGGCAACTCTACCTG GATGGTGGCCTGCATAGTCT	83 bp	62°C
RPL4	DQ845176	Forward Reverse	CAAGAGTAACTACAACCTTC GAACTCTACGATGAATCTTC	122 bp	60°C
TBP I	DQ845178	Forward Reverse	AACAGTTCAGTAGTTATGAGC CAGA AGATGTTCTCAAACGCTTCG	153 bp	60°C

[a]*OR51E1*, olfactory receptor, family 51, subfamily E, member 1; *HMBS*, hydroxymethylbilane synthase; *RPL4*, ribosomal protein L4; *TBP,* TATA box binding protein.

Statistics

Statistical analysis of data (S1, S2 and S3 Tables) was carried out using the MIXED procedure of SAS (version 9.3; SAS Institute Inc., Cary, NC, USA). In study A, the effect of the point of measurement was calculated considering the repeated measures inside each pig. In study B, the effects of sow's treatment (against an error calculated between litters), offspring age at slaughter (error within litters), point of measurement (FU and PL, error within pigs), and the relative first order interaction terms were tested. For the effect of age at slaughter, the following contrasts were calculated: "Linear effect during suckling", inside un-weaned groups (that is: offspring aged 14, 21 and 28 days), and "suckled *vs*. weaned" (that is: un-weaned pigs aged 14, 21 and 28 days *versus* weaned piglets aged 42 days). In study C, the effects of early microbiota

association (against an error calculated between pigs), of loop treatment (error within pigs), and the interaction term between early microbiota association and loop treatment were tested. For the loop experiment, treatments were contrasted against control infusion. Results are presented as least-squares means and pooled SEM. Least-squares means comparisons for each interaction were made only when a tendency ($p \leq 0.10$) for an interaction between these terms was observed. Effects were considered significant at $p \leq 0.05$ and as a trend at $p \leq 0.10$.

Results

Trial A

The immunohistochemical visualizations for *OR51E1* staining in each tissue and for co-localizations with CgA, 5HT and PYY are presented in Fig. 1 and Fig. 2, respectively.

All tissues samples from the various segments of GIT displayed cells staining for OR51E1. Tissue densities of OR51E1+ cells were in the decreasing order: PL=DU>CA=FU> JE=IL=CE=CO=RE ($p<0.01$) (Fig. 3, A). OR51E1+ cells stained also for CGA, with co-staining varying from 100% (CA, JE) to 73% (IL) (Fig. 3, B). In PL and CO, 56% and 91% OR51E1+ cells co-stained with 5HT and PYY, respectively. Conversely, 95% 5HT+ and 85% PYY+ cells co-stained with OR51E1 (Fig. 3, C and D).

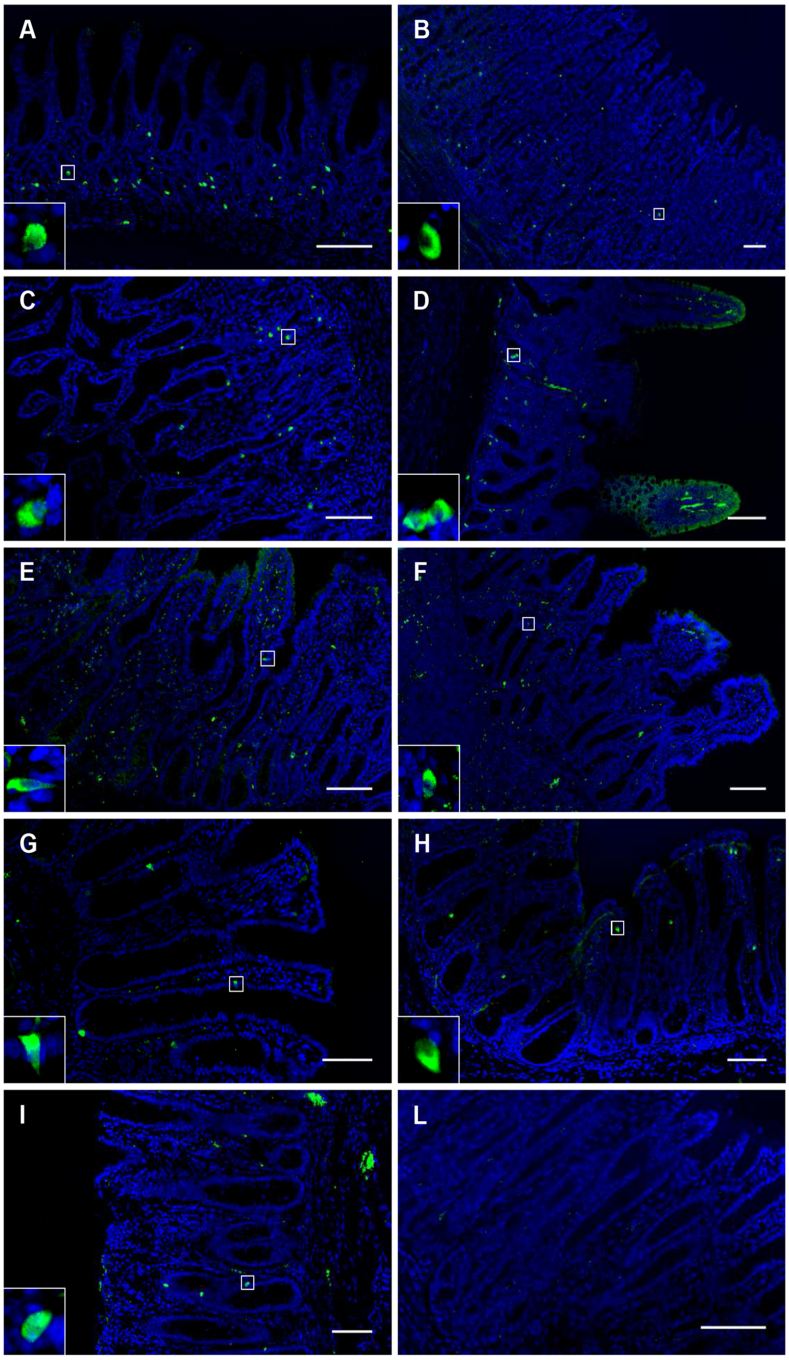
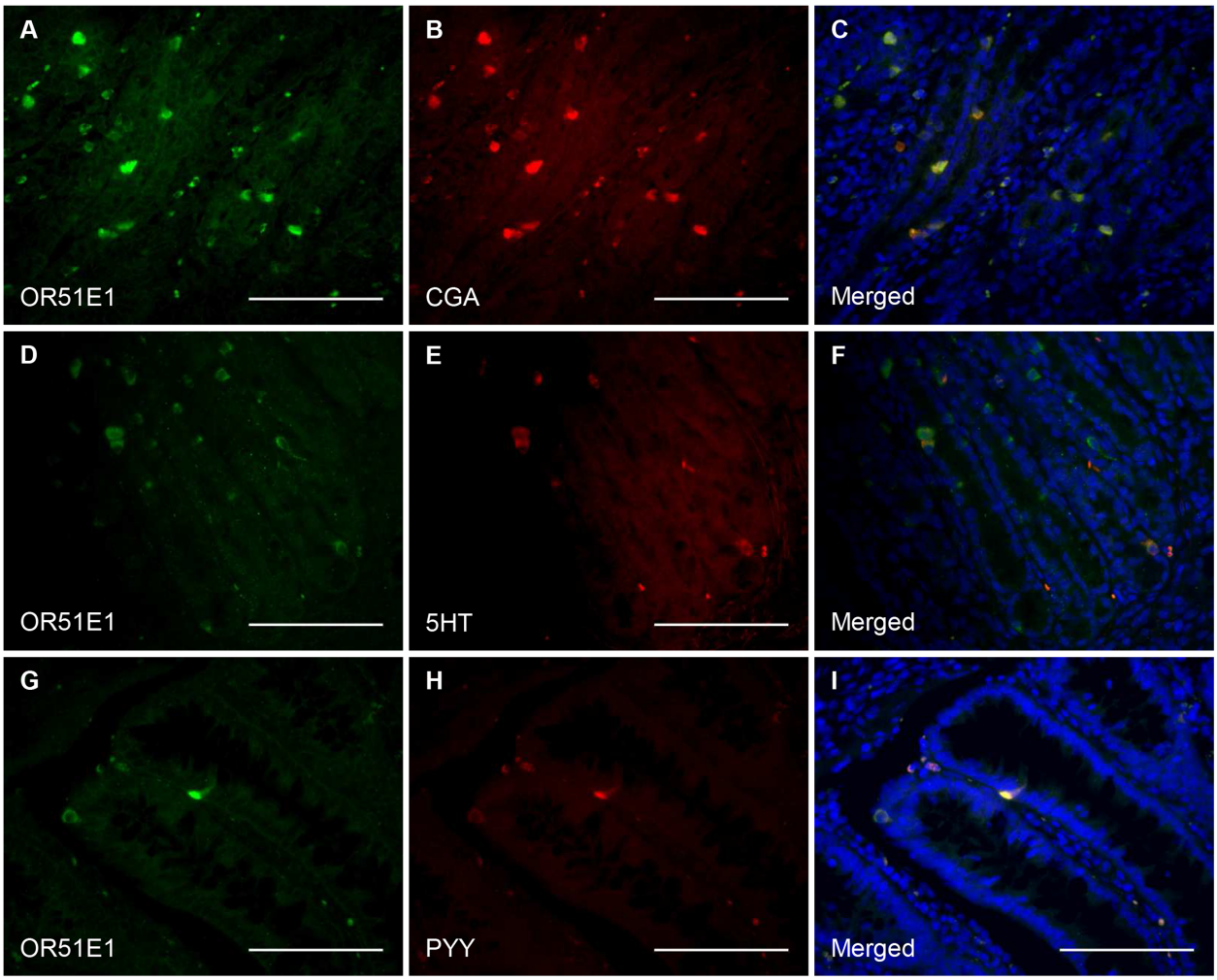


Fig. 1. Visualization of OR51E1 tissue distribution in the gastrointestinal tract. A = cardia, B = fundus, C = pylorus, D = duodenum, E = jejunum, F = ileum, G = cecum, H = colon, I = rectum, L = control. Scale bar = 100 μm. The OR51E1 immunostaining distribution is mostly in the bottom half of the mucosa in each tissue. There is a higher density of OR51E1+cells found in the gastric mucosa (A-C) with a peak density in the pylorus (C). The morphology of the OR51E1+ cells, as magnified in a small square on each picture is generally of the close-type with a round shape but sometimes they show an open-type morphology with a triangular shape (e.g. Fig. 1, E, G, H), particularly in the top half of the mucosa, closer to the lumen.

Fig. 2. Visualization of OR51E1 co-localization with enteroendocrine cells and some hormones.



A-F = pylorus, G-I = colon. Scale bars = 100 μm. Almost all the OR51E1+ cells have an overlapping staining with the cells positive for the enteroendocrine marker chromogranin A (A, B, merged in C) showing the endocrine nature of these cells. A subset of these cells also contain hormones such as serotonin (C, D, merged in E) and peptide YY (G, H, merged in I) suggesting a possible role for OR51E1 in the control of hormone release.

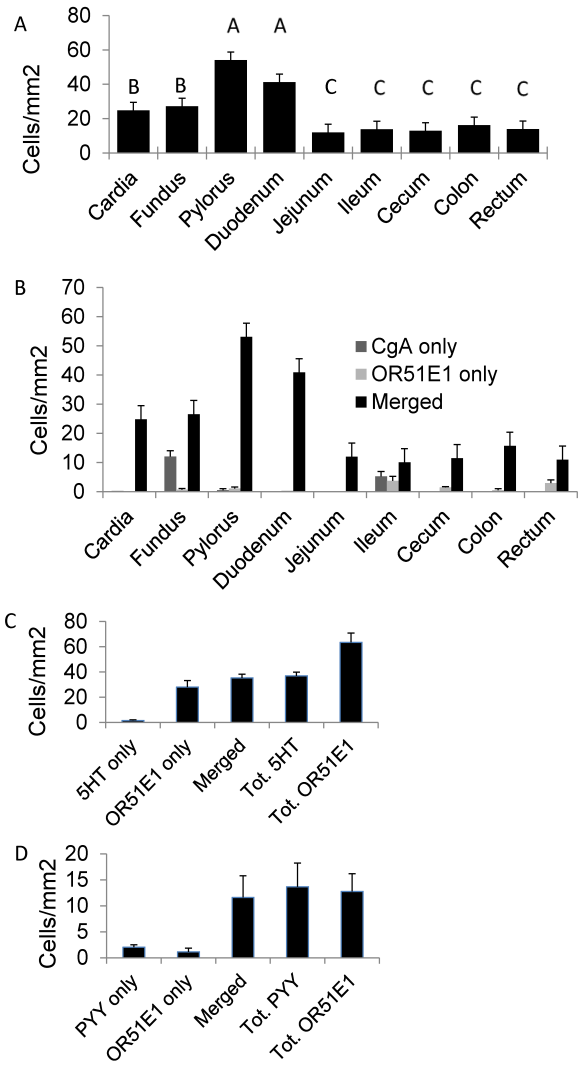


Fig. 3. Immunostainings for OR51E1, chromogranin A (CgA), serotonin (5HT) and peptide YY (PYY) in different mucosae of the GIT. Counts (with standard errors of means) of: A) OR51E+ cells. PL=DU>CA=FU> JE=IL=CE=CO=RE (A,B,C: $p<0.01$); B) cells co-staining (= Merged) or not, for OR51E1 and CgA; C) cells co-staining (= Merged) or not, for OR51E1 and 5HT in jejunum; D) cells co-staining (= Merged) or not, for OR51E1 and PYY in colon.

Trial B

The results showed that OR51E1 gene expression in small intestinal tissue differed about similarly for intestinal loops imposed to the various perfusion treatments in both microbiota association groups of pigs, as confirmed by the lack of significant interactions between these factors. Therefore, the effects of these factors are presented independently. The antibiotic treatment to the sows in the end gestation – lactation phase marginally reduced *OR51E1* gene expression in offspring gastric PL and FU regions (tendency, p=0.073) (Fig. 4, A) *vs*. control sows. Moreover, *OR51E1* gene expression was higher in PL than in FU (p=0.005) (Fig.4, B).

OR51E1 gene expression in PL and FU increased during the suckling phase (p=0.032), that was covered by samplings at d18, d21 and d28, and was higher in the weaned pigs (sampled at d42) compared with the whole pre-weaning period (p=0.047) (Fig. 3, C).

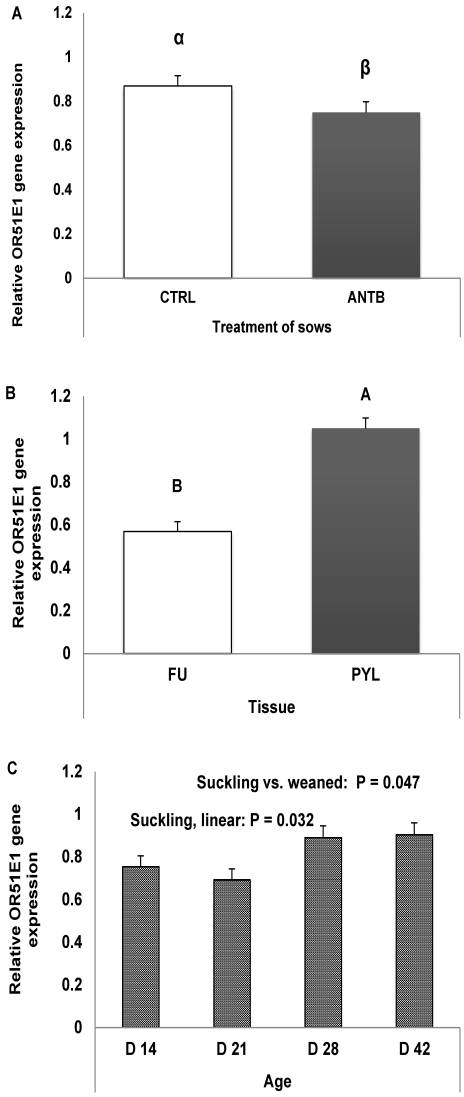


Fig. 4. Effects of antibiotic treatment of sows in gestation – lactation (A), offspring gastric tissue location (FU=fundic; PL=pyloric) (B), and offspring age (weaning at 28 days) (C), on relative gene expression of *OR51E1* in the stomach. Bars represent standard errors. No statistically significant interaction between the factors was seen. Orthogonal contrast analyses were conducted as follows: 4A: control vs. Antibiotic treatment (all ages and gastric sites considered) (α;β: p=0.073); 4B: Fundic vs. Pyloric region (all sows' treatments (CTL and ANTB) and age at slaughter considered) (A,B: p=0.005); and 4C: Suckling (day 18 + day 21 + day 28) vs. weaned (day 42) period (all sows' treatments (CTL and ANTB), age at slaughter and gastric site considered).

Trial C

The results showed that *OR51E1* gene expression in small intestinal tissue differed about similarly in both microbiota association groups of pigs and in intestinal loops imposed to the various perfusion treatments within each pig, as confirmed by the lack of significant interactions

between these factors. *OR51E1* gene expression was lower in piglets early associated with SA compared with CA (p=0.003, Fig. 5A), and in ETEC-perfused jejunal loops *vs*. CTRL (p<0.001, Fig. 5B). LAM perfusion had no effect on *OR51E1* gene expression.

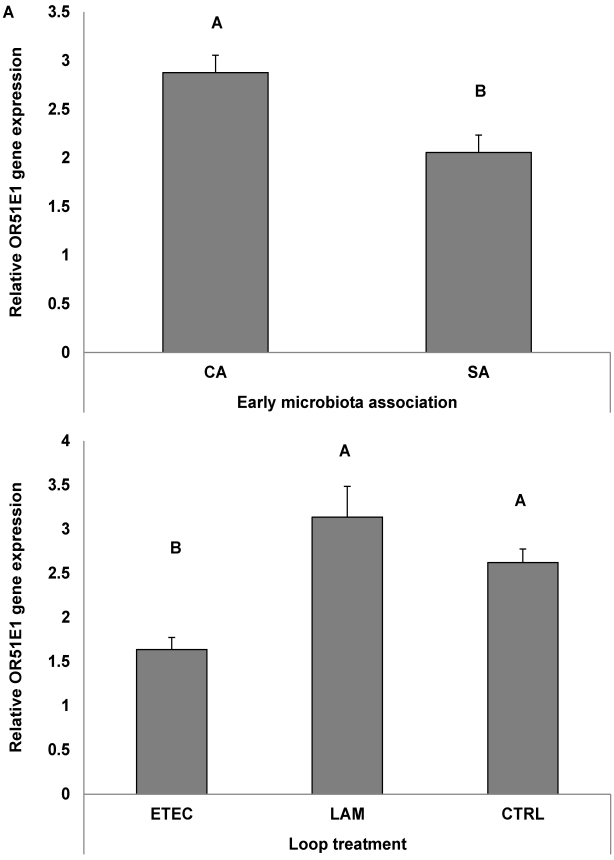


Fig. 5. Effects of early association with simple (SA) or complex microbiota (CA) to piglets in the post natal phase, and of intestinal loop treatment (pre-perfusion with saline, *Lactobacillus amylovorus* or ETEC) at 4 to 5 weeks of age on the relative gene expression of *OR51E1*, after 8 h of loop perfusion. Bars represent standard errors**.** No statistically significant interaction between the factors was observed. 4A) Effect of early microbiota association (A, B: p=0.003); 4B) Effect of loop pre-perfusion treatment; statistical significance is against saline treatment (A, B: p<0.001), ETEC = enterotoxigenic *Escherichia coli* K88; LAM = *Lactobacillus amylovorus*; CTRL = saline.

Discussion

Olfactory receptors belong to the largest G protein-coupled receptor family in mammals and are generally thought to be expressed in the olfactory epithelium, to detect volatile odorants [30-33]. However, recent studies reported the presence of ORs in non-olfactory tissues, where their roles remain unclear [34,35]. Our previous transcriptomic study [4] indicated the abundance of *OR51E1* mRNA in the gastric mucosa of the pig, an observation never reported for this tissue before. Inside this investigation, exploring the differential gene expression between oxyntic and pyloric tissues by microarray, we found that *OR51E1* gene expression was similar to the expression of one of the two gastric housekeeping genes (*HMBS*) and also 3.5 times higher than the expression of the second higher expressed OR gene (*OR4X1*) (unpublished data).

We hypothesize that OR51E1 could have a chemosensing role in the detection of volatile substances along the GIT, could be involved in some important endocrine functions, and finally may be modulated by several factors related to the gut environment including the residing microbiota and challenge by pathogenic bacteria.

The protein expression of *OR51E1* was detected all along the GIT (Fig.3-A), from the gastric cardia to the rectum, and this wide distribution suggests an important, still undiscovered role for this receptor. The highest relative number of OR51E1+ cells in the pylorus is consistent with a role for the distal stomach in sensing nutrients and xenobiotics [36]. Comparing the fundic and pyloric mucosae in growing pigs, it was also evidenced that several genes related to antimicrobial peptide secretion and cytoprotection were highly expressed in the pylorus: lysozyme, polymeric immunoglobulin receptor, cytochrome P450 family 3 subfamily A, polypeptide 46, secretoglobin, family 1A, member 1 (uteroglobin) [4]. The pyloric region is an interface between different micro environments in the stomach and in the proximal small intestine. Our results show that OR51E1 co-localizes with the enteroendocrine cell marker CgA all along the GIT for a percentage varying from 78 % to 100% (Fig.3-B). This suggests that this olfactory receptor is constitutive to this type of cells and reveals their enteroendocrine nature. Therefore, our findings support the hypothesis that the function of OR51E1 may be related to an endocrine response, though this requires further confirmation. In addition, 96% and 85% of the enteroendocrine cell subsets containing 5HT and PYY respectively, co-localize with OR51E1 (Fig.3-B, 3-C). Enterochromaffin cells are widely distributed along the mucosal surface of the GIT and respond to chemical and physical signals, thus releasing 5HT to activate intrinsic and

extrinsic nerves, and producing physiological responses such as GIT motility [37] and immune activation [38]. Serotonin has been shown to co-localize with olfactory receptors in solitary pulmonary neuroendocrine cells where a volatile stimulation-dependent release of 5HT is detected [39]. Conversely, in FU mucosa, co-staining between OR51E1 and CgA was not so high (69 % merged cells of total CgA+ cells). In FU different enteroendocrine cell types are present, e.g. D and P/D1 cells secreting somatostatin and ghrelin, respectively, and enterochromaffin-like cells, secreting histamine.

The second tissue with high density of OR51E1+ cells revealed to be the duodenum. Also for this location there was an almost full overlap (99%) with CgA staining. Duodenal enteroendocrine cells are primarily sources of gastric inhibitory peptide (GIP, secreted by K cells) [40] and cholecystokinin (CKK, secreted by I cells) [36]. Furthermore, these cells were shown to be equipped with several receptors linked to sweet and bitter tastes [36]. They also displayed the necessary proteins (α-transducin and α-gusducin) to translate this signal into the endocrine secretion. Collectively, these observations may suggest a possible link between *OR51E1* expression and modulation of endocrine activity.

Bacterial metabolites as 3- and 4- methyl-valeric acids, nonanoic acid and butyrate were recently proposed to be activators of OR51E1 [5, 6, 7]. It is already established that the sensing of bacterial metabolites participates to the local hormonal control of the gut function. For example, propionate has been shown to cause the release of PYY and to slow down intestinal transit [41]. Our observation that colonic PYY+ cells harbored also OR51E1 adds to what has been seen for the two fatty acid receptors GPR43 and GPR41 in human colon [42,43]. However, OR51E1 receptor was more densely located in the gastric than in the intestinal mucosae (Fig. 1, fig.3-B). In gastric digesta of young pigs, butyrate was detected at different concentrations largely depending on dietary condition [44-46] which may have affected the presence of butyrate-producing bacteria. In the stomach of suckling pigs, lactate-utilizing butyrate-producing bacteria may take benefit of the lactate producers growing on lactose from milk or of the cross-feeding of partial breakdown products from other substrates, as proposed for human gut [47]. Furthermore, butyrate-producing Clostridia isolated from pigs can use mucins for growth [48].

With trial B and C we provide evidence that several experimental factors modulate *OR51E1* gene expression along the gut. This led to variations in the numbers of OR51E1-expressing cells and/or in the transcript level in the expressing cells. Feedback mechanisms, differential activation of transcription factors as well as epigenetic regulation have been evidenced for *OR51E1* gene expression in olfactory bulbs [3]. Several circulating hormones, mostly related

to the control of food intake and energy balance can modulate olfactory epithelium [3]. Conversely, there is scarce knowledge on regulation of *OR* genes beyond the nose. Postnatal GIT colonization is favored by the multiple encounters of a large variable pool of bacteria from the environment. The progressive raise of gene expression during the suckling period and from this phase to the post-weaning period suggests a dependence of *OR51E1* gene regulation on mucosa or microbiota maturation (Fig.4-C). In an earlier experiment, caesarean-derived piglets that were orally dosed with an inoculant consisting of sow's diluted feces in the first days after birth had a more complex intestinal and fecal microbiota for the first four weeks of life, compared to those receiving only the simplified pool of bacteria (as the one used in our study) [49]. The effect of microbiota association treatment (simple or complex) on the jejunal microbiota community has been confirmed in contemporary piglets in the same study (A.J.M. Jansman et al, manuscript in preparation). Furthermore, it was evidenced that an early microbiota association treatment also influenced gastric mucosa transcriptome at two weeks of age, with down-regulation of several metabolic pathways related to cell replication and immune response, with SA treatment [50]. Early association with a complex microflora (study C) stimulated the intestine to have a high *OR51E1* gene expression (Fig.5-A), and this may have been related to the higher complexity of the gastric microbiota. Furthermore, the same early imprinting from the environment can also help explain the trend for decreased *OR51E1* gene expression in piglets that were reared from sows treated with an antibiotic, as compared to those from control sows (study B) (Fig. 4-A). In fact, sow's antibiotic treatment affected offspring ileal microbiota composition (approximate genus-level microbial groups) during the first weeks of life and reduced microbiota diversity on day 14 of age [18]. Unfortunately, gastric digesta was not sampled in the present study, so possible changes in microbiota composition, as induced by antibiotic treatment, could established and not be linked to changes *OR51E1* gene expression in stomach tissue.

The second goal of the study C was to investigate the effects of beneficial or harmful conditions coupled to differences in intestinal microbiota composition on *OR51E1* gene expression in the gut. The *OR51E1* gene expression was also strongly decreased in a pathogenic condition induced by ETEC challenge (fig.5-B). Previous research had demonstrated that ETEC challenge in the SISP test increased the number of ETEC attached to the small intestinal mucosa in ETEC perfused loops, causing a decrease in net fluid absorption [51]. This is certainly related to the strong damage of the tissue always observed during ETEC infection [52] and likely by the lower microbiota complexity induced by an early association treatment with a limited number of microbial species (SA). Conversely, *Lactobacillus amylovorus* did not affect *OR51E1* gene expression compared to CTRL treatment, suggesting that this supposed beneficial strain did not

interfere with normal OR51E1 receptor expression. Nevertheless, the results obtained with SISP model in study C allows to hypothesize the existence of a lumen-driven regulation of *OR51E1* gene expression. Conversely, systemic-driven regulation should not be hypothesized because *OR51E1* down regulation was not observed in intestinal loops perfused with *Lactobacillus* or saline. In fact in the SISP model, all the loops share the same systemic blood supply. Thus, paracrine regulatory mechanism for *OR51E1* gene expression can be envisaged.

Conclusions

Our results indicate that age, pathogen challenge and dietary manipulations affecting the gut luminal micro-environment and the intestinal microbiota modulate *OR51E1* gene expression in GIT tissues. The expression of this receptor seems be related particularly to the factors that affect complexity of the microbiota. Moreover, we showed that OR51E1 is a receptor often located in enteroendocrine cells all along the GIT in which this receptor could have an important role to modulate the secretion of some gastrointestinal hormones. Further investigations are needed to elucidate the functional implications of these findings and to identify which bacterial species are directly involved in *OR51E1* modulation.

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Supporting Information

S1 Table 1. Counts of OR51E1+ cells per each subject and point of measure in study 1. Density values, n cells/mm^2.

S2 Table 2. Individual expression of *OR51E1* and of housekeeping genes for study 2. Suckling sow number, its treatment, and the final piglet age are reported for each subject. Values are per each point of sampling and in gene copies/mg RNA.

S3 Table 3. Individual expression of *OR51E1* and of housekeeping genes for study 3. Early microbiota association treatment of the pig and intestinal loop treatment are reported for each observation. Values are in gene copies/mg RNA.

Chapter five

Differential gene expression in the oxyntic and pyloric mucosa of the young pig

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Abstract

The stomach is often considered a single compartment, although morphological differences among specific areas are well known. Oxyntic mucosa (OXY) and pyloric mucosa (PYL, in other species called antral mucosa) are primarily equipped for acid secretion and gastrin production, respectively, while it is not yet clear how the remainder of genes expressed differs in these areas. Here, the differential gene expression between OXY and PYL mucosa was assessed in seven starter pigs. Total RNA expression was analyzed by whole genome Affymetrix Porcine Gene 1.1_ST array strips. Exploratory functional analysis of gene expression values was done by Gene Set Enrichment Analysis, comparing OXY and PYL. Normalized enrichment scores (NESs) were calculated for each gene (statistical significance defined when False Discovery Rate % < 25 and *P*-values of NES< 0.05). Expression values were selected for a set of 44 genes and the effect of point of gastric sample was tested by analysis of variance with the procedure for repeated measures. In OXY, HYDROGEN ION TRANSMEMBRANE TRANSPORTER ACTIVITY gene set was the most enriched set compared to PYL, including the two genes for H^+/K^+-ATPase. Pathways related to mitochondrial activity and feeding behavior were also enriched (primarily cholecystokinin receptors and ghrelin). Aquaporin 4 was the top-ranking gene. In PYL, two gene sets were enriched compared with OXY: LYMPHOCYTE ACTIVATION and LIPID RAFT, a gene set involved in cholesterol-rich microdomains of the plasma membrane. The single most differentially expressed genes were gastrin and secretoglobin 1A, member 1, presumably located in the epithelial line, to inactivate inflammatory mediators. Several genes related to mucosal integrity, immune response, detoxification and epithelium renewal were also enriched in PYL ($P < 0.05$). The data indicate that there is significant differential gene expression between OXY of the young pig and PYL and further functional studies are needed to confirm their physiological importance.

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INTRODUCTION

The stomach essentially is devoted to the preparation of the bolus for the best digestion conditions in the downstream digestive tracts. Neural, hormonal, paracrine signals resulting from luminal content sensing (chemicals and nutrients, xenobiota components), are integrated in the stomach [1] to adjust the intake, passage rate and metabolism in collaboration with the intestine.

In the pig, oxyntic glands are found in the cardia gland and fundic gland regions (**OXY**), while antral-type mucous glands are found in the pyloric gland region (**PYL**).

This is reflected by their main functions of acid secretion and gastrin secretion, respectively. Differential expressions of numerous gene groups highlight the different specializations of the gastric mucosa compared with the small and large intestines [2]. However, it is not documented if these differences are unique to the whole stomach or two functional mucosal compartments.

Additional knowledge about the compartmentalization between OXY and PYL would help to identify markers of gastric areas [3] and provide models to investigate the developmental process of the gastric mucosa in normal or specific conditions, such as during weaning.

The development of specific physiological functions of the stomach is relevant for the young pig to rapidly adapt to post-weaning diets and also to control the gastro-intestinal microbiota using acid secretion or other defenses. Several feeding strategies have been proposed to improve the health of piglets [4] and improved knowledge of the differential gene expression in the two specialized gastric mucosal areas would help to improve feeding practices and provide further markers in addition to those already used [5-8].

Our aim is to assess the differential gene expression between OXY and PYL in young pigs.

MATERIALS AND METHODS

The procedures carried out on the pigs were conducted in compliance with Italian laws on experimental animals and were approved by the Ethic-Scientific Committee for Experiments on Animals of the University of Bologna (Permit number: ARIC- 47357).

Animals and sample collection

Seven crossbred (Large White × Landrace) male weaned pigs (5–6 weeks of age, 11.1 kg body weight, on average) were individually housed in cages and a standard post-weaning feed for five days. Then, after the morning meal, pigs were slaughtered by intracardiac injection (Tanax, 0.5 mL/kg body weight; Intervet Italia, Peschiera Borromeo, Italy), after being anaesthetized with sodium thiopental (10 mg/kg body weight). For each subject, the stomach was removed, opened

along the greater curvature and washed in ice-cold PBS, and two samples with transmural sections were collected respectively for OXY in the great curvature between the cardiac gland region and for PYL in the pyloric gland region close to the pyloric sphincter. Samples were immediately frozen in liquid nitrogen and stored at -80°C until use.

RNA Isolation, Microarray Processing, Quality Control

Total RNA was isolated from oxyntic and PYL collected from each subject, according to Qiagen RNeasy Midi Kit protocol (Qiagen, Hilden, Germany). To reduce the viscosity of the lysate, specimens (50 to 100 mg) were homogenized directly in the buffer RTL containing guanidine thiocyanate. All the other procedures were in agreement with the manufacturer protocol. Purity and integrity evaluation was assessed just before analysis by Agilent Bioanalyzer 2100. Total RNA was hybridized on Affymetrix Porcine Gene 1.1 ST array strips. Hybridized arrays were scanned on a GeneAtlas imaging station (Affymetrix, Santa Clara, CA, USA). Performance quality tests of the arrays including the labelling, hybridization, scanning and background signals by a Robust Multichip Analysis were performed on the CEL files using Affymetrix Expression Console. The intensity records were log2-transformed. Transcript data have been submitted to the National Center for Biotechnology Information's Gene Expression Omnibus (NCBI GEO) with GEO accession number GSE57620.

Gene quantification by real-time RT-PCR

Samples were validated by the quantification of the expression of H+/K+ Atpase α (***ATP4A)***; gastrin ***(GAST)***; ghrelin/obestatin prepropeptide (***GHRL***); polymeric immunoglobulin receptor (***PIGR)*** genes by real-time quantitative PCR (RT-qPCR). 1 μg of total 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 primers sequences, amplicon length and annealing/extension temperatures are given in Table 1.

The RT-qPCR reaction was performed in a LightCycler Real-Time PCR Systems (Roche Applied Science) by a shuttle PCR (2 steps) following the procedure described by Trevisi *et al.* [9]. The 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 housekeeping genes are reported in Table 1.

Pathway Analysis and other statistics

Affymetrix Trascripts IDs, each one in general characterized by several exonic sequences, were associated with 13,406 human gene names based on the *Sus scrofa* Ensembl database (release 69, www.ensembl.org). For the processed gene expression values, exploratory functional analysis was done with Gene Set Enrichment Analysis using the C5.BP catalog of the gene sets (based on Gene Ontology) from Molecular Signatures Database v3.1(http://www.broadinstitute.org/gsea/msigdb/Index.jsp), comparing OXY with PYL. Normalized enrichment scores (**NES**s) were calculated for each gene set and statistical significance was defined when the False Discovery Rate % was < 25 and the *P*-values of the NES were < 0.05, as suggested by the program. Enrichment score *P*-values were estimated using a gene set-based permutation test procedure.

From microarray analysis, expression values were obtained for a preselected set of 45 genes, identified on the basis of the literature [2] and our previous observations. The effect of the kind of gastric mucosa (OXY or PYL) was tested on these data by analysis of variance with the SAS GLM (SAS Inst. Inc., Cary, NC, USA) procedure for repeated measures (each pig).

RESULTS AND DISCUSSION

In OXY, a total of 18 gene sets were significantly enriched compared with PYL (Table 2). HYDROGEN ION TRANSMEMBRANE TRANSPORTER ACTIVITY was the gene set most differentially enriched, which includes the two genes for H^+/K^+-ATPase, fundamental enzyme for acid secretion; pathways related to mitochondrial activity and feeding behavior were also enriched (the last involving primarily cholecystokinin receptors, GHRL and the anorexigenic neuropeptide W). Aquaporin 4, water-selective channel protein present in the plasma membranes, was the top-ranking gene.

In PYL, only two gene sets were significantly enriched compared with OXY: LYMPHOCYTE ACTIVATION, with interleukin 7 ranking first, and LIPID RAFT, a gene set involved in specialized membrane domains composed mainly of cholesterol and sphingolipids.

The single gene most differentially expressed was *GAST*, the peptide hormone produced in pylorus by G cells. The second most differentially expressed gene was ***SCGB1A1***, secretoglobin, family 1A, member 1, presumably located in the epithelial line, to inactivate inflammatory mediators.

Among the set of pre-selected genes, 16 genes were more expressed in OXY compared with PYL (Table 3). These genes were related to acid secretion and pH homeostasis (ATP4A; anion exchanger 2) and were ion and water channels (potassium voltage-gated channel, isk-related family, member 2 – ***KCNE2***; potassium inwardly rectifying channel, subfamily J, member 13 and member 15 – **KCNJ13** and ***KCNJ15***; chloride intracellular channel 6 - ***CLIC6***; aquaporin 4), endocrine mediators, growth factors, receptors and binding proteins (insulin-like growth factor binding protein 5 – ***IGFBP5***; *GHRL*; epidermal growth factor - ***EGF***) or related to digestion, nutrient uptake and transport (pepsinogen B and C; chitinase, acidic; lipoprotein lipase; solute carrier family 2, facilitated glucose transporter, member 4).

Among the set of pre-selected genes, 16 genes were more expressed in PYL compared with OXY (Table 4). These genes were cell adhesion factors and regulators of tight junctions (Olfactomedin 4; Claudin 2 and 7) and were related to epithelial protection, immunity and detoxifying enzymes (lysozyme; polymeric immunoglobulin receptor; cytochrome P450, family 3, subfamily A, polypeptide 4 - ***CYP3A46***; secretoglobin, family 1A, member 1 (uteroglobin) - ***SCGB1A1***); transcription factors (Meis homeobox 2; SRY (sex determining region Y)-box 21; leucine-rich repeat containing G protein-coupled receptor 5 - ***LGR5***), endocrin mediators, growth factors, receptors and binding proteins (somatostatin; *GAST*), digestive enzymes, or nutrient transporters (gastric intrinsic factor), and others (aldo-keto reductase family 1, member C1; cysteine dioxygenase 1; adenylate kinase 5).

Other genes, characterizing the gastric mucosa versus the intestinal mucosae in mice, were not affected by the type of gastric mucosa (Table 5): ATPase, class V, type 10D – ***ATP10D***; carbonic anhydrase 2 & 11; gastrokine 1; protein disulfide isomerase (PDI) family A, 3 & 4; Mucin 1; glutathione peroxidase 1; glutathione S-transferase alpha 4; fatty acid binding protein 5; sodium iodide symporter, member 5; pancreatic amylase.

The validation of the microarray data by quantitative real-time PCR analysis of four representative genes (*ATP4A*, *GAST*, *GHRL*, *PIGR*) is reported in table 6. The variations for the two different mucosae were confirmed for all the genes by the quantitative real-time PCR analysis.

DISCUSSION

Expression in oxyntic mucosa. As expected, the comparison of OXY with PYL mucosa revealed a greater differentiation of OXY due to the presence of parietal cells responsible for the gastric hydrochloric acid secretion into the lumen of the stomach. This finding implies the powerful activation of H^+/K^+-ATPase to transport H^+ across the apical membrane of parietal cells and explains the differential enrichment of genes collected in the pathway HYDROGEN ION TRANSMEMBRANE TRANSPORTER ACTIVITY. The enrichment of many MITOCHONDRIAL genes and pathways involved in membranes activity, oxidative phosphorylation and respiratory chain show the high ATP production and the massive use of energy primarily for proton pumping in oxyntic paretial cells. Interestingly, the gene for nicotinamide nucleotide transhydrogenase was enriched in OXY and present in both of these groups of genes and its activity is likely related to ATP biosynthesis [10] or free radical detoxification [11] in the cell. The hydrochloric acid secretion requires the exchange of bicarbonate for chloride ions; anion exchanger 2 contributes to basolateral membrane HCO3- transport [12]. Its observed gene upgrade confirms the relevance of this transporter for the ionic balance in the oxyntic mucosa, in agreement with observations of knock-out mice for this gene [13].

Class V *ATP10D*, which encodes phospholipid-translocating ATPase, was enriched in both in OXY and PYL mucosae while previously it has been shown to be localized in murine parietal cells [2]. However, the similar gene expression observed in our survey for OXY and PYL may indicate that this enzyme involved in the phospholipid translocation may be not only related to H^+/K^++-ATPase membranes in parietal cells but also to the formation or reorganization of cellular or intracellular membranes or vesicular trafficking in the PYL [14]. Carbonic anhydrases catalyze the bidirectional conversion of carbon dioxide and water to bicarbonate and protons required for acid secretion. The enrichment in the transcripts for carbonic anhydrase 2 (cytosolic) and 11 (catalytic), which was observed in gastric corpus of mice in confront with intestinal segments [2], is observed in both gastric mucosae here. This result suggests that, beside the involvement of acid secretion, these carbonic anhydrases also serve to maintain the mucus-bicarbonate barrier by the mucus-producing epithelial cells in both gastric areas.

Regulation of the K^+ balance in gastric surface cells and parietal cells is also relevant. Several genes transcribing for K^+ channels were more strongly expressed in OXY than in PYL. *KCNE2*,

which was the most expressed in parietal cells, in mice is associated with potassium the voltage-gated channel, KQT-like subfamily, member 1 (***KCNQ1***), forming an heterodimeric potassium channel [15]. The control of K^+ fluxes to cytosol by this complex has relevance for acid secretion and is independent of H^+/K^+-ATPase activity in mice [15]. KCNQ1 was not present on our microarray chip. Therefore no data are available. However, KCNE2, which forms a complex with KCNQ1 to provide K-efflux for acid secretion in stimulated parietal cells [15] was highly expressed in OXY in our analysis confirming similar results in other species. In mice, He *et al.* [16] revealed the relevance of *KCNJ15* transcript in OXY and *KCNJ15* product in parietal cells, where it is stimulated by acid secretion and can cooperate with *KCNQ1*. *KCNJ15* is also present in chief cells, but not in mucous neck cells. A gene expression study of isolated parietal cells revealed that *KCNJ13* is present in significant amounts but shows the same or lower expression levels compared with whole gastric epithelium [17]. The relevance of total fluid excretion in OXY is finally outlined by the greater activation of *CLIC6*, which presumably creates a chloride ion gradient for water movement in parietal cells [18], in connection with water-selective channels like Aquaporin 4 in the plasma membranes to secrete water in the lumen and produce a more fluid bolus. Finally, these processes require the timely provision of energy substrates, which is evidenced by the increased transcription for genes related to lipolytic activity (*LPL*) and glucose transport (*SLC2A4*) in OXY compared with PYL. Nevertheless, the similar gene expression of *FABP5* in both gastric tissues may indicate that in PYL there is also an important need for fatty acids uptake, as has been evidenced for OXY in comparison with intestinal tissues in mice [2].

We also found that OXY shows increased expression of a gene related to digestion, the acidic chitinase (*CHIA*), which has already been identified in the stomach of other species, although with a variable degree of expression [19]. The activity of CHIA may be addressed to chitin-containing feeds (as may be also true for wild boar), to insects and other live organisms and be favored by the peculiar pH in OXY, which explains also the reduced gene expression in PYL.

Our results showed that OXY is more specialized for the control of feeding behavior given by the greater expression of genes of the FEEDING_BEHAVIOR group, where specifically ghrelin was enriched. Other endocrine mediators were found out to be more specifically involved in growth; EGF has a regulatory function on H^+/K^+-ATPase activity in parietal cells [20], thus its greater gene expression in OXY may be related to a paracrine control based on this growth factor that is not present in PYL. It is known that EGF expression and secretion in the stomach is regulated by capsaicin sensitive peripheral neurons and results in cytoprotective and antiulcerogenic activity together with the increase of CGRP and NO release [21, 22].

Furthermore, an upregulated gene expression of *IGFBP5* in OXY may be required for regulating cellular growth, differentiation and turnover in parietal cells [23].

GPX1 and *GSTA4*, which were enriched in the stomach compared with the intestine [2], were not differentially expressed between OXY and PYL, supporting the detoxification role for all mucosa regions against reactive oxygen species [24] and xenobiotics [25]. Mucus is also important for the protection of the stomach, which is supported by the similar expression of the mucin 1 gene in OXY and PYL..

Expression in pyloric mucosa. Other relevant gastric control mechanisms are also resident in PYL, including greater expression for GAST and its paracrine negative regulator, somatostatin, released from G-cells and D-cells, respectively. However, in PYL, all of the gastric content is forced to pass toward the intestine. Thus, the significant expression of genes of the LYMPHOCYTE ACTIVATION group in PYL is not surprising. It is furthermore reasonable to find that some proteins involved in mucosal defense such as antibacterial lysozymes and polymeric immunoglobulin receptors, required for IgA transepithelial basal-to-apical transport to the epithelial surface, were more expressed in PYL. The greater gene expression of polymeric immunoglobulin receptor in PYL versus OXY is consistent with previous observations using pigs at different ages [9].Our data reveal that other defense genes encoding for for protein disulfide isomerases (**PDI**) *PDIA3* and *PDIA4* are presumably not only highly expressed in chief cells, as reported previously [26], but also in PYL. *PDIA3* and *PDIA4* are involved in protein folding in rough endoplasmic reticulum and reported to be related to various function, in particular the assembly of major histocompatibility complex class I [27] and redox homeostasis [28], respectively.

It is also worth noting that the genes for the xenobiotic metabolizing cytochrome P450 enzyme, *CYP3A46* [29] and a secretoglobin (*SCGB1A1*), were more expressed in PYL. *CYP3A46* may have relevance for protecting the gut against T-2 toxin [30], the mold byproduct of *Fusarium* spp fungus, that, among other effects, causes vomit. Interestingly, SCGB1A1 is known for its anti-inflammatory properties and for the predominant localization in Clara cells of distal conducting pulmonary airways [31, 32]. Finally, the upregulation of genes related to lymphocyte activation may be also linked to the second genes set upregulated in PYL, LIPID_RAFTS. In fact, lipid rafts are cholesterol-rich microdomains of the plasma membrane known to be also involved in the activation of cytokine signaling [33] and T lymphocytes differentiation [34].

PYL mucosa also shows greater expression of some relevant genes related to the barrier defense of the mucosa: claudin 7 is, for example, necessary for the epithelial barrier integrity and to avoid bacterial translocation [35]. Furthermore, PYL seems to be better equipped than OXY to sustain a much greater turnover rate [36] and may be related to the constant mechanical stress caused by the passage of the feed bolus. This hypothesis is supported by the greater gene expression of the marker of gastro-intestinal stem cells *LGR5* [37]. Conversely, *GKN1*, another gene that is more involved in the renewal of gastric epithelium [38] compared with intestinal epithelium [2], was similarly expressed in both gastric areas, confirming that it is in general relevant to the replication of gastric surface mucous cells [37].

In conclusion, OXY and PYL mucosae show high expression of genes other than known functional genes related to hydrochloric acid and gastrin secretion. In general, the data indicate that OXY has a higher specialization than the PYL, useful for new marker detections. The pylorus expressed some gene transcripts that may merit additional studies, particularly those related to mucosal defense function. In addition, the research suggests that several genes are shared between OXY and PYL. These new observations should be addressed in further studies considering the different compartments of the stomach separately, as is usually the case for the intestine, to reveal novel functions.

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

Gene[1]	NCBI accession number	Oligo sequence (5'→3')	Amplicon length	Annealing T	
ATP4A	M22724	Forward	GCATATGAGAAGGCCGAGAG	151 pb	57°C
		Reverse	TGGCCGTGAAGTAGTCAGTG		
GAST	NM_001004036	Forward	GACTCTGCGCCTATGTCCTG	133 bp	60°C
		Reverse	GCTCTTTGCCCCTGTTGG		
GHRL	NM213807	Forward	GAACAGAGGTGGCTGGTCTC	202 pb	62°C
		Reverse	ACAGGGGAGACAAGGAAAGG		
PIGR	NM_214159.1	Forward	AGCCAACCTCACCAACTTCC	105 bp	62°C
		Reverse	CTGCTAATGCCCAGACCAC		
HMBS	DQ845174	Forward	AGGATGGGCAACTCTACCTG	83 bp	62°C
		Reverse	GATGGTGGCCTGCATAGTCT		
RPL4	DQ845176	Forward	CAAGAGTAACTACAACCTTC	122 bp	60°C
		Reverse	GAACTCTACGATGAATCTTC		

[1]*ATP4A*, H+/K+ Atpase α; *GAST*, gastrin; *GHRL*, ghrelin/obestatin prepropeptide; *PIGR*, polymeric immunoglobulin receptor; *HMBS*, hydroxymethylbilane synthase; *RPL4*, ribosomal protein L4.

Table 2. Gene sets enriched in oxyntic and pyloric mucosae of young pigs

Name	Size[1]	NES[2]	FDR[3] q-value
Oxyntic mucosa			
HYDROGEN_ION_TRANSMEMBRANE_ TRANSPORTER_ACTIVITY	20	2.172	0.002
MITOCHONDRION	268	2.149	0.002
MITOCHONDRIAL_MEMBRANE_PART	39	2.094	0.002
MITOCHONDRIAL_RESPIRATORY_CHAIN	18	2.029	0.009
MITOCHONDRIAL_INNER_MEMBRANE	50	2.029	0.008
CELLULAR_RESPIRATION	16	1.990	0.014
MONOVALENT_INORGANIC_CATION_ TRANSMEMBRANE_TRANSPORTER_ACTIVITY	24	1.981	0.014
ORGANELLE_INNER_MEMBRANE	56	1.956	0.019
FEEDING_BEHAVIOR	21	1.955	0.018
MITOCHONDRIAL_PART	104	1.896	0.040
INORGANIC_CATION_TRANSMEMBRANE_ TRANSPORTER_ACTIVITY	44	1.877	0.050
MITOCHONDRIAL_MEMBRANE	64	1.847	0.071
ENERGY_DERIVATION_BY_OXIDATION_OF_ ORGANIC_COMPOUNDS	31	1.823	0.088
MITOCHONDRIAL_ENVELOPE	73	1.797	0.108
CHEMOKINE_ACTIVITY	28	1.760	0.150
CHEMOKINE_RECEPTOR_BINDING	29	1.740	0.176
HYDROLASE_ACTIVITY_ACTING_ON_CARBON_NITROGEN_NOT_PEPTIDEBONDSIN_LINEAR_ AMIDES	17	1.692	0.246
LIGAND_DEPENDENT_NUCLEAR_RECEPTOR_ ACTIVITY	22	1.679	0.248
ATPASE_ACTIVITY_COUPLED_TO_ TRANSMEMBRANE_MOVEMENT_OF_IONS	21	1.660	0.247
Pyloric mucosa			
LYMPHOCYTE_ACTIVATION	49	-1.770	0.236
LIPID_ RAFT	24	-1.760	0.221

[1]Number of genes in the set

[2]Normalized enrichment score

[3]False discovery rate

Table 3. Genes that were more expressed ($P < 0.05$) in OXY mucosa, a priori selected for their relevance in the stomach, compared with PYL mucosa[1]

		Gastric mucosa[2]		
Gene product	Gene name	OXY	PYL	SEM
Acid secretion and pH homeostasis				
H^+/K^+ Atpase α	*ATP4A*	12.1	4.6	0.09
Anion exchanger 2	*SLC4A2*	10.0	7.9	0.12
Ion and water channels				
Potassium voltage-gated channel, Isk-related family, member 2	*KCNE2*	10.2	3.9	0.20
Potassium inwardly rectifying channel, subfamily J, member 13	*KCNJ13*	8.0	3.3	0.27
Potassium inwardly rectifying channel, subfamily J, member 15	*KCNJ15*	8.8	3.7	0.17
Chloride intracellular channel 6	*CLIC6*	10.7	4.0	0.14
Aquaporin 4	*AQP4*	9.5	3.0	0.26
Endocrine mediators, growth factors, receptors and binding proteins				
Insulin-like growth factor	*IGFBP5*	10.1	8.5	0.25

binding protein 5				
Ghrelin/Obestatin Prepropeptide	*GHRL*	9.9	7.8	0.38
Epidermal growth factor	*EGF*	9.4	5.1	0.41
Digestive enzymes, nutrient uptake and transport				
Pepsinogen B	*PGB*	11.9	10.4	0.13
Pepsinogen C	*PGC*	12.2	11.3	0.14
Chitinase, acidic	*CHIA*	12.2	7.8	0.19
Lipoprotein lipase	*LPL*	9.8	7.6	0.46
Solute carrier family 2 (facilitated glucose transporter), member 4	*SLC2A4*	8.7	6.4	0.36
Others				
Alcohol dehydrogenase, iron containing, 1	*ADHFE1*	6.9	5.6	0.32

[1]OXY = oxyntic mucosa; PYL = pyloric mucosa.

[2]Mean values, expressed as log2 of intensity values.

Table 4. Genes that were more expressed ($P < 0.05$) in PYL mucosa, a priori selected for their relevance in the stomach, compared with OXY mucosa[1]

		Gastric mucosa[2]		
Gene product	Gene name	OXY	PYL	SEM
Cell adhesion factors and tight junction regulation				
Olfactomedin 4	*OLFM4*	4.8	9.4	0.69
Claudin 7	*CLDN7*	5.8	9.1	0.33
Claudin 2	*CLDN2*	5.0	7.5	0.48
Epithelial protection, immunity and detoxifying enzymes				
Lysozyme	*LYZ*	10.3	11.7	0.22
Polymeric immunoglobulin receptor	*PIGR*	8.3	10.4	0.23
Cytochrome P450, family 3, subfamily A, polypeptide 4	*CYP3A46*	4.3	7.9	0.52
Secretoglobin, family 1A, member 1 (uteroglobin)	*SCGB1A1*	3.8	8.0	0.57
Transcription factors				
Meis homeobox 2	*MEIS2*	7.0	9.1	0.17
SRY (sex determining region Y)-box 21	*SOX21*	5.5	6.5	0.18
Leucine-rich repeat containing G protein-coupled receptor 5	*LGR5*	3.6	5.6	0.29
Endocrin mediators, growth				

factors, receptors and binding proteins				
Somatostatin	*SST*	9.4	11.4	0.24
Gastrin	*GAST*	5.5	10.6	0.30
Digestive enzymes, nutrient uptake and transport				
Gastric intrinsic factor	*GIF*	10.0	11.4	0.37
Others				
Aldo-keto reductase family 1, member C1	*AKR1C1*	4.3	8.0	0.48
Cysteine dioxygenase 1	*CDO1*	5.1	7.9	0.50
Adenylate Kinase 5	*AK5*	4.1	7.3	0.25

[1]OXY = oxyntic mucosa; PYL = pyloric mucosa.

[2]Mean values, expressed as log2 of intensity values.

Table 5. Genes that did not differ for expression in OXY and PYL mucosa, a priori selected for their relevance in the stomach[1]

		Gastric mucosa[2]		
Gene product	Gene name	OXY	PYL	SEM
Acid secretion and pH homeostasis				
ATPase, class V, type 10D	*ATP10D*	6.3	6.5	0.66
Carbonic anhydrase 2	*CA2*	11.8	11.6	0.11
Carbonic anhydrase 11	*CA11*	5.4	6.0	0.30
Epithelial protection and detoxifying enzymes				
Gastrokine 1	*GKN1*	11.8	11.9	0.44
PDI family A, 3	*PDIA3*	10.9	10.9	0.23
PDI family A, 4	*PDIA4*	9.8	9.8	0.16
Mucin 1	*MUC1*	10.8	10.7	0.745
Glutathione peroxidase 1	*GPX1*	9.1	9.3	0.45
Glutathione S-transferase alpha 4	*GSTA4*	8.8	8.2	0.34
Endocrin mediators, growth factors, receptors and binding proteins				
Fatty acid binding protein 5	*FABP5*	8.0	7.2	0.35
Digestive enzymes, nutrient uptake and transport				
Solute carrier family 5 (sodium iodide symporter), member 5.	*SLC5A5*	7.8	8.4	0.27

Pancreatic amylase	*AMY2*	5.7	5.1	0.33
Others				
Cysteine sulfinic acid decarboxylase.	*CSAD*	5.8	5.4	0.18

[1]OXY = oxyntic mucosa; PYL = pyloric mucosa.

[2]Mean values, expressed as log2 of intensity values.

Table 6. Validation of the microarray data by quantitative real-time PCR (qRT-PCR) analysis of four representative genes

	Real Time RT-PCR[1]			Microarray[1]		
Gene name[2]	OXY	PYL	SEM[3]	OXY	PYL	SEM[3]
ATP4A	120.6	0.03	12.4	33.7	0.2	2.3
GAST	0.2	316.2	65.6	0.3	15.9	2.6
GHRL	12.84	4.2	2.6	7.5	2.6	1.1
PIGR	2.0	18.6	3.9	2.6	12.6	1.7

[1]Values normalized for *hydroxymethylbilane synthase* and *ribosomal protein L4* gene expression.

[2]*ATP4A*, H+/K+ Atpase α; *GAST*, gastrin; *GHRL*, ghrelin/obestatin prepropeptide; *PIGR*, polymeric immunoglobulin receptor.

[3]*All gene values differed for the different mucosae inside each analysis method ($P < 0.05$).*

Chapter six

Effect of free thymol on differential gene expression in gastric mucosa of the young pig

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ABSTRACT

Abstract

Thymol is the most representative molecule of thyme and is proposed as an oral alternative to antibiotics in the feeding of pigs and broilers. Knowledge of in-vivo physiological effects of thymol on tissues is scarce, particularly for its impact on gastric mucosa, where it is largely absorbed when it is orally supplied. Thymol (TH, 50 mg per kg body weight) or a Placebo (CO) were inoculated in the stomach of eight weaned pigs, that were slaughtered after 12 hours and sampled for gastric oxyntic and pyloric mucosa. The analysis of whole transcript expression was done by Affymetrix©Porcine Gene 1.1ST array strips. Affymetrix Transcripts ID's were associated with 13,406 Human gene names, based on Sus scrofa Ensemble. Gene Set Enrichment Analysis was done, comparing TH and CO. For each gene set, normalized enrichment score (NES) was defined significant when False Discovery Rate % < 25 and P-values of NES<0.05. With TH, 72 and 19 gene sets were significantly enriched in oxyntic and pyloric mucosa, respectively. Several gene sets involved in mitosis and in its regulation ranked the top, primarily in the oxyntic mucosa; the gene set DIGESTION ranked first and ninth in pyloric and oxyntic mucosa. Inside this group, somatostatin (SST), SST receptors, peptide transporter 1 (SLC15A1), calpain 9 (calpain specific for gastrointestinal tract) were the most upgraded genes. Thymol reduced the enrichment in 120 and 59 gene sets for oxyntic and pyloric mucosa, respectively. Several gene sets related to ion transports and channeling, and aqueous pores across membranes, were less enriched. Among these, there were short transient receptor potential (TRP) channel 4, potassium voltage-gated channel members 1 & 2 and ryanodine receptors 2 & 3 . The down-regulation of these genes sensitive in vitro to thymol could depend on the dose of thymol and on the contact with gastric tissues that caused an adaptive response with their reduced activation. Conversely, the activation of TRPA1 gene (ranked 1072 and 128 among all the genes in oxyntic and pyloric mucosa, respectively) indicates the involvement of another TRP's regulating cellular calcium storage.

In conclusion, the stimulation of gastric proliferative activity and of the controls of digestive activity by thymol can influence positively gastric maturation and function of the post-weaning pig. These properties should be considered in addition to thymol antimicrobial properties when the option of supplementation of this molecule to the feed is evaluated.

Key words: gene expression, pig, stomach, thymol

I**mplications**

The study provides critical support for an effect of luminal thymol on oxyntic and gastric mucosa, inducing intense proliferative activity and expression of several genes involved in the control of digestive activity. In the present research thymol was given intra-gastrically, but these observed actions could be positive when thymol is provided in the feed to post-weaning pigs, helping them to rapidly stimulate the maturation and the functional activity of stomach. The results provide also evidence that thymol gastric detection affects genes involved in the control of cellular storage of calcium (and other ions).

Introduction

Thymol is a phenolic compound, the principal constituent of the extract oil of thyme and has antimicrobial activity. Thymol has been proposed in animal feeding to positively influence the gut microbiota and, consequently, health and growth performance (Lallès *et al.*, 2009; Khan *et al.*, 2012). However research evidence suggests that thymol rapidly gets to the blood after its oral supply (Kohlert *et al.*, 2002) and that in pig it is absorbed in the stomach (Michiels *et al.*, 2008). Thus, after mouth, gastric mucosa could be an important site of action of thymol added to the feed. Toxicological effects of thymol were studied and in general thymol is considered safe (U.S. Environmental Protection Agency, 2009). Nevertheless, when considering oral supplementation of a molecule, potential luminal effect should be considered. Thymol has an intense odour and taste, and this is a sign that it can interact with the mucosae, having possible local stimulatory effects. In Ussing chamber system, anion secretion was detected in human and rat colonic epithelia after luminal thymol applications (Kaji *et al.*, 2011); this may be mediated by the sensing of thymol by olfactory receptors or by transient receptor potential (TRP) channels (Kaji *et al.*, 2011).

Stomach has several important functions that are mainly played in the oxyntic mucosa (hydrochloric acid secretion and pH control, endocrine secretion, intrinsic factor for vitamin B12 absorption, production of growth factors, Goebel *et al.*, 2011). However other important activities are emerging and some of these seem to involve other functional districts of porcine stomach, like the pyloric mucosa, including taste sensing (Colombo *et al.*, 2012), translation of chemosensory signals (Mazzoni *et al.*, 2013) and activation of mucosa-associated lymphoid tissue (Mazzoni *et al.*, 2011).

Thus we aimed at assessing the effect of intragastric thymol on the differential gene expression in the oxyntic and pyloric mucosa of the young pig.

MATERIALS AND METHODS

Material and Methods

Animals and Treatment

The procedures carried out on the pigs were conducted in compliance with Italian laws on experimental animals and were approved by the Ethic-Scientific Committee for Experiments on Animals of the University of Bologna. Eight crossbred (Large White x Landrace) male weaned pigs (six weeks of age, averagely 11.3 kg body weight - BW), previously individually housed in cages and fed *ad libitum* a post-weaning standard diet, were randomly assigned to the Thymol (TH) treatment or to the Control (CO) (Four pigs per treatment). Pigs were healthy and were not littermate.

Natural identical free Thymol was a kind gift of A.W.P. s.r.l. (Reggio Emilia, Italy).

After the morning meal (restricted to 5 g per kg BW, to allow a full rapid intake), pigs received 50 mg per kg BWt of thymol or a Placebo, 5ml of a sterile physiological saline solution, directly in the stomach through intragastric gavage by medical catheter. We adopted this protocol to standardize the quantity for each pig and avoid the risk that part of thymol was not consumed, due to its pungent taste. A single dose of thymol was used to evidence acute effects of thymol on the stomach.

Sample collection

Pigs were slaughtered after 12 h from the thymol administration by an intracardiac injection (Tanax, 0.5 mL per kg BW; Intervet Italia, Peschiera Borromeo, Italy), after being anaesthetized with sodium thiopental (10 mg per kg BW). For each subject, the stomach was removed, opened along the greater curvature and washed in ice-cold PBS. Tissue samples from the oxyntic gland area close to the greater curvature and from pyloric mucosa were collected and immediately frozen in liquid nitrogen and stored at -80°C until use.

RNA Isolation, Microarray Processing, Quality Control

Total mRNA was isolated from oxyntic and pyloric mucosa collected from each subject, according to Qiagen RNeasy® Midi Kit protocol (Qiagen, Hilden, Germany). Purity and integrity evaluation was assessed just before analysis by Agilent Bioanalyzer 2100. Total mRNA was hybridised on Affymetrix©Porcine Gene 1.1 ST array strips. Hybridized arrays were scanned on a GeneAtlas imaging station (Affymetrix, Santa Clara, CA, USA). Performance quality tests of the arrays including the labelling, hybridization, scanning and background signals by a Robust Multichip Analysis were performed on the CEL files using Affymetrix Expression Console™.

Pathway Analysis

Affymetrix Trascripts ID's, in general characterized each one by several exonic sequences, were associated to 13,406 Human gene names, based on Sus scrofa Ensembl database (release 69, www.ensembl.org). On processed gene expression values, exploratory functional analysis was done with Gene Set Enrichment Analysis using the C5.BP catalog of gene sets (based on Gene Ontology) from Molecular Signatures Database v3.1(http://www.broadinstitute.org/gsea/msigdb/Index.jsp), comparing TH with CO. Normalized enrichment score (NES) was calculated for each gene set, and statistical significance was defined when False Discovery Rate % < 25 and p-values of NES < 0.05. Enrichment score p values were estimated using a gene set-based permutation test procedure.

Results

With TH, 72 and 19 gene sets were significantly enriched in oxyntic and pyloric mucosa, respectively (Table 1). In the oxyntic mucosa, several gene sets involved in mitosis and in its regulation ranked the top. The first gene set not involved in the regulation of cell cycle was DIGESTION. Pancreatic lipase-related protein 2 (PNLIPRP2 - galactolipase) ranked first in the whole list of genes by TH.

In pyloric mucosa, the gene set DIGESTION was the most enriched, and then gene sets related to serine hydrolase activity and cellular turnover were significantly enriched by TH. Aldo-keto reductase family 1, member C-like 1 (AKR1CL1) ranked first in the whole list of genes upgraded by TH.

The ranking of up-regulated genes inside DIGESTION gene set by TH in oxyntic and pylori mucosae is summarized in Figure 1. Peptide transporter 1 (SLC15A1), somatostatin (SST) (only in PYL), gastricsin, also known as pepsinogen C (PGC) (only in PYL), pancreatic polypeptide (PPY), somatostatin receptors (SSTR1, SSTR2), calpain 9 (a calpain specific for gastrointestinal tract) were the most up-regulated genes.

Thymol reduced the enrichment in 120 and 59 gene sets, for oxyntic and pyloric mucosa respectively (Table 2). In oxyntic mucosa, several gene sets related to ion transports channels and aqueous pores across membranes, were less enriched. Inside CATION_CHANNEL_ACTIVITY and GATED_CHANNEL_ACTIVITY gene sets, the first down-regulated genes were short transient receptor potential channel (TRPC) 4 and 5, ryanodine receptors (RYR) 2 and 3 and some calcium channel, voltage-dependent (CACN) genes. Other

core enriched genes in these sets in CO pigs were potassium voltage-gated channel members (KCNA) 1 and 2 and some potassium large conductance calcium-activated channel (KCN) genes. SYNAPTIC_TRASMISSION set ranked 1st among enriched genes in pyloric mucosa of CO pigs. On the whole gene dataset 5-hydroxytryptamine receptor 2A (HTR2A) ranked first in pylorus mucosa of CO pigs, as compared with TH. However the comparative expression observed for this gene was low (roughly lower than 75% of the whole gene dataset) (data not in table).

Discussion

Here, we identify for the first time the molecular signaling pathways regulated by the direct contact of thymol on the oxyntic and pyloric mucosa of the young pig. Firstly, we give strong evidence that thymol activates mRNA for mitosis and for its regulation. No direct evidence was seen in the inspected literature about this effect. However an indirect confirmation can be found in the in vitro ability of thymol of reducing gamma radiation-induced apoptosis in V79 cells (Archana *et al.*, 2011), primarily by modulation of intracellular antioxidant levels and free radical scavenging.

Likely, carvacrol, another monoterpenic phenol structurally similar to thymol, stimulated mitosis and liver tissue regeneration after partial hepatectomy (Uyanoglu *et al.*, 2008).

The differential effect of genes involved in digestive function of the stomach induced by thymol has never been shown. Among the genes included in the DIGESTION set SLC15A1 was the most upgraded gene. This gene transcribes for a little selective oligopeptide transporter that is widespread in the gut and that is stimulated by fasting, drugs, hormones and circadian rhythm (Shimakura *et al.*, 2006a). We inspected the possible parallel activation of peroxisome proliferator-activated receptor α and caudal type homeobox 2, two transcription factor that were found to specifically act on SLC15A1 gene expression (Shimakura *et al.*, 2006a and 2006b), but they were not differentially expressed.

Also pathogenic bacteria (Nguyen *et al.*, 2009) and lipopolysaccharide (Shu *et al.*, 2002), the major component of the outer layer of Gram-negative bacteria, can affect expression of SLC15A1, that can also transport bacterial peptides. Our trial was not designed to assess the impact of thymol on gastro-intestinal microbiota, its antibacterial properties are well known, but we cannot speculate here on possible variations on the gastric bacteria profile. Nevertheless it is worth mentioning that the SLC15a1 overexpression after infection of mice with *Citrobacter rodentium* reduced colonic colonization by this microbe (Nguyen *et al.*, 2009). Intriguingly, in a previous in vivo trial we found that *Citrobacter freundii*, a microbe closely related to *Citrobacter rodentium*, was increased in the intestinal content of pigs fed thymol, as compared with control

(Jankzyk *et al.*, 2008). Finally, the activation of SLC15A1 could be relevant for the pig ingestive behaviour because this can elicit release of cholecystokinin from enteroendocrine cells and inhibit gastric motility, via vagal afferent activation (Darcel *et al.*, 2005).

Another gene overexpressed in pigs inoculated with thymol was gastric lipase (PNLIPRP2), that in human can hydrolyse up to 17.5% of dietary triglycerides (Carrière *et al.*, 1993). The preduodenal lipase have been identified also in pigs (Bauer *et al.*, 2005). PNLIPRP2 is important for newborn and milk-fed mammals (Andersson *et al.*, 2011). Furthermore, recently, inhibitory action of carvacrol against murin lipase was observed (Yamada *et al.*, 2010). Thus it can be hypothesize that thymol could have the same action and could increase mRNA production for PNLIPRP2.

Other genes were overexpressed only in pylorus with thymol treatment: SST and CAPN9; both are activated by peroxisome proliferator-activated receptor γ, but this gene was not differentially expressed in pylorus with thymol treatment. Interestingly it was shown that both SST and CAPN9 were down-regulated in human gastric cancer in two different populations (Junnila *et al.*, 2010).

Thymol has pungent odour and induces a thermal sensation (warm) in the contact with tongue. The diffuse presence of different sensors related to taste, olfactory and thermal sensation along the digestive tract makes reasonable the activation of some of these by thymol. Research evidence show that several different mechanism can be involved, but in general they are related to sensory detection and/or implicate cation regulation by direct or indirect mechanism. Ion fluxes along the cell membrane are gated by pore-forming proteins (channels). Among these, receptors belonging to Cation channels of TRP family act also as cellular sensor; in cell cultures, thymol activates the thermal/irritant-responsive TRPA1 (Lee *et al.*, 2009; Kaji *et al.*, 2011), the warm-sensing TRPV3 (Lee *et al.*, 2006) and the cold-sensing TRPM8 (Ortar *et al.*, 2012). Activation of TRPA1 by luminal stimuli induces anion secretion in colon enterocytes (Kaji *et al.,* 2012*)* and thymol induced also anion secretion from porcine jejunal epithelium in Ussing chambers (Bourdy and Perrier, 2008). Although TRPA1 was not included in the gene sets related to channel activity that we tested for enrichment analysis, it ranked 1072 and 128 position in the list of genes upgraded after thymol inoculation, in oxyntic and pyloric mucosa respectively. Thus our data confirm for TRPA1 mRNA in porcine stomach the effect of thymol on TRPA1 previously seen in human or rat colon or kidney cell cultures. Conversely TRPV3 and TRPM8 gene expression was not affected by thymol in our trial, contrary to what was seen for other species and tissues, presumably because in pig stomach its value was in general very low.

It was shown that thymol triggers the olfactory receptors OR73 and OR1G1 in colon epithelial cells (Kaji *et al.*, 2011) but we did not find any of these receptors in the array of porcine genes

orthologous to human, thus we cannot make any conclusion possible effect of thymol on olfactory receptors. The thymol-induced downregulation of several genes related to the cation channeling activity, could be related to a physiological desensitization of the tissues in response to thymol. Particularly, this could be for the SYNAPTIC_TRANSMISSION gene dataset in both oxyntic and pyloric mucosae. Our observation seems partially conflicting with previous physiological evidences showing an effect of thymol in calcium release from sarcoplasmic reticulum (SR) vesicles isolated from pig skeletal muscle by the channel protein RYR2 (Sárközi *et al.*, 2007) However, our results could depend on the dose of thymol and on the contact with gastric tissues that give to rise an adaptive response reducing activation of genes sensitive to thymol. In fact a dose-dependent desensitization of thymol on some TRP channels was already seen (6.25 to 25 μM thymol, Lee *et al.*, 2008; 50 to 100 μM thymol, Ortar *et al.*, 2012). The desentization can also explain the practical observation that weaned pigs fed for several days a high dose of thymol in the feed (1% of the diet, Trevisi *et al.*, 2007), after an initial reduction of feed intake, progressively were able to eat like control pigs..

TRPC4, which here was the first down-regulated gene by thymol, encodes a non-selective cation channel mediating Ca^{2+} entry to maintain intracellular Ca^{2+} stores (Birnbaumer, 2009) but a direct effect of thymol on TRPC4 is not documented yet. TPRC4 is normally activated by a pathway involving a G-protein coupled membrane receptor (GCPR),with the following stimulation of phospholipase C and diacylglycerol (Birnbaumer, 2009). Among GCPR's, there is 5HTR2a, another gene here down-regulated by thymol. Previous studies showed that the stomach has the potential ability to chemical sensing by several taste receptors including those involved in bitter taste (Colombo *et al.*, 2012) or by olfactory sensing cells. It can thus be hypothesized that the sensing of thymol, presumably in the stomach by bitter taste or olfactory sensing, could stimulate enterochromaffin cells to produce serotonin (5HT). Indeed the tryptophan hydroxylase (TPH) enzyme gene, involved in 5HT production, ranked 341 and 7 in the list of genes upgraded by thymol, in oxyntic and pyloric mucosae respectively. This could have in turn down-regulated 5HTR2A and genes regulated by this receptor, including several gated channels. It is possible also that under the activation of the irritant TRPA1 (and may be other TRP's) in the stomach of our pigs, the increased release of calcium elicited a compensative response in other calcium store operating TRP's, including particularly TRPC4. This in practice can explain the differentially higher gene expression of TRPC4, RYR2 and other calcium channels in control pigs.

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Table 1 *First gene sets enriched in oxyntic (OXY) and pyloric (PYL) mucosa with Thymol compared to Control*

Name	Size	FDR[1] q-value	Rank in OXY	Rank in PYL
In OXY				
SPINDLE	30	0.000	1	32
M_PHASE_OF_MITOTIC_CELL_CYCLE[2]	70	0.000	2	8
REGULATION_OF_MITOSIS	34	0.001	7	13
DIGESTION	26	0.001	9	1
CHROMOSOME[3]	95	0.001	10	149
CENTROSOME	39	0.001	11	184
APOPTOTIC_PROGRAM[4]	49	0.007	15	5
KINETOCHORE	18	0.016	18	-
MICROTUBULE_CYTOSKELETON	110	0.027	20	-
NUCLEAR_ENVELOPE_ENDOPLASMIC_ RETICULUM_NETWORK	75	0.027	21	55
ENDOPLASMIC_RETICULUM_MEMBRANE	68	0.030	22	53
STRUCTURAL_CONSTITUENT_OF_RIBOSOME	56	0.044	25	21
Other first gene sets in PYL:				
HYDROLASE_ACTIVITY_HYDROLYZING_O_GLYCOSYL_COMPOUNDS[5]	27	0.049	103	4
COENZYME_BINDING	16	0.108	166	15

[1] False discovery rate

[2]MITOSIS, MITOTIC_CELL_CYCLE, M_PHASE, CELL_CYCLE_PROCESS, CELL_CYCLE_CHECKPOINT, CELL_CYCLE_PHASE and MITOTIC_CELL_CYCLE_CHECKPOINT were also significantly enriched.

[3]CHROMOSOMEPERICENTRIC_REGION, CHROMOSOME_SEGREGATION and CHROMOSOMAL_PART were also significantly enriched.

[4]CELLULAR_COMPONENT_DISASSEMBLY and CELL_STRUCTURE_DISASSEMBLY_DURING_APOPTOSIS were also significantly enriched.

[5]SERINE_HYDROLASE_ACTIVITY, SERINE_TYPE_ENDOPEPTIDASE_ACTIVITY and SERINE_TYPE_PEPTIDASE_ ACTIVITY were also significantly enriched.

Table 2 *First gene sets enriched in oxyntic (OXY) and pyloric (PYL) mucosa with Control compared to Thymol*

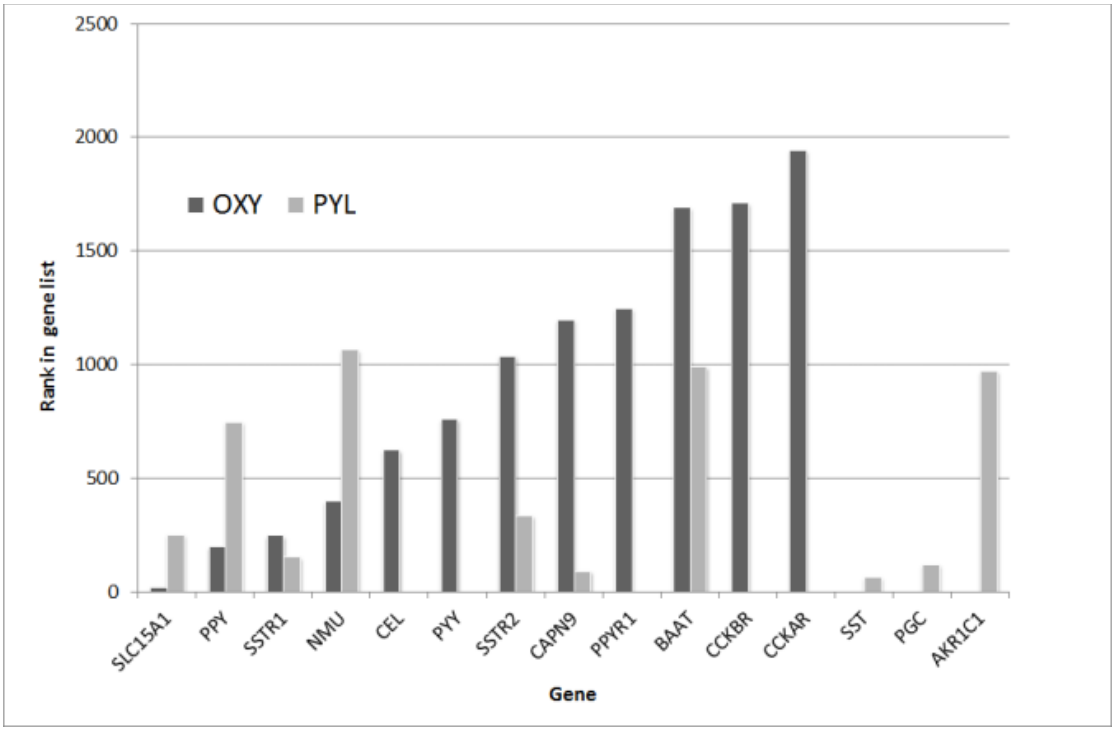
Name	Size	FDR[1] q-value	Rank in OXY	Rank in PYL
In OXY:				
CATION_CHANNEL_ACTIVITY[2]	88	0.000	1	9
GATED_CHANNEL_ACTIVITY[2]	94	0.000	2	12
EXTRACELLULAR_MATRIX[3]	74	0.000	3	4
CALCIUM_CHANNEL_ACTIVITY[2]	25	0.000	4	11
METAL_ION_TRANSMEMBRANE_TRANSPORTER_ ACTIVITY[2]	109	0.000	6	10
SYNAPTIC_TRANSMISSION	128	0.000	7	1
VOLTAGE_GATED_CATION_CHANNEL_ACTIVITY[2]	51	0.000	11	16
TRANSMISSION_OF_NERVE_IMPULSE	141	0.000	12	5
ADHERENS_JUNCTION	16	0.002	21	69
STRUCTURAL_CONSTITUENT_OF_MUSCLE	27	0.002	23	-
CELL_MATRIX_ADHESION	32	0.004	24	34
Other first gene sets in PYL:				
BASEMENT_MEMBRANE	28	0.001	15	6
COLLAGEN	17	0.003	31	7
REGULATION_OF_NEUROTRANSMITTER_LEVELS	21	0.003	43	8
NEUROLOGICAL_SYSTEM_PROCESS	274	0.017	34	13
SYSTEM_PROCESS	416	0.033	27	14
GROWTH_FACTOR_ACTIVITY	44	0.042	76	17
CELL_MIGRATION	77	0.072	29	21
GLUTAMATE_RECEPTOR_ACTIVITY	15	0.097	-	25

[1] False discovery rate

[2]METAL_ION_TRANSPORT, CALCIUM_ION_TRANSPORT, VOLTAGE_GATED_ CHANNEL_ACTIVITY, CATION_TRANSPORT, ION_CHANNEL_ACTIVITY, DI_TRI_VALENT_INORGANIC_CATION_TRANSPORT, LIGAND_GATED _CHANNEL_ACTIVITY, CATION_ TRANSMEMBRANE_TRANSPORTER_ ACTIVITY and ION_TRANSPORT were also significantly enriched.

[3]PROTEINACEOUS_EXTRACELLULAR_MATRIX, EXTRACELLULAR_MATRIX_PART, BASEMENT_MEMBRANE and EXTRACELLULAR_STRUCTURE_ORGANIZATION_ AND_BIOGENESIS were also significantly enriched.

Figure 1 Rank of core enriched genes inside the gene set DIGESTION and on the whole gene set, in oxyntic (OXY) and pyloric (PYL) mucosa of pigs intragastrically inoculated with thymol, compared with control.



CONCLUSIONS

In this thesis two approaches were applied to achieve a double general objective. The two investigation on the piglet chemosensory system along the gastrointestinal tract, on the one hand, identify for the first time the presence of the bitter and fat receptor and on the other hand, show that age, pathogen challenge and dietary manipulations affecti the gut luminal micro-environment. Moreover the intestinal microbiota modulate gene expression in OR51E1 gastrointestinal tissues, presumably in association with the release of microbial metabolites. This results represent the first step for understanding the relationship between luminal contents of the intestine and pig.

The expression study on the stomach shows an high expression, in oxyntic and pyloric mucosae, of genes other than those known for their function related to hydrochloric acid and gastrin secretion. In general, data indicate that the oxyntic mucosa has a higher specialization than the pyloric, that conversely was useful for the detection of new markers. The pylorus expressed some gene transcripts that may merit additional studies, particularly those related to mucosal defense function. In addition, the research suggests that several genes are shared between oxyntic and pyloric. The stimulation of gastric proliferative activity and of the controls of digestive activity by thymol can influence positively gastric maturation and function of the post-weaning pig. These properties should be considered in addition to thymol antimicrobial properties when the option of supplementation of this molecule to the feed is evaluated. These new observations should be addressed in further studies considering the different compartments of the stomach separately, as is usually the case for the intestine, to reveal novel functions.