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**Dietary interventions to improve healthy status  
in pig intensive farm conditions: a morpho-  
functional study of target organs.**

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# CHAPTER 1

## Foreword





# 1. Foreword

Keeping farm animals in healthy condition appears now necessary for both humanitarian reasons and to obtain healthy animal products. In addition healthy problems often result in bad growth performances and as a consequence in severe economic losses. Morbidity rates are often based upon (or worsened by) the presence of stressors in rearing farms. Major problems in piggeries can be ascribed to stress factors during two periods well distinguished in the life of pig: acute stress in weaning phase and chronic stress factors related to intensive rearing, such as those ones environmental (space, crowding, temperature), and hierarchical.

Weaning is generally precociously (21-28d) applied in the pig rearing farms. It is a complex step involving dietary, environmental, social and psychological stresses, which deeply interfere with feed intake, gut development and adaptation to the weaning diet (reviews by Pluske et al, 1997; Lallès et al. 2004). Transient post-weaning (PW) anorexia leads to under-nutrition and growth check. Feed intake resumption after weaning is highly variable among individuals and it takes up to 2 weeks for piglets to recover pre-weaning levels of energy intake (review by Le Dividich and Sève, 2000). The first organ suffering from nutrient shortage in concomitance with the PW phase is the alimentary canal that partly modifies its anatomy and functions, including barrier function efficiency against harmful antigens and pathogens. For this reason any intervention aimed at improving gut trophism appears welcome. Another organ that may suffer in this phase is the liver, which is involved in the alimentary canal metabolism and receives from it endotoxins and catabolites. The liver in addition serves as the key organ for the removal and detoxification of bacterial toxins that are continuously absorbed in small amounts from the gastrointestinal tract when its barrier function is altered in a critical phase like weaning: this in turn can lead to a general oxidative stress status, which may finally damage tissues and cells of the organism.

Moreover, in the growing-finishing phase the chronic stress related to the intensive rearing conditions may lead to the occurrence of pathological problems and consequently to an economic loss. Because 80% of the total feed cost of pig production is associated with the growing/fattening phase, control of porcine health and welfare status during this period can lead to improved feed efficiency and generate significant cost savings, thereby increasing profits (Duhamel, 1996). Stressors existing in swine production systems during growing-finishing phase would include cold/hot environmental temperatures (Stahly and Cromwell, 1979), microbial infections (Webel et al., 1997), insufficient space allowances (Brumm and Miller, 1996; Wolter et al., 2000), social mixing (Barnett et al., 1993; Marchant et al., 1995) and nutritional deficiencies or imbalances (NRC, 1998). These stressors cause growth retardation, changes in hormone release, increases

in disease susceptibility, and/or behavioral changes, but primarily afflict the gut and produce dramatic consequences on the performance of pig, that reduces feed intake, and as a consequence reduces body weight (Breinekova et al., 2006) with an increase of costs for the owner.

For all these reasons diets appropriately formulated to meet target nutrient requirements are necessary to overcome with success the above mentioned critical phases.

## **1.1 Acute stress: weaning**

### *1.1.1 Early development, nutrition and the gut of pig*

In the lifespan of the pig, the neonatal and weaning phases represent critical periods of growth and development. Preweaning mortality is influenced by individual birth weight, litter size, duration of farrowing, dystocia, birth order, thermal environment, nutritional status, disease, sow and piglet behavior, sex, and genetics (Lay et al., 2002). At weaning, the young pig that survived the neonatal period, is subjected to a myriad of stressors (e.g. change in nutrition, separation from mother and littermates, new environment), which very often cause reduced growth. The preweaning mortality rates and post-weaning 'growth check' continue to represent a major source of production loss in many commercial piggeries (Pluske et al., 1997). During neonatal and weaning phases, the survival, health and consequent growth rates of young piglets depend on their ability to physiologically adapt to significant changes in nutrition and environment (Pluske et al., 1997; Lay et al., 2002). Of particular importance to this adaptation process is the correct morpho-functional development of the gastrointestinal tract, with the assessment of its digestive, absorptive and immune functions. The morpho-functional post-natal development of the gastrointestinal (GI) tract is a complex of dramatic processes: first, the gastrointestinal tract must be able to support a shift from parenteral nutrition in the foetus to enteral nutrition in the neonate, but the interactions among diet, intestinal growth and digestive functions are critical during the entire perinatal phase. A critical determinant in the functional development and growth of the gastrointestinal tract is nutrition. Nutrition plays a key role in the modulation of the evolving gut influencing all the main components of the intestinal ecosystem. Nutrients like glycoproteins, nucleotides and lipids operate as substrates for oxidative energy and as precursors of constitutive and secretive proteins. Indirectly they stimulate the production of endocrine as well as paracrine hormones, growth factors and a variety of metabolites that affect gastrointestinal physiology, either positively or negatively. In addition, nutrients are important

for the presence and the increase of intestinal beneficial microbiota that, at the end of their life cycle, can be an additional source of simple molecules usable for the functional assessment of the GI tract. In the neonatal and weaning periods, nutrition and the gastrointestinal tract morpho-functional assessment play crucial roles in the overall growth and survival of pigs. Providing the appropriate amount and composition of dietary nutrients is necessary to maintain a correct gastrointestinal function and development. The appropriately weaned pig provides the potential for fast, lean, and efficient growth after weaning. Really, the weaning phase continues to have a large influence on the performance of pigs at the finishing stage, because they enter the finishing barn at a higher weight, and consequently continue to grow faster and more efficiently. So, the results in terms of good health and positive behavior in the weaning phase have positive repercussions in growing-finishing period, crucial for pig rearing too. Death loss among growing-finishing pigs can have an impact on the profitability of the entire swine rearing (Holden 1991). Enteric problems in the finishing period can result in poorer performance in average daily gain, feed conversion and weight variations in the group, with important economic effects.

### *1.1.2 Gut growth and adaptation*

The gastrointestinal tract of the piglet is approximately 2% of body weight at birth and increases nearly three folds to more than 6% two weeks after weaning. Most of the growth occurs during the first 6 h of suckling and is caused by a rapid increase in the mucosal components of all three regions of the small intestine. In newborn piglets, sow's colostrum and subsequently milk operate a trophic effect on the gut through the numerous growth factors contained in them. The onset of suckling causes marked increases of intestinal dimension (Widdowson et al., 1976) and structure (Xu et al., 1992). This is evident from the increase in the intestinal wall thickness, which results in a 100% increase in absorptive area at the microscopic level (Burrin et al., 1994; Xu et al., 1992) and greatly exceeds the 27% increase in nominal surface area (Zhang et al., 1997). These dimensional aspects are accompanied by an increased synthesis of functional proteins linked to brush border membrane of enterocytes (Zhang et al., 1998). These integrated processes of intestinal growth, development and functional maturation continue until the weaning period that is generally precociously (21-28 days of age) applied when pigs are reared, and constitutes for them a dramatically critical period. This generalized precocious weaning is accompanied by important modifications of the small intestinal structure (Lallés et al., 2004). Despite the considerable amount of research that has been conducted in this field, many questions still remain to be clarified, as the precise aetiology of these changes in gut structure and function immediately after

weaning, during which several key nutritional and environmental factors are described (see in Table 1) that contribute to significant changes in structure and function of the gastrointestinal tract (Pluske et al., 1997; Dréau and Lallès 1999; Burrin et al., 2003; Lalles et al., 2004). During weaning these factors act simultaneously with each other to produce temporal changes in gastrointestinal structure and function.

**Table 1.** (modified from Lallès et al., 2004)

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**Context: weaning = immaturity + stress**

- immature animal for
  - behaviour (general and feeding)
  - gut functions (secretions, motility, digestion, absorption, defence, etc.)
  - immune system (intestinal and general)
- psychological stress
  - abrupt separation from the mother
  - mixing with pigs from other litters
  - new environment (room, building, farm, etc.)
- dietary stress
  - withdrawal of milk (liquid, highly palatable and digestible, etc.)
  - access to dry feed (solid, less palatable and digestible, etc)
  - separate access to drinking water

**Induced small intestinal disorders**

- alterations in intestinal architecture and function
  - morphology: villus atrophy followed by crypt hyperplasia
  - reduced activities of intestinal digestive enzymes
  - disturbed intestinal absorption, secretion and permeability
- feed-associated enteric pathogens
  - bacteria (*Escherichia coli*, enterotoxigenic or enteropathogenic)
  - viruses: rotavirus

**Main risk factors**

- dietary factors
  - low or erratic feed intake
  - presence of antinutritional factors (antitryptic factors, lectins, antigens, etc.)
  - diets with high component complexity and low digestibility (protein, complex carbohydrates)
  - high level of protein (+ high buffering capacity)
- rearing factors
  - large litter size / low weaning weight

- high density of post-weaning piglets
  - low level of hygiene
  - inadapted environment (low temperature, low air quality, etc.)
- 

Weaning involves complex psychological, social, environmental and dietary stresses that interfere with gut development and adaptation. First of all, during weaning, the switch from liquid (mother's milk) to solid food takes place. In the intensive production the switch is abrupt, without any or with an inadequate period of adaptation. Weaning of farmed pigs occurs early, when the GI motor, digestive and absorptive functions are not yet fully assessed for food other than milk. In intensive livestock production short suckling periods have beneficial consequences for the farmer, as the increased number of piglets born per year, but also display detrimental aspects for the animals, such as a generally observed increased number of gastrointestinal disorders in weaning piglets. When pigs are weaned, they suffer of many stressors, including the sudden separation from their mother, mixing with unfamiliar piglets, and changing from a liquid diet (highly-digestible milk) to a new solid feed. The temporal changes induced by a precocious weaning on the gastrointestinal structure and function can be divided into the acute and adaptive phases. The acute phase is in a short time period, from day 1 to day 5 after weaning, and is followed by a more progressive adaptation phase, from day 5 to day 15 (Montagne et al., 2007). The distinction between acute and adaptation phases is based primarily on the changes in feed intake, since it takes about seven days for weaning pigs to learn how to eat out of a feeder and resume an intake that is comparable to that during the pre-weaning period (Pluske et al., 1997). However the changes in feed intake that lead to the distinction between acute and adaptation phases may have their causes in parallel changes in the small intestine aspect, too.

The mucosal epithelium of the small intestine is regarded as anatomically and functionally immature in neonatal pigs (Gaskins and Kelly, 1995), a feature that is exacerbated at weaning where there are the above described dramatic changes in gut anatomy and function. The early phase consists of an acute deterioration of the gut structure and function. Immediately post-weaning days constitute the "acute" phase of weaning, during which the feed (and water) intake is lower or more variable than in pre-weaning period, and this seems to be the main reason for the observed growth stasis immediately after weaning (Pluske et al., 1997). Moreover, a decrease of overall mass and mucosal architecture of the small intestine with a consequent decreased activity of enzymes (i.e. peptidase) can occur (Pluske et al., 1997; Lallès et al., 2004). There is a decrease in the villous length and in the total brush border enzymes activities and an increase in the paracellular permeability. Such changes leading to a depressed digestive capacity of the gut have been clearly identified as the main factors predisposing for post-

weaning digestive disorders in piglets (Hampson, 1994). The effect of fasting or a low feed intake is most likely multifactorial. It includes a deprivation of luminal substrates for mucosal epithelial cell growth, and a reduced secretion and expression of gut growth factors such as glucagon-like peptide 2, insulin-like growth factor I (Burrin et al., 2000) and CCK (Montagne et al., 2007).

The late post-weaning phase, from day 5 to day 15, corresponds to an adaptation of the gut to the weaning diet. The main factors of this adaptation are the re-feeding and the time (Montagne et al., 2007). The adaptation phase is marked by a partial regeneration of the structural characteristics of the proximal intestine. The re-feeding and consequently the arrival of nutrients in the intestinal lumen is the driving factor for the structural development of the mucosa. However the activities of the enzymes of the proximal jejunal mucosa are not fully restored until day 15, although they are significantly correlated with the villus length (McCracken et al., 1995). The adaptation period corresponds to a phase of development and maturation of the distal part of the small intestine, and variables measuring the functions of the distal ileal mucosa and colonic bacteria might be considered as markers of maturation of the gut. The adaptive phase of weaning is also characterized by a substantial increase in digestive enzyme secretion, particularly those ones referred to gastric and pancreatic proteases and alpha-amylase activities.

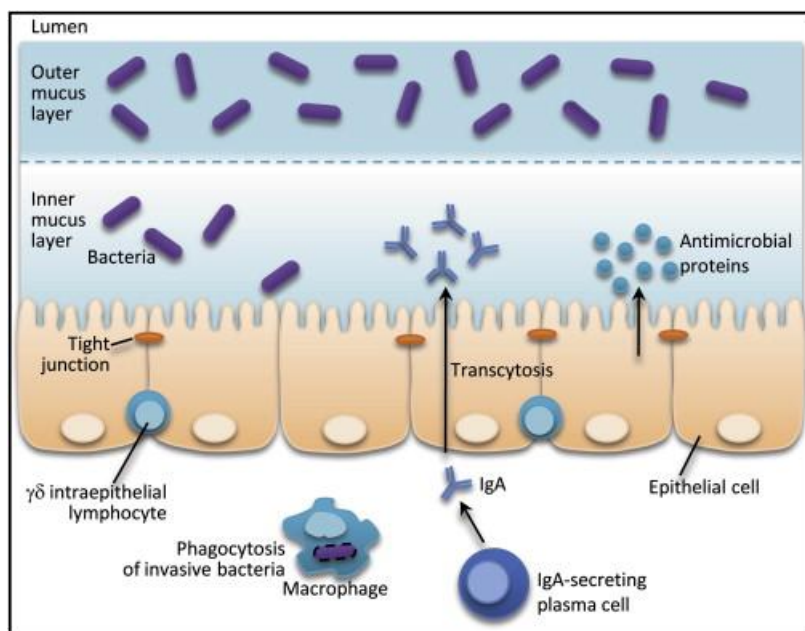
### *1.1.3 Anatomy and physiology of the intestinal barrier*

The intestine serves as an interface between the external and the internal environments of the pig and its main functions are: digestion and absorption of nutrients, absorption and secretion of electrolytes, secretion of mucins and immunoglobulins, and protection against pathogenic microorganisms, toxins and potentially allergenic macromolecules. The gastrointestinal mucosa contains a highly specialized defensive system, which is exposed to a wide variety of antigens derived from foods, resident bacteria and invading micro-organism. These need to be limited by a barrier that allows absorption of nutrients but also provides immune defence from harmful antigens.

#### *The mucous biofilm*

The mucous gel layer (Figure 1) is an integrated structural component of the gastro-intestinal mucosal surface, that covers the epithelium of the gastrointestinal tract, and protects the mucosa from the mechanical damage possibly exerted by hard dietary constituents and from chemical, enzymatic, physical and bacterial aggressors, lubricates the mucosal epithelium, and regulates transport of ions and small molecules between luminal contents and the epithelial lining.

It is composed by an outer and an inner layer, whose composition and physico-chemical properties are in part different (Matsuo et al., 1997, Atuma et al., 2001).



**Figure 1:** The mucous gel layer

Gastro-intestinal mucus exerts several functions linked to the local and general defence (Table 2).

**Table 2.** Functional Properties of Digestive Mucins (modified from Montagne et al., 2004)

---

**Roles in relation with gut physiology**

- Lubrication of the gut epithelium
- Protection of the epithelium against acidic environment (stomach and duodenum)
- Protection against endogenous and bacterial proteases
- Selective diffusion barrier permeable to nutrients (small molecules)

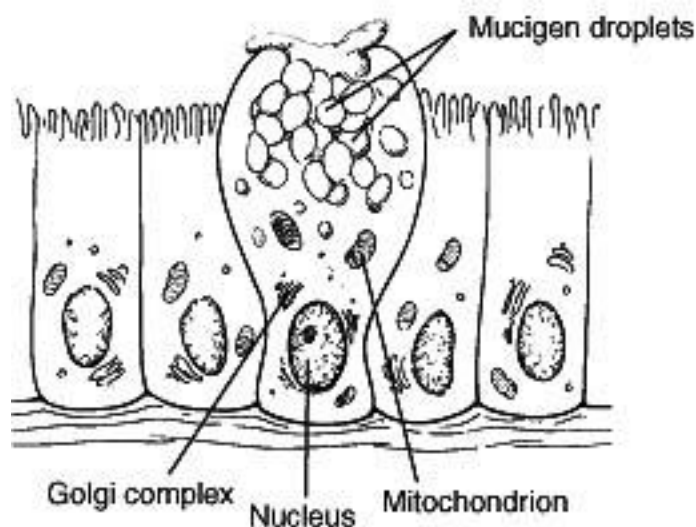
**Roles in relation with gut health**

- Fixation of commensal bacteria permitting colonization resistance
  - Fixation of pathogen bacteria, viruses, and parasites
  - Component of the gut-associated lymphoid tissue (GALT)
  - Epithelium repairation (synergic action with trefoil peptides)
  - Substrate for bacterial fermentation
-

The mucous gel layer is intimately adherent to the epithelial lining of the gastrointestinal mucosa, so that it is usually identified with the term “adherent mucous gel layer”. It is a heterogeneous mixture made up of approximately 95% of water and containing electrolytes ( $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Mg}^{2+}$ , and  $\text{Ca}^{2+}$ ), carbohydrates, proteins, glycoproteins, peptides, amino acids, and lipids (Verdugo, 1990). The active viscoelastic property of mucus derives from filamentous glycoproteins called mucins or glycoconjugates secreted by goblet cells. Mucins are also structural, because are membrane-associated. Mucins are polypeptide structures with branching carbohydrates side-chains (hexosamines), often having either sialic acid or sulphate radicals in a terminal position. Each subunit of mucin contains a central region rich in threonine and serine, which are the O-oligosaccharides attachment sites (Montagne et al., 2000). The coat of oligosaccharides, that covers up to 80% of the proteine backbone, protects mucin in the lumen from proteolysis.

Mucins are classified into neutral and acidic subtypes, depending on the monosaccharide composition, and on the different type of radicals located in terminal positions. Neutral mucins do not contain acidic reactive groups, and are usually present in both the alimentary and respiratory tract goblet cells, as well as in Brunner’s glands and gastric lining cells. Acidic mucins are subdivided into sulfated (sulfomucins) and nonsulfated (sialomucins) mucins. The sulfomucins are polysaccharide sulphate esters, in which the sulphate radical is linked to various hexosamines, the sialomucins contain derivatives of neuraminic acids, which is terminally located on oligosaccharide side-chains of polypeptides. The proportions of these three classes vary spatially along the normal gastrointestinal tract (GIT) and temporally during postnatal development of mammals. In the small and large intestine, mucins are synthesized and secreted at a relatively constant rate by specialized glandular cells called goblet cells, (so-named by the characteristic “goblet” shape), and released in the gut lumen (Figure 2), where they exert their articulate functions and are in addition utilized by resident microflora. So, the intestinal adherent mucous gel is in a continuous dynamic state of renewal.





**Figure 2.** Goblet cell: a unicellular mucous gland situated in the epithelium of various mucous membranes, especially that of respiratory tracts and intestines. Droplets of mucigen collect in the upper part of the cell and distend it, while the basal, containing nucleus, remains slender, then the cell assumes the shape of a goblet.

The composition of the mucous layer is importantly changed in gastrointestinal pathologies, such as tumours and inflammatory and parasitic diseases (Moncada et al., 2003; Lievin-Le Moal and Servin, 2006; Dharmani et al., 2009).

Ontogenic changes in the composition of the mucus layer, that are correlated with changes in the intestinal microbiota and with maturation of acquired immune functions of intestine, are consistent with mucus-secreting goblet cells playing a crucial role in intestinal homeostasis (Neutra and Forstner, 1987; Deplancke and Gaskins, 2001) and support the idea that the mucous layer is considered a dynamically bioactive component of intestinal defence. Really, the adherent mucous gel exerts not only physico-chemical protective functions, but also prevents infections by binding viruses or bacteria through specific interactions with the carbohydrate chains. Mucin carbohydrates have the capacity to either repel or bind to microbial receptors (Neutra and Forstner, 1987) thus some microorganisms, which have the ability to bind to intestinal mucins, can colonize the mucus layer and utilize this surface link to penetrate the mucosa and possibly interact with its defensive actors. Intestinal bacteria, which are unable to bind mucus may be able to temporarily survive in the intestinal lumen (possibly eliciting a defensive answer), but earlier or later are ended to be destroyed (and their small molecules utilized) and expelled via peristalsis and defecation. It is a general belief that mucus-resistant resident bacteria may prevent the attachment of pathogenic organisms by competing with occupied binding sites. In addition, intestinal mucus has a crucial role in the digestive process by immobilizing enzymes near the epithelium surface, so that it is possible to prevent their rapid removal by peristalsis and to place them in a

favourable position for hydrolysis and consequent absorption of small molecules through the epithelial layer. The production of mucins may be nutritionally significant, because they are not readily digested, and thus the colonic fermentation of their constitutive essential amino acids represents an obligatory loss to the animal, however contributing to the feeding of the resident microflora.

For all these properties, the adherent mucous gel plays an important role in animal nutrition and health, and its own integrity at the surface of gut epithelium is necessary for protection and a correct functioning of the intestinal mucosa. Qualitative and quantitative alterations of the mucous layer are expression of inflammatory diseases and tumours.

#### *Weaning mucin biology*

In unweaned piglets, goblet cell numerical density increases in the villi and crypts of duodenal and jejunal mucosa between 9 and 36 days of age (Dunsford et al. 1990). Moreover, the goblet cells of the villi, which prevalently synthesize acidic mucins, are more numerous than those ones that synthesize neutral mucins, which are predominant in the intestinal crypts (Brown et al., 1988). These observations can be explained by the functional maturation of the intestinal goblet cells during their migration from the crypts up to the top of the villi (Brown et al., 1988).

In the weaning period, the young pigs begin to assume dietary fibres, and several studies have reported that this dietary intervention increases the release of mucin in the gut lumen (Lallès et al., 2004). This is prevalently due to the abrasive action exerted upon the adherent mucous gel by the insoluble fibres. The improved capacity of mucin secretion can be regarded as one of the multiple gut adaptations to a chronic mechanical irritation.

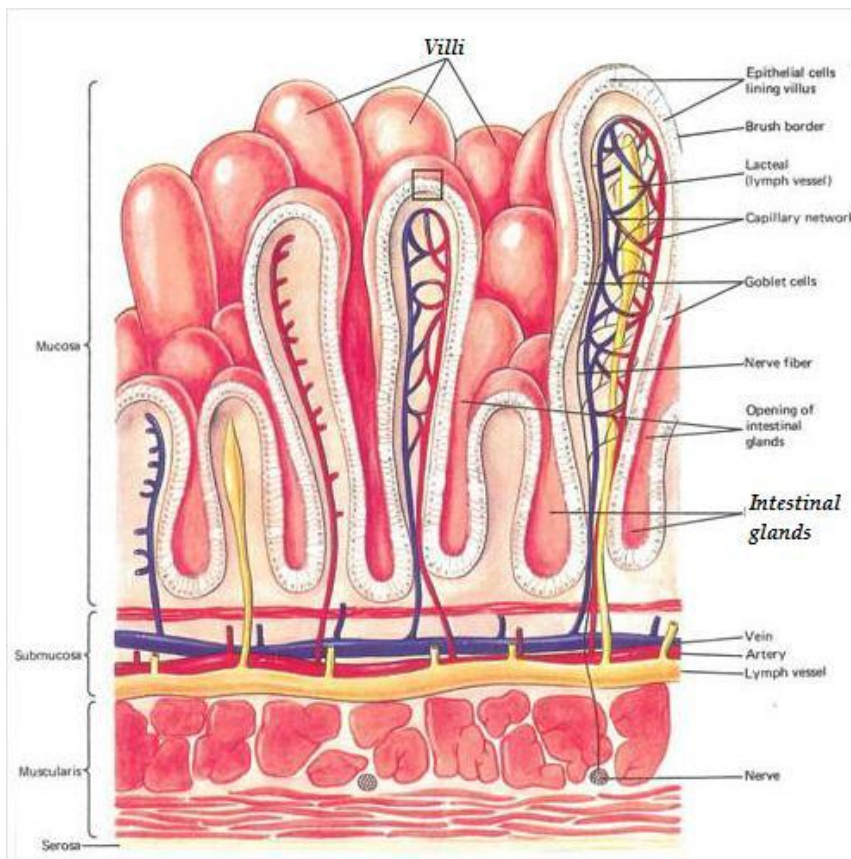
Some studies have shown an early decrease in goblet cell number in the intestinal villi in the initial phase of weaning, which tends to increase again at 3 and 15 days post weaning (Dunsford et al., 1991). This pattern can be linked to the transient post-weaning anorexia described by several studies, since underfeeding lower both levels of mucins (Lopez Pedrosa et al., 1998) and densities of goblet cells in the villi (Nuñez et al., 1996; Brown et al., 1988). This apparently did not influence the neutral to acidic goblet cell ratio in the villi but cell with sulphated mucins had increased densities in the crypts. However an increased concentration of mucin in ileal digesta is shown in newly weaned pigs in comparison to unweaned littermates (Pluske et al., 1997). An increase in the mucin synthesis would likely increase the maintenance requirements for amino acids (mainly threonine) and energy and, therefore, decrease their availability for animal growth and production (Lallès et al., 2004). In the large intestine also, a

drop and a subsequent rise in the tree types of secreted mucins was observed during post-weaning (Brunsgaard, 1997).

The diet can influence also the composition of secreted mucins. Dietary proteins seem to stimulate the synthesis and secretion of sialomucins, as illustrated by Turck et al. (1993). Brown et al. (1988) observed no changes in the proportion of neutral and acidic mucins in the intestinal villi of weaned pigs fed with a diet containing soya protein; on the contrary they noted a relative increase in the number and distribution of goblet cells producing sulphated mucins in the small intestine of the same animals. More recent Baum et al., (2002) observed that the number of goblet cells and the type of secreted mucins appeared in part modified in the large bowel of pigs dietary supplemented with *Bacillus cereus toyoi* or *Saccharomyces boulardii* (Baum et al., 2002). An increased quantity of acid glycoconjugates in yeast-supplemented piglets was observed by Bontempo et al. (2006), and this aspect may confer a greater resistance to bacterial infection in the gut.

#### *The Intestinal Epithelium*

A correct development of mammalian intestine should result in the formation of a specialized mature epithelium. Intestinal epithelial cells have the capacity to accomplish different roles, including classical digestive and absorptive functions, and the maintenance of a barrier against noxious antigens, components and bacteria by keeping proper viscosity of the luminal contents. The epithelial cell monolayer (simple columnar epithelium), which is situated immediately under the mucus layer, is organised into two morphologically and functionally distinct compartments: a deep region, which is aimed at originating new cells because contains stem cells, and to display anti-microorganism activity because contains Paneth cells, and a surface region (the villi in the small intestine, the apex of intestinal glands in the large intestine), which is devoted to apoptotic processes (McCracken et al., 1995) (Figure 3).



**Figure 3.** Mucosal structure of the mammalian small intestine shows the presence of villi and crypts or intestinal glands.

The predominant cell type of the epithelium is the columnar cell (or absorbing cell) called enterocyte in small intestine and colonocyte in the large intestine. Each one is equipped with microvilli at its luminal surface, which appear in the light microscope as the fuzzy striated border on the surface of the villi.

Enterocytes are specialized for digesting molecules and macromolecules, and transporting small molecules coming from digestion: amino acids and monosaccharides are absorbed by active transport, monoglycerides and fatty acids cross the microvilli membranes passively. Absorbed substances enter either the fenestrated capillaries in the lamina propria just below the epithelium, or the lymphatic lacteal. They may also form a substrate for the digestive process because it has been found that pancreatic or intraluminal enzymes are adsorbed.

Colonocytes have short, irregular microvilli, and although they secrete a glycocalyx, it has not been shown that they synthesize digestive enzymes. They are merely absorbing cells, which actively transport electrolytes, and water is also absorbed as it passively follows the electrolytes.

In all intestinal tracts, goblet cells (see above) are present throughout the intestine, but increase in density along the proximal-distal axis. These cell are in different numbers intermingled with columnar cells, and the epithelial layer,

which these cell types compose, is resistant to mucolytic and proteolytic microorganisms, thus contributing to display protective functions.

Epithelial cells, which belong to the Diffuse Endocrine System (DES), are also abundantly distributed throughout the gastrointestinal epithelium. Their presence, due their pale cytoplasm, is hardly discernible applying the usual histological stains, like the Hematoxylin-Eosin sequential stain. On the contrary, due the presence of specific, basally located, secretory granules, they can be easily identified utilizing histochemical methods, like those ones, which use various silver stains. It is on these bases that these cells are also known as argentaffin and argyrophilic cells. Historical names for this cell type include enterochromaffin cells (due the affinity to chrome salts of their secretory granules) and APUD (Amine Precursor Uptake and Decarboxylation) cells. Enteroendocrine cells synthesize and secrete peptide hormones and/or serotonin in either a true endocrine (via the blood) or paracrine (via intracellular spaces) manner; before the realising, the chemical messengers are stored in basally located secretory granules. Enteroendocrine cell integrate the intestine with the systemic nervous system and are dispersed throughout the small and large intestine (Furness et al., 1999). Morpho-functional identification of several types of (entero) endocrine cells depends on differences in size, shape, electron-density of the secretory granules but mainly on their amine or peptide products (serotonin, somatostatin, glucagon/ glicentin, cholecystokinin, gastrin, motilin, secretin, neurotensin, substance-P, gastric inhibitory poly-peptide, and  $\beta$ -endorphin), which may be immunohistochemically identified.

Paneth cells are found in the deep third of the small intestine glands. These cells contain in their apical cytoplasm large and round acidophilic granules, which are the site of storage of a number of substances like lysozyme, tumor necrosis factor, cryptins (small molecular weight peptides, related to defensins) that act in the unspecific anti-bacterial activity.

Undifferentiated cells (stem cells) are found at the base of the crypts, and give rise to all the other cell types. Cells destined to be a goblet cell, enteroendocrine cell or enterocyte undergo about 2 divisions after leaving the pool of stem cells, and subsequently migrate upwards in columns from the crypt along to the villus, and in the meanwhile they display their specific functions (Sierro et al., 2000). When they reach the tip of the villous, cells enter programmed cell death (apoptosis) and are shed into the intestinal lumen (Sierro et al., 2000). Epithelial cells are bound each to each other and to other cell types by junctional complexes, in this way building a crucial physical barrier to external environment. Moreover enterocytes are always in a dynamic status, their process of continual desquamation and renewal considerably limit the opportunities for pathogens to colonize epithelial cells (Potten and Loeffler, 1990). The intestinal

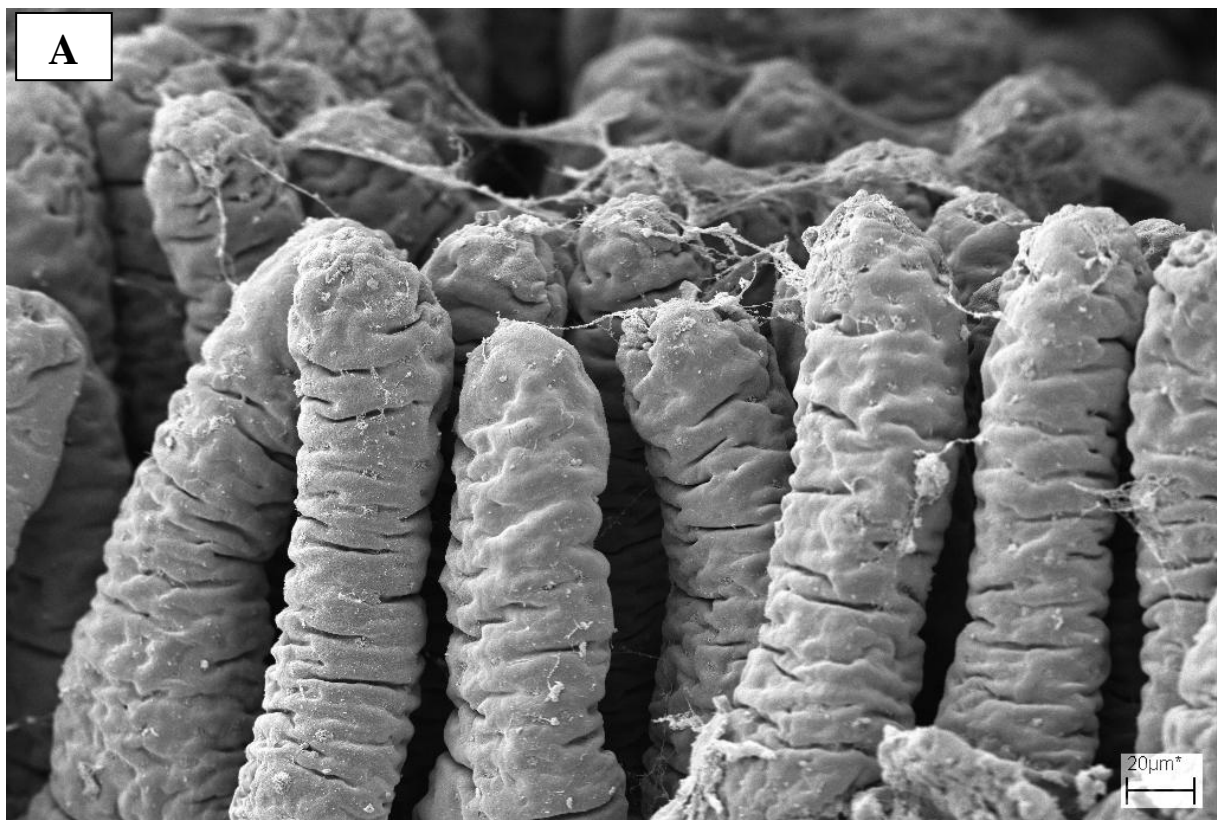
epithelial cells serve a key role in integrating the signals from luminal microorganism with host development and local mucosal defence.

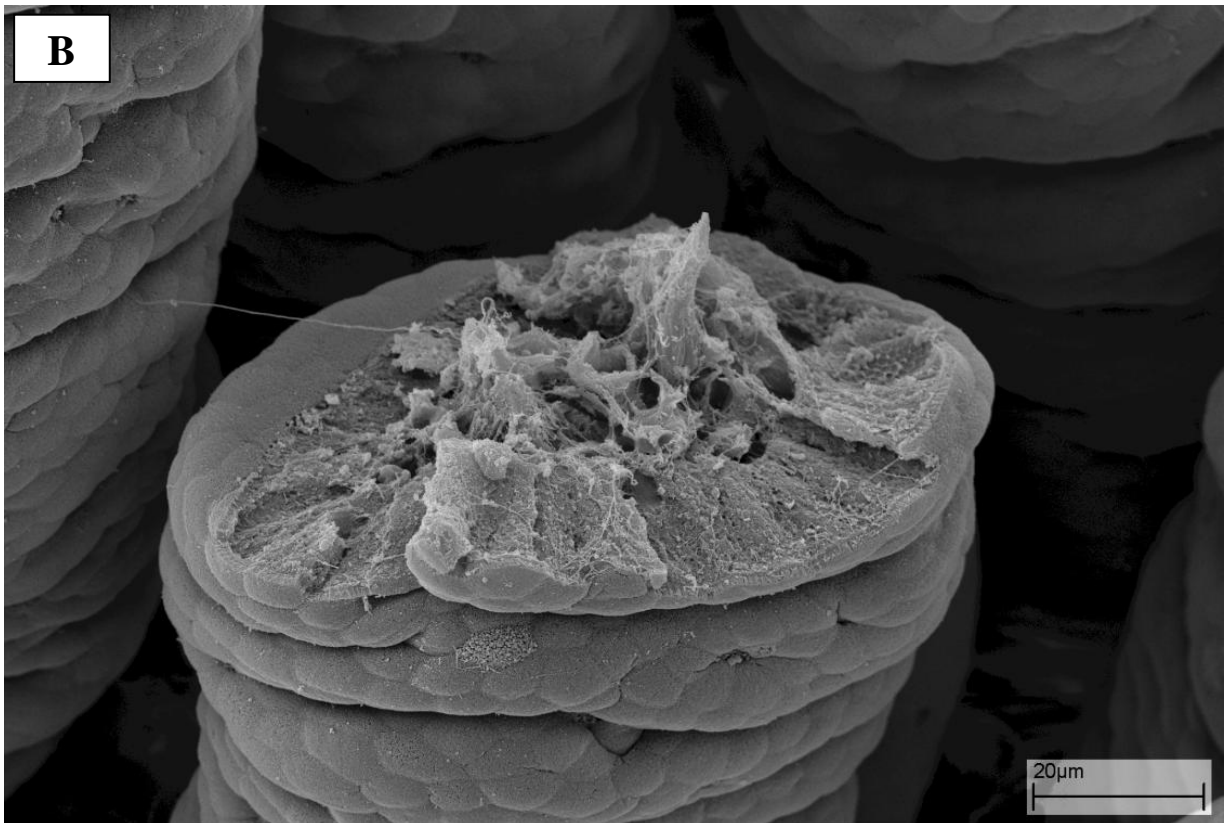
#### *The intestinal epithelium at weaning*

Intestinal alterations often seen in the post-weaning period in piglets include primarily changes in villus\crypt morphology and in brush border enzyme activities, and implication of enteric pathogens (like *Escherichia coli* and rotaviruses) has also been addressed (Lalles et al., 2004).

During the post-weaning 2 days the small intestinal mucosa loses 20-30% of its relative weight, and it needs 5-10 days for a structural regeneration and a full functional recovery (Lallès et al., 2004).

During the “degenerating or acute phase” of post-weaning, a marked villi atrophy is described (from - 45% to - 70% of pre-weaning values), particularly in the proximal and mid-small intestine (Figure 4)(Pluske et al., 1997; Boudry et al., 2004).





**Figure 4.** A: The surface of duodenal villi at birth with characteristic transverse furrows in neonatal pig (top). **B:** Villus fracture, which identifies the villus atrophy described at 2-4 days post-weaning. (modified from Zabielski et al., 2008).

Different observations are done about intestinal crypt depth, which may (McCracken et al., 1999; Spreeuwenberg et al., 2001) or may not (Hedemann et al., 2003; Van Beers-Schreurs et al., 1998) appear to be reduced, concomitantly with the beginning of weaning. Intestinal crypt cell proliferation and cell migration rates appear to be strongly dependent on energy availability during the first 2 days post-weaning (Pluske et al., 1997; Spreeuwenberg et al., 2001). Crypt depth may also reflect gut pathogen exposure, because the presence of cytokines, induced by the invasion of a possible pathogen, increases proliferation of crypt cells and apoptosis of enterocytes at the top of villi (Rafferty et al., 1994; Piguet et al., 1999). The rate of intestinal epithelium renewal in response to invasion of pathogens that damage epithelial cells may play a key role in the efficacy of the global defensive response of the intestine (Gaskins and Kelley, 1995). In fact, Tang et al (1999) have observed significantly shorter crypts and a higher villi/crypts (V/C) ratio in jejunum and ileum of segregate early weaned compared with normal weaned pigs that had a major pathogen exposure. A fasting period or a low fed intake that happen especially during 2 days after weaning are responsible for a deprivation of luminal substrates necessary for epithelial cell growth, and a reduced secretion and releasing of growth factors

such as glucagon-like peptide 2, insulin-like growth factor 1 (Burrin et al., 2000; Stoll et al., 2000) and CCK (Montagne et al., 2007). The weaning induced dramatic transient as well as long-lasting changes in intestinal physiology, mainly at the small intestinal level. Both psychological stress and food deprivation dramatically affect intestinal architecture and physiology.

The above described changes in the intestinal mucosa architecture and the consequent decreased enzyme activities are correlated with many functional changes, which are traceable till 2 weeks post weaning. Basal short-circuit current (parameter of intestinal secretory capacity) in jejunum and colon increased in 2 days post-weaning. Food deprivation in piglets was also shown to decrease transmucosal resistance and to increase short-circuit current, the Na<sup>+</sup>- dependent glucose absorption. A dramatic drop (68% lower than pre-weaning values) of transmucosal resistance is observed in the proximal jejunum. Permeability also to macromolecules drops in the jejunum at 2 days post-weaning (Boudry et al., 2004). Pre-weaning values are usually detected 5 days after weaning, except for the permeability to macromolecules, which remains low. The Na<sup>+</sup>- dependent glucose absorption in the ileum and ileal transmucosal electric resistance decline for 5 days post-weaning. In colon the basal intestinal secretory capacity is higher in 2 days post-weaning piglets than in 14 days unweaned piglets (Bach and Carey, 1994)

The observed villus atrophy and the consequent decreased enzyme activities, which characterize the pig small intestine in the post-weaning period, are likely responsible for an enhanced flow of nutrients undigested or only in part digested arriving in the distal part of small intestine, where, on the other hand, the ileal epithelium has a low absorptive capacity. As a consequence, either an osmotic or a secretory diarrhoea could occur at the same time in a number of piglets. Really, a compromised epithelial barrier function with augmented para-cellular (between cells) and trans-cellular (through cells) permeabilities could let an access in the lamina propria for toxins, allergenic compounds or bacteria, resulting in the occurrence of physio-pathological conditions as well as inflammatory diseases (Spreeuwenberg et al., 2001). These latter in turn are associated with a poor performance with a body growth stasis.

#### *1.1.4. Microbiology of the gastrointestinal tract*

The porcine gastrointestinal tract represents a dynamic ecosystem containing a complex community of micro-aerophilic and anaerobic microbes that are involved in the fermentation of ingested feed and the components secreted by the host into the GI tract (Konstantinov et al., 2004). The gastrointestinal tract of an adult pig contains approximately 10<sup>14</sup> prokaryotic and eukaryotic microorganisms belonging to around 400 different species: this is an immense



load of commensal microorganisms, which represent an extremely diverse community (Shirkey et al., 2006). This population of bacteria has marked effects on host intestinal activities and structural patterns, including morpho-functional aspects, mucus secretion, nutrient digestion and metabolism, and immune functions. In addition, considering the strict interaction (via blood and lymphatic vessels) between the local defensive functions and those ones systemic, the correct interplay between the intestinal barrier and the resident microflora is clearly linked to an overall health status.

Immediately at birth the mammalian gut is essentially sterile, and initial bacterial colonisation is coming from mother- and environment-derived microorganisms, which enter the neonatal intestine by suckling and nursing just after birth. The development of the intestinal microbiota undergoes a rapid ecological succession during the first period from birth to weaning (Lallès et al., 2004). At the beginning of colonization the microbiota of the newborn is heterogeneous (Fanaro et al., 2003). During the first days after birth, a large number ( $10^8$ - $10^{10}$  g in faeces) of coliforms and streptococci initiate intestinal colonisation, which produces an environment favourable for the establishment of anaerobic phyla, such as *Bacterioides*, *Bifidobacterium* and *Clostridium* (Konstantinov et al., 2006). In the ileum of 2-day-old piglets a lot of *Lactobacillus* spp. are present and proliferate: they have a strong potential to prevent the colonisation of pathogens by competition for nutrients and epithelial binding sites, as well as the production of antimicrobial factors, such as lactic acid and bacteriocins (Konstantinov et al., 2006). *Lactobacillus* species (*L. sobrius*, *L. reuteri* and *L. acidophilus*) are abundant and remain stable before weaning in the intestine of neonatal piglets and may play a crucial role in the establishment and the maintenance of the gastrointestinal tract bacterial homeostasis after birth.

During and after birth, the young animal alimentary canal becomes colonised with a variety of microbes from the birth canal and the immediate environment. The microflora remains enough stable in terms of species composition after initial colonisation and as long as the piglets receive sow's milk. In fact during the first few days post-birth often only one phylotype is dominant in the faeces, generally *Lactobacillus* (Konstantinov et al., 2006). The introduction of a solid diet causes major qualitative and quantitative alterations in the microflora; induces obligate anaerobes to increase in quantity and quality until an adult-type pattern is achieved (Inoue et al., 2005; Konstantinov et al., 2006). When the adult-type pattern is reached, the commensal microbiota populations can entirely display their functions to synthesize vitamins and to protect the host from pathogens, forming a front line of mucosal defence. The displayed protective functions need the utilization of energy from otherwise indigestible carbohydrates, and are based upon the elicited local (and systemic) defensive responses (Zoetendal et al., 2004).

It is well-known that microbial fermentation in the gastrointestinal tract is very important for the pig (Williams et al. 2001), because it produces fundamental intermediates, like volatile fatty acids (VFAs). Carbohydrate microbial fermentation leads to the production of straight-chain VFAs. Protein microbial fermentation may result in end products like  $\text{NH}_3$ , branched-chain VFA and in potentially toxic substances (Williams et al. 2001) like amines, volatile phenols and indoles. VFAs play essential roles in energy production (because are quickly “breathed” by the mitochondria), as well as in the water (and  $\text{Na}^+$ ) absorption, the pH control, and the inhibition of pathogens. In addition, microbial fermentation is also important for the gastrointestinal health of host, modulating the gut motility, enhancing the vitamin synthesis, and stimulating the gut immunity. As a consequence, VFAs indirectly contribute to the defence against pathogens, the prevention of diarrhoea and the enhancement of resistance processes to pathogen colonisation (van der Waaij et al., 1971). Then, the composition of the diet is essential for determining and maintaining the composition and the activity of the intestinal microflora.

#### Microbiology of the gut at weaning

Piglets at weaning become vulnerable to a higher incidence of gastro-intestinal and systemic diseases, on the bases of both the changes in the diet and in the composition of the gastrointestinal tract microbial community (Hopwood and Hampson, 2003). In commercial swine production, piglets are weaned early, usually after their transport to a production farm, followed by an abrupt introduction of a solid diet. This combination of stress factors often leads to diarrhoea and growth reduction (Spreeuwenberg et al., 2001). Important changes in the intestinal microbiota composition are caused by the new diet, with its passage from maternal milk to a solid diet rich in plant polysaccharides (Konstantinov et al., 2004). The porcine intestinal *Lactobacillus* community can decrease after the exposure of the animal host to stress factors such as mixing with other piglets, suboptimal feed intake and transportation (Hammes et al., 1991), and the microbial populations of *Lactobacillus* colonizing the porcine intestine in the early post-natal period diminish significantly during weaning (Konstantinov et al., 2006). *Clostridium* spp and *E. coli* populations are abundantly found in the ileum of 23 days old weaned piglets (Konstantinov et al., 2006). The ileum shows in addition a measured higher level of lactate, one of the principal metabolites of the carbohydrate fermentation performed by many microorganism groups including Clostridia, and a lower level of ammonia (Konstantinov et al., 2005). Ammonia is used by bacteria for their growth, and so a reduction of its concentration is likely due to the rapid bacterial proliferation that occurs in the intestinal lumen in the immediate post-weaning period. An unbalance in the microbial proliferation and an unstable bacterial community can

promote the occurrence of bacterial intestinal diseases. Either anorexia or low feed intake that especially occur during the first 2 days post-weaning may cause a scarce dietary supply of carbohydrates, and consequently not-digested proteins or proteins of endogenous origin are fermented as energy sources. This in turn results in the production of branched-chain VFAs and potentially toxic end-products, such as amines, volatile phenols and indoles (Lallès et al., 2007).

For the above mentioned arguments, the weaning period is a critical phase in the development of appropriate responses to either pathogens or harmless dietary and commensal antigens. The mucosal immune system is not completely mature, but both the colonisation of the gut by the new microbial populations, and the abrupt absence of the maternal milk necessarily induce a quick morpho-functional maturation of the defensive mucosal system with frequently occurring unbalanced relations between the different components of GALT. Thus at weaning the until now balanced specific and non-specific ways of action may be disturbed: when a component of immune system develops inappropriately, inappropriate effector responses to harmless antigens, such as food protein (allergy), or inadequate responses to pathogens (disease susceptibility) may occur (Bailey et al., 2005). In conclusion, this ascertained instability of the gut microbial community populations of piglets at weaning may considerably contribute to the observed porcine intestinal morbidity problems in the immediate post-weaning period. On the other hand, a stable and articulate commensal bacterial community should be considered a necessary pre-requisite for a gut healthy ecosystem, which in turn is linked to good producing performance.

#### 1.1.5 Liver

The liver is an accessory gland of the gastrointestinal tract but it has a remarkable diversity of other functions unrelated to alimentation. The organ is closely associated with the small intestine, processing the nutrient-enriched venous blood that leaves the digestive tract.

The liver plays a unique role as a metabolic center of the body, and also performs other important functions (Table 3).

**Table 3** Main functions of the liver (Kmiec 2001)

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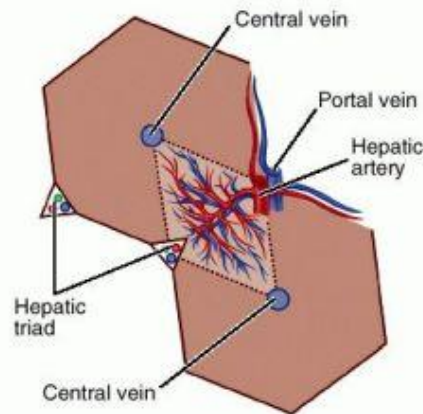
1	Service functions for non-hepatic organs
1.1	Center of metabolism
	Energy supply of the organism
	Glucose uptake and release
	Amino acids uptake and release
	Urea formation
	Lipid processing and synthesis

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- 
- Ketone bodies synthesis
  - Biosynthesis and biodegradation
  - Plasma protein synthesis and degradation
  - Bile formation (excretion of endobiotics and xenobiotics)
  - 1.2 Center of defense
    - Xenobiotic metabolism, scavenging of ROS
    - Phagocytosis
    - Uptake and destruction of bacteria, viruses, parasites, particulate material and macromolecules
    - Elimination of tumor cells
    - Acute phase reaction
  - 1.3 Control station of the hormonal system
    - Inactivation and elimination of hormones and mediators
    - Synthesis and release of (pro)hormones
    - Synthesis of hormone-binding proteins
  - 1.4 Blood reservoir: passive and active blood storage
  - 1.5 Involvement in the regulation of blood pH
  - 1.6 Hematopoietic function in fetal and probably postnatal life
  - 1.7 Immunoregulatory functions
    - Antigen presentation to lymphocytes
    - Clearance of apoptotic lymphocytes
    - Tolerance induction towards oral antigens
  - 1.8 Formation and maintenance of organ structure
    - Synthesis and degradation of extracellular matrix components
- 

The macroscopic and microscopic structure of the mammalian liver has been recognized relatively early. In 1833 Kiernan proposed that lobes of the pig liver organized around main branches of the portal vein were built up of small polyhedron morphological units of parenchyma, called lobules, with boundaries made of connective tissue (Kiernan 1833). This classical liver lobule is characterized by the presence of a central vein (terminal hepatic vein) located in the middle of the unit, and of areas of connective tissue at its corners, called portal tracts or hepatic triads, that contain interlobular branches of hepatic artery and portal vein, biliary ductules, lymphatic vessels and nerves (Kmiec, 2001).

In 1954, Rappaport and co-workers proposed that the functional unit of the liver parenchyma may be represented by the smallest hepatic microcirculatory unit called simple acinus (Fig. 5). Acinus contains the area of liver parenchyma supplied by terminal afferent vessels, portal venule and hepatic arteriole, present in the connective tissue septa between adjacent classical lobules (Rappaport 1954). Terminal afferent vessels, together with bile ductules, form the axis of the acinus, from which it is supplied with blood.



**Fig.5** Liver acinus

The liver tissue is organized at the microscopic level in lobules that consist of muralium made of anatomizing plates one cell thick, which extend from the portal tract in linear fashion to the central vein, and transmural spaces that contain tortuous hepatic sinusoids that are separated from hepatocytes by the perisinusoidal space of Disse. Hepatic sinusoids lead the mixed blood of the terminal branches of hepatic artery and portal vein to the terminal branch of the hepatic vein, i.e., central vein. The sinusoid represents a unique form of capillary bed characterized by a fenestrated endothelial lining, and by the presence of hepatic macrophages (Kupffer cells) inside the lumen or as a part of sinusoid lining, the lack of genuine basement lamina, and the presence of perisinusoidal space (Disse's space) at its external aspect. The perisinusoidal space contains many cytoplasmic dendritic projections and the cell body of hepatic stellate cells, abundant microvilli of hepatocytes, nerve endings, and a non-electron dense complex extracellular matrix (ECM) (Kmiec 2001).

The bulk of liver parenchyma is made up of hepatocytes that occupy more than ten times the volumes of non-hepatocytes. Non parenchymal liver cells contribute 26.5% to the total cell membrane surface (mainly endothelial cells), 58% to the volume of pinocytotic vesicles (mainly endothelial cells), 43% to the total lysosomal volume (mainly Kupffer and endothelial cells), and 55% to the total fat droplet volume (mainly stellate cells).

So the liver parenchyma presents different cell types: hepatocytes, sinusoidal endothelial cells, Kupffer cells, Ito cells, and pit cells and other intrahepatic lymphocytes (Fig 6).

### *Hepatocytes*

Parenchymal liver cells, hepatocytes, constitute the major cellular compartment of the liver and may be regarded as key hepatic effector cells, since the majority of liver functions could be attributed to their activities. The hepatocyte is a

polyhedral multifaceted cell with eight or more surfaces. Several structure surface components participate in two or three major domains of the hepatocytes: (a) the basolateral surface (perisinusoidal and pericellular), which is highly enlarged through the presence of many short microvilli; (b) the straight or contiguous domain between adjacent cells, and (c) the bile canalicular domain (Kmiec, 2001).

Parenchymal liver cells participate in almost all functions that have been attributed to the liver. They confer to the liver an important function of the body's glucostat by releasing glucose through the process of glycogenolysis, and producing glucose from noncarbohydrate substrates via the pathway of gluconeogenesis. Hepatocytes are the only cells that inactivate toxic ammonia in the urea cycle. Parenchymal liver cells synthesize the bulk of serum proteins, including components of the complement system and acute-phase proteins, important compounds of the innate immunological system. Hepatocytes play a substantial role in the metabolism of exogenous and endogenous lipids through the synthesis of many classes of lipoproteins and catabolism of blood-derived cholesterol-enriched proteins. Parenchymal liver cells are well equipped for the defense against oxidative stress, and are responsible for the detoxification of numerous endo- and exogenous substances. The production of bile components such as bile acids, cholesterol, phospholipids and conjugated bilirubin, takes place also in hepatocytes. Hepatocytes also synthesize and release some (pro)hormones, and many intercellular mediators into the circulation.

The metabolic heterogeneity of hepatocytes may be explained through their distance from the terminal portal and arterial branches that determines their relation to the nutritional and oxygen gradients (Jungermann 1995). Parenchymal liver cells localized at the periphery of the classical liver lobule, which can be termed as periportal hepatocytes, function in the microenvironment rich in oxygen, substrates, and hormones contained in the blood of terminal afferent vessels. In contrast, parenchymal cells located around the central vein, called perivenous hepatocytes, contact the perfusate of blood partially depleted of oxygen and substrates, but enriched in CO<sub>2</sub> and other products of metabolism (Rappaport 1976; Sasse et al., 1992).

#### *Sinusoidal endothelial cells*

Endothelial cells of liver sinusoids (SECs) differ in many structural and functional aspects from other endothelial cells of the body. They do not have a regular basement membrane, and are often embraced by the cytoplasmic processes of underlying hepatic stellate cells. SECs constitute an important filtration barrier between macromolecules and blood cells present in the sinusoidal lumen and hepatocytes that prevents their direct contact, and determines the exchange of various substances. SECs possess a large pinocytotic

and endocytotic capacity due to the presence of numerous plasma membrane receptors. They also actively participate in the immunological functions of the liver. Liver sinusoidal endothelial cells form a continuous lining of the liver sinusoids, separating parenchymal cells and hepatic stellate cells from sinusoidal blood. These flat, elongated cells with a small cell body and numerous thin cytoplasmic processes form the lining of the sinusoidal wall.

The main function of SECs is believed to be formation of general barrier against pathogenic agents and they serve as a selective sieve for substances passing from the blood to parenchymal and stellate cells, and in the opposite direction.

The filtering effect is greatly enhanced by a huge transport and clearance capacity of SECs based on the receptor-mediated endocytosis and intercellular metabolism of a wide range of macromolecules, including glycoproteins, lipoproteins, extracellular matrix components, and inert colloids. Liver sinusoidal endothelial cells constitute an important component of the complex network of cellular interactions in the liver through the secretion of many substances and reactivity to many signals received from neighboring cells. The sieving function of SECs has a major effect on the hepatic uptake of particles or rigid droplets. The ultrastructural features indicate that SECs possess a high pinocytotic and endocytotic capacity for fluids, molecules, and small particles (Wisse 1972).

Vast amounts of particulate and soluble material generated in the gut, such as food antigens and bacterial products, continuously enter the liver through the portal vein. Due to the presence of Kupffer cells, and sinusoidal endothelial cells that show prominent endocytic and phagocytic capabilities, the liver removes many of the foreign molecules both in nonspecific and specific ways. However many exogenous antigens arriving in the liver may induce specific immunological reactions that lead to the development of immune tolerance rather than immune responses. Liver sinusoidal endothelial cells have the capacity to function as antigen presenting cells (APCs) as they constitutively express MHC class I and II, CD4, CD11, CD54 (ICAM-1), CD106 (VCAM-1) molecules, and also costimulatory molecules CD89, CD40 and CD86, necessary for the presentation of antigens to T cells.

Another role of SECs in the modulation of the immunological responses appears from the selective retention in the liver of activated, but neither rested nor apoptosing, CD8<sup>+</sup> T cells, which occurs via mechanism mediated primarily by ICAM-1 constitutively expressed on SECs and Kupffer cells.

Sinusoidal endothelial cells synthesize many substances that affect neighboring cells in a paracrine way, or, after release into the circulation, may influence distant cells. Some of these substances are produced constitutively, whereas others are released after induction by specific factors. To the main secretory products of sinusoidal endothelial cells belong prostanoids, nitric oxide, endothelin-1, and various cytokines (Kmiec 2001).

Endothelial cells of the liver were shown to participate in the control of hemostasis, coagulation, sinusoidal blood flow, tissue proliferation and repair via the production of growth factors, and antitumoral activity.

#### *Kupffer cells*

Kupffer cells, the resident macrophages of the liver, are located within the lumen of the sinusoids overlapping the tinned endothelial wall, and their cellular extensions contribute to a kind of double lining of the sinusoid (Motta 1984). Kupffer cells represent the largest population of macrophages in the mammalian body (Bouwens et al., 1986), and are largely responsible for clearing the post-mesenteric blood of gut-derived bacteria and potent bacterial toxins.

Kupffer cells closely resemble other macrophages: their cell body shows numerous microvillous projections, blebs, lamellipodia and occasionally filipodia. In many instances their cytoplasmic processes penetrate through the fenestrae of endothelial cells and reach out into the subendothelial Disse's space being in direct contact with hepatic stellate cells and hepatocytes (Wisse 1974). Kupffer cells, by virtue of their location in the mainstream of splanchnic blood flow, are positioned to receive a constant exposure to gut-derived mediators known to activate macrophages.

Similarly to other macrophages, Kupffer cells are very active in endocytosis and phagocytosis due to the presence of numerous cell membrane receptors. Activated liver macrophages release many substances that have paracrine and autocrine effects within the liver, and endocrine effects throughout the body. In contrast to the liver SECs that take up soluble molecules from the circulation, Kupffer cells play an essential role in the elimination of both soluble and blood-borne particulate material derived from portal circulation (Kmiec, 2001).

Kupffer cells play an important role in the clearance of senescent and damaged erythrocytes by binding them to the members of the scavenger receptor family and in subsequent degradation of haemoglobin and recirculation of iron.

Kupffer cells play an important role in intrahepatic immunosuppression and induction of immunological tolerance (Terabe et al., 2000). Because activated Kupffer cells also acts as antigen-presenting cells toward intrahepatic lymphocytes due to the expression of high levels of class I and class II MHC molecules, and CD89 and CD40 co-stimulatory molecules, they may play a role in the regulation of hepatic immune responses.

#### *Hepatic stellate cells*

These cells are known with a variety of synonyms (Ito cells, vitamin A-storing cells, lipocytes, fat-storing cells, liver-specific pericytes, perisinusoidal cells) reflecting their functions, however, the term "hepatic stellate cells", HSCs, has been recently most often use (Kmiec , 2001).



HSCs are located in the perisinusoidal (Disse) space in direct vicinity to the endothelial cell layer with their cell bodies often compressed into the recesses between hepatocytes. They have two types of cytoplasmic extensions: the long intersinusoidal or interhepatocellular processes, which penetrate the hepatic cell plates and may reach the nearby sinusoids, and the shorter perisinusoidal or subendothelial processes that encircle the sinusoid, thus reinforcing its wall.

In vivo, hepatic stellate cells exhibit a dual phenotype: a quiescent one in normal liver, and an activated phenotype in chronically diseased liver, especially in liver fibrosis. In the quiescent state the cells show abundant lipid droplets, a low proliferative rate, and a low synthetic capacity. The activated or myofibroblast-like phenotype is characterized by the loss of lipid vacuoles, increased cell proliferation, and enhanced synthesis of extracellular matrix components (Friedmann 1993).

The following functions can be attributed to quiescent (non-activated) stellate cells:

- retinoid metabolism: more than 85% of vitamin A in the body is stored in the liver and 80-90% of the retinoids in the liver are stored in the lipid droplets of hepatic stellate cells;
- modulation of sinusoidal blood flow;
- synthesis of extracellular matrix components;
- intercellular communication through the synthesis of cytokines and growth factors;
- erythropoietin synthesis;
- synthesis of plasminogen activation system.

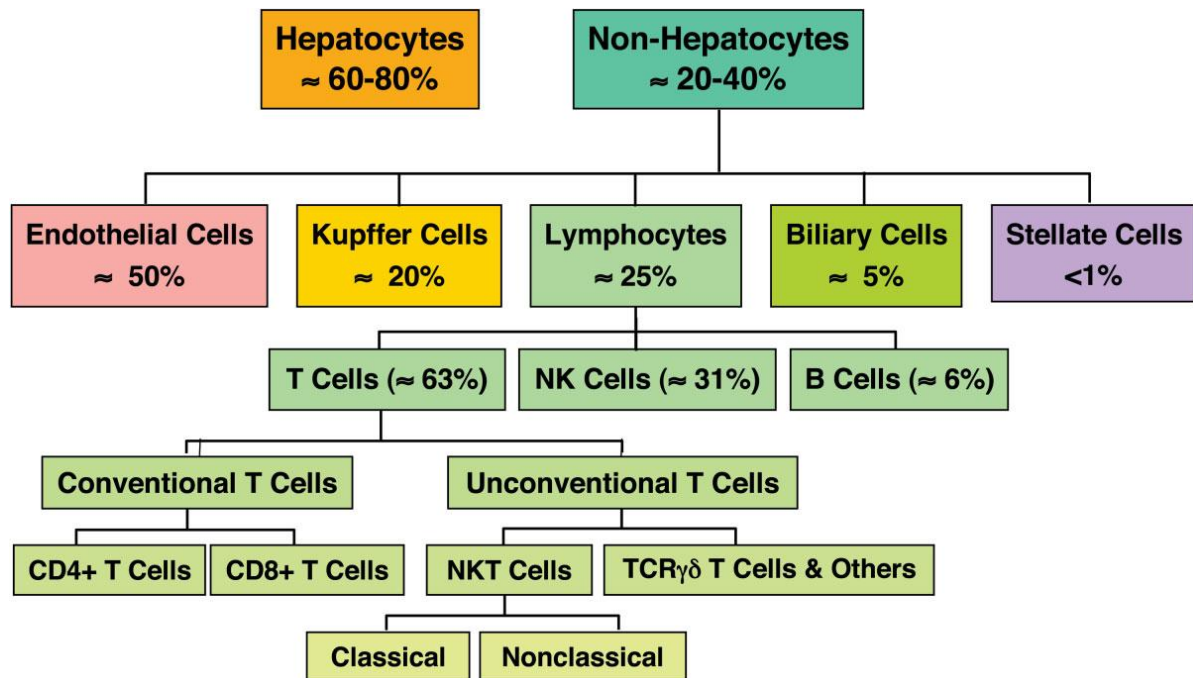
In their activated phenotype, stellate cells are characterized by pronounced synthetic, proliferative, chemotactic, and contractile properties. Several structural changes characterize the transdifferentiation of the liver stellate cells into myofibroblast-like cells. They include loss of vitamin A-containing fat droplets, and increased cell spreading accompanied by the formation of cellular processes expressing prominent actin microfilaments, and focal adhesion.

In the liver damage, activated stellate cells or liver myofibroblasts play a pivotal role in the initiating and progression of liver fibrosis. In the liver injury, HSCs show increased contractile activity, and in this way they may affect sinusoidal blood flow. The secretion of leptin by activated HSCs may be involved in the modulation of action of many proinflammatory cytokines. In recent years there is an increasing interest in the critical role of leptin in hepatic inflammation and fibrogenesis. Aleffi et al. (2005) observed that leptin increases the expression of proinflammatory and angiogenic cytokines by human HSCs, demonstrating a complex regulation of the liver wound-healing response.

*Pit cells and other intrahepatic lymphocytes*

Liver sinusoids contain a very large and heterogeneous population of resident lymphocytes, which comprise liver-specific natural killer (NK) cells, and different subpopulation of T cells. It has been suggested that the term “liver-associated lymphocytes” LALs, which encompasses all sinusoidal lymphocytes, can be used for this type of sinusoidal cells. LALs are defined as those lymphocytes that remain in sinusoids after liver perfusion with physiological solution at physiological pressure to remove circulating hematopoietic cells (Winnock et al., 1995).

However, because liver contains also numerous extrasinusoidal lymphocytes that reside in portal tracts or between parenchymal cells, term such as “intrahepatic lymphocytes” IHLs or “liver-resident lymphocytes” that encompass many subpopulations of liver lymphocytes have recently been introduced. The main groups of liver lymphocytes include: pit cells, large  $\gamma\delta$ T lymphocytes; T cells with conventional level of T cell receptor ( $\alpha\beta$ T cells) expressing either CD4 or CD 8 molecules; unconventional  $\alpha\beta$ T cells (TCRintIL-2R $\beta$ +) expressing IL-2 $\beta$  receptor and lower levels of CD3 that conventional T cells; TCRintIL-2R $\beta$ + cells that exhibit dual T cell and NK cell phenotype (NK1.1+) and function (cytotoxicity), denoted NK T cells; small population of c-kit+ cells (stem cells) lacking lineage markers; and small numbers of B lymphocytes (and monocytes).

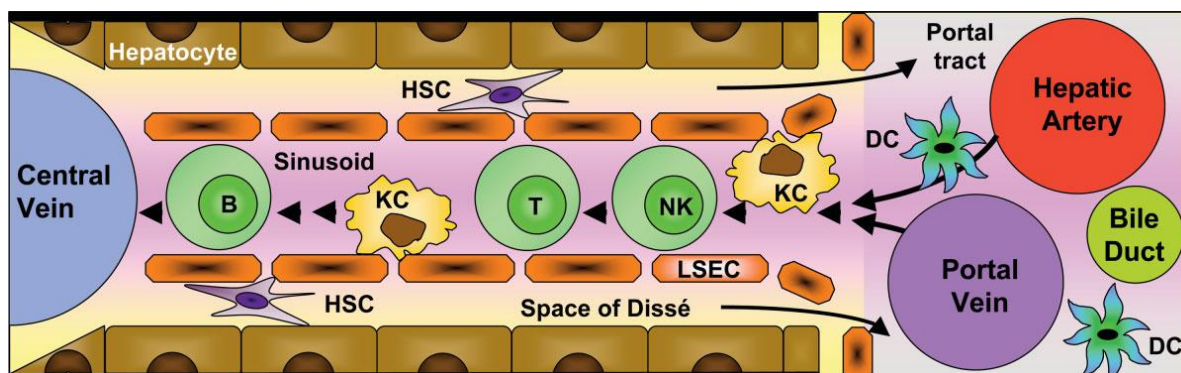


**Fig 6** Cell composition of the healthy liver

### *1.1.6 The immunological function of the liver*

The structural organization of the liver has profound implications for its immune function (Fig.7). The liver's blood supply depends on a conventional arterial system from the abdominal aorta that supports predominantly bile ducts and other tissues in the portal tracts, and on two venous systems: a minor system from the arterial plexus within portal tracts (peribiliary plexus) and a major system from the splanchnic organs. About 30% of the total blood passes through the liver every minute carrying about  $10^8$  peripheral blood lymphocytes in 24 hours. Blood enters the hepatic parenchyma mainly via terminal portal vessels, then passes through a network of liver sinusoids, leaving the parenchyma via the central hepatic veins.

The liver is a unique anatomical and immunological site in which antigen-rich blood from the gastrointestinal tract is pressed through a network of sinusoids and scanned by antigen-presenting cells and lymphocytes. The liver's lymphocyte population is selectively enriched in natural killer and natural killer T cells, which play critical roles in the first line immune defense against invading pathogens, the modulation of liver injury and the recruitment of circulating lymphocytes. Circulating lymphocytes come in close contact in the sinusoids to antigens displayed by endothelial cells, Kupffer cells and liver resident dendritic cells (DCs), resident antigen-presenting cells. DCs are derived from the bone marrow and are typically located around the central veins and portal tracts. In the healthy liver, DCs are predominantly immature cells, prone to capture and process antigens. Once activated, they downregulate some receptors and increase their capacity to migrate via the space of Disse to the lymphatics in the portal tracts and ultimately to extrahepatic lymph nodes. Circulating lymphocytes can also contact hepatocytes directly, because the sinusoidal endothelium is fenestrated and lacks a basement membrane. This unique anatomy of the liver may facilitate direct or indirect priming of lymphocytes, modulate the immune response to hepatotropic pathogens and contribute to some of the unique immunological properties of this organ, particularly its capacity to induce antigen-specific tolerance (Racanelli and Rehermann 2006).



**Fig 7 Immune cells in the healthy liver (from Racanelli and Rehermann 2006).** Liver sinusoids are lined by a fenestrated monolayer of sinusoidal endothelial cells (LSEC). Note that each lymphocyte that passes through the sinusoids is in close contact to the endothelial cells flanking the sinusoids, because of the small diameter of the liver sinusoids (approx. 6-15  $\mu\text{m}$ ) and the comparably large diameter of lymphocytes ( $\approx 7\text{-}12 \mu\text{m}$ ). The Space of Disse contains stellate cells in a loose extracellular matrix. B, B cell; DC, dendritic cell; HSC, hepatic stellate cell; KC, Kupffer cell; NK, natural killer cell; T, T cell.

The liver has a number of major functions in both innate and adaptive immunity, which can be outlined as follows:

*-Innate Immunity Involvement*

- Production of acute phase proteins
- Nonspecific phagocytosis
- Nonspecific cell killing
- Disposal of waste molecules

*-Adaptive Immunity Involvement*

- Deletion of activated T cells
- Induction of tolerance to ingested and self antigens
- Extrathymic proliferation of T cells
- Disposal of waste molecules

*1.1.7 Liver at weaning*

As already underlined, the early weaning of pigs is stressful and is characterized by poor growth, decreased feed intake and diarrhea.

Postweaning nutritional stress may affect the liver because the source of nutrients changes from a liquid to a *dry* diet, as above mentioned in describing intestinal alterations. The rapid change of pancreatic enzyme production occurring at weaning (Friend et al., 1970; Shields et al., 1980; Efirid et al., 1982a) indicates that the digestive system is underprepared to digest typical starter diets. Environmental stress may occur when newly weaned pigs are taken from a warm farrowing house to a nursery with temperatures outside a pig's zone of thermoneutrality (LeDividich et al., 1980).

Weaning is a complex step involving dietary, environmental, social and psychological stresses, which interfere deeply with feed intake, digestive development and adaptation. Moreover, the gastrointestinal tract is a reservoir of viable bacterial organisms and biologically active microbiological products, such as endotoxins. The stressors of the weaning period can affect the welfare and production of pigs because they can create oxidative stress and an excessive production of ROS that damage tissue and cell of the organism.

In literature there are few data on oxidative stress in farm animals but in recent years has increased the interest in this parameter having it correlations with the onset of many diseases. Sauerwein et al. (2005) showed that in the post-weaning period there is an increased production of ROS concomitant with the serum increase of haptoglobin, index of inflammatory processes. Also Zhu et al. (2012) observed an increase of the concentration of NO, and H<sub>2</sub>O<sub>2</sub> after weaning with a decrease of antioxidant enzymes.

Oxidative stress occurs when the cell antioxidant system is overwhelmed by the production of an excess of reactive oxygen species, which can lead to increased prevalence of infectious disease via impaired immune cell function, and perhaps various sudden death syndromes. The production of ROS in excess is able to initiate uncontrolled oxidative cascades that damage cellular membranes and other cellular components resulting in oxidative stress and eventually cell death. The term ROS is used to cover both the free radical and non-radical oxidants; a free radical contains at least one unpaired electron in the shells around the atomic nucleus and is capable of independent existence. The free radical group includes species such as hydroxyl radical (OH $\cdot$ ), nitric oxide (NO $\cdot$ ) and superoxide (O $2^{\cdot-}$ ). Compounds can also be highly reactive without being radicals. Such non-radical oxidants include peroxyxynitrite (ONOO $\cdot$ ), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and hypochlorous acid (HOCl).

Oxidative stress is commonly and frequently defined as an imbalance between oxidants and reductants (antioxidants) at the cellular or tissutal levels.

Oxidative damage is one result of such an imbalance and includes oxidative modification of cellular macromolecules, cell death by apoptosis or necrosis, as well as structural tissue damage. The presence of free radicals and non-radical reactive molecules at high concentrations is dangerous because of their ability to damage cell organelles. Nitric monoxide (NO), superoxide anions, ROS and nitrogen species (RNS), however, also play important modulating roles in certain signal transduction pathways. Several ROS-mediated reactions protect the cell from oxidative stress and serve to stabilize redox homeostasis. NO and ROS act as signal transducing molecules, modulating vascular tone, monitoring oxygen pressure and production of erythropoietin, as well as playing a role in signal transduction pathways involving membrane receptors as part of various physiological processes (Somogyi et al., 2007).

The high reactivity of free radicals and their short life span illustrate the potential toxic effect and difficulties in preventing oxidative damage. The cellular macromolecules, in particular DNA, proteins and lipids, are natural targets of oxidation.

The digestive system may be affected by an abnormal ROS production. Under normal conditions, hepatic aerobic metabolism involves a steady-state production of pro-oxidants such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are balanced by a similar rate of their consumption by antioxidants. Imbalance in the pro-oxidant/antioxidant equilibrium in favor of pro-oxidants constitutes the oxidative stress phenomenon, a condition that may induce a number of pathophysiological events in the liver. Hepatotoxicity by oxidative stress may be achieved through a direct attack of ROS and RNS on essential biomolecules with loss of their biological functions and cell viability.

Cellular defense systems including antioxidative enzymes, such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSHPx), and non-enzymatic scavengers, such as glutathione, vitamins A and E, protect the organism against oxyradical damage, which may result in DNA strand breaks, protein oxidation and the induction of lipid peroxidation. Glutathione peroxidase (GPX), along with superoxide dismutase (SOD) and catalase (CAT), are considered the main antioxidant enzymes in mammals: superoxide dismutase (SOD) transforms superoxide radical ( $O_2^{\cdot-}$ ) into hydrogen peroxide ( $H_2O_2$ ), catalase (CAT) and glutathione peroxidase (GPx) remove hydrogen peroxide and limit the hydroxyl radical (OH $\cdot$ ) formation, and glutathione-S-transferases (GST) removes toxic products of ROS damage (Halliwell and Gutteridge, 2001).

An increase of the activated form of molecular oxygen species due to overproduction and /or to inability to destroy them may lead to damage in the DNA structure and thus may cause mutations, chromosomal aberrations and carcinogenesis (Akyol et al., 1995).

Free radicals may also stimulate cell growth by damaging specific genes that control the growth rate and differentiation during the promotion phase. To counter the increase in superoxide radical levels, cells increase activities of SOD and CAT (Winzer et al., 2000).

### *1.1.8 Post-weaning pathophysiology alterations: any solutions?*



Immediate PW anorexia is now largely recognized as a major aetiological factor in gut disorders in piglets (Pluske 1997). Many factors interact with each other to affect the alterations seen in both mucosal architecture and biochemistry. Since there is an abrupt change of nutrition associated with the weaning process, it is likely that interactions between dietary growth, protective factors and microorganism with the cell epithelium are important determinants of the way the weaned pig digests and absorbs the food it consumes. A number of advances have been made in the fields of GIT physiology, microbiology and immunology in relation to weaning and nutrition in young pigs. Also the manipulation of the developing gut with an appropriate dietary composition and amount of nutrients are studied in recent years for obtaining an efficient determinant optimal growth of animals, in particular after the banning (2006) from EU countries of the use of chemotherapeutics as growing substances. Various nutritional approaches with different dietary integrations have been tested in the recent years for finding alternatives to the limited use of chemotherapeutics, as well as for enhancing the intrinsic quality of a food product of animal origin, with the aim to optimize the weaning transition period and minimise the occurrence of enteric diseases in the pig farms. Probiotics are an important nutritional option but prebiotics also, as well as synbiotics, organic acids, proteins (spray-dried plasma derivatives) and amino acids, natural plant or herbal extracts can be considered as useful nutritional approaches, thoroughly applied and studied in the practical management of the pig farms. These aspects are necessarily to be carefully evaluated for improving and preserving health status during the entire producing process of pigs above all when intensively reared.

## 1.2 Chronic stress: growing-finishing phase

The growing-finishing phase in pig industries is a key point to be approached with the aim of improving healthy animal status and products (i.e. lean growth at least cost). This necessitates feeding diets formulated to meet target nutrient requirements, with energy and protein being the major components. Because 80% of the total feed cost is associated with the growing/fattening phase of pig production, control of porcine welfare status during this period can lead to improved feed efficiency and generate significant cost savings, thereby increasing profits (Duhamel, 1999).

The major problems in piggeries worldwide are referred to digestive and respiratory diseases, which cause bad growth performances and/or bad meat quality. Two are the critical periods for the production of piggeries: i) weaning, that produce an acute stress response, provoked by abrupt social, nutritional, and environmental changes (this in turn may disrupt homeostasis and thus compromise well-being); and ii) growing-finishing phase, in which environmental factors may cause chronic stress, which is able to impair efficiency of feed utilization and result in significantly reduced growth rates.

Commercial pig production presents a multitude of potentially stressful challenges to the reared animals. Distress is a threat to animal welfare and may impair productivity in both growing and reproducing pigs.

The chronic stress responses include the occurrence of a large number of pathologies such as muscle wasting, gastric ulcers, suppression of growth, alterations in immune and reproductive functions (Sapolsky, 1992; Chrousos and Gold, 1992). However, such deleterious effects tend to be neglected when intensifying production.

Stressors existing in swine production systems during growing- finishing phase would include cold/hot environmental temperatures (Stahly and Cromwell, 1979), microbial infections (Webel et al., 1997), insufficient space allowances (Brumm and Miller, 1996; Wolter et al., 2000), social mixing (Barnett et al., 1993; Marchant et al., 1995) and nutritional deficiencies or imbalances (NRC, 1998). As above mentioned, these stressors may cause growth retardation, changes in hormone release, increases in disease susceptibility, and/or behavioral changes. A physiological response to a chronic presence of stressors (such as heat and spatial restriction) results in activation of the sympathetic nervous system, and the consequent release of catecholamines and glucocorticoids reduces body weight (Breinekova et al., 2006). Indeed, pig performance being subjected to stressors is common in commercial swine production. Stress may cause oxidative changes due to an increase in reactive oxygen species (ROS) or a decrease in the antioxidant status (Lykkesfeldt and Svendsen, 2007).



Among many factors, stocking density increases social stress and influences pig performance. Many studies have reported that reducing space allowance could induce decreases in growth performance of pigs. In addition, at higher stocking densities, the likelihood of heightened aggression, competition and disease outbreak rapidly rises, and when this happens, the negative relationship between space and growth becomes even worse (Lebret et al., 2006). A high level of crowding also results in behavioral changes, which may be indicative of reduced welfare.

Curtis (1996) reported that the reduction in feed intake found in large groups may be caused by an increase in social pressure in larger as compared to smaller groups. This stressful factor becomes complicated not only by heat temperature of the farm but also by the type of the floor, where the animals stay. The heat-induced economic burden is due to increased morbidity, mortality, suboptimal growth, inefficient nutrient utilization, poor sow performance, decreased carcass value and carcass processing problems (St- Pierre et al., 2003). Heat stress compromises intestinal integrity, but this appears to be due to heat-induced reductions in nutrient intake parameters in young pigs. Heat stress directly and indirectly (via reduced feed intake) affects post-absorptive metabolism and intestinal integrity and both variables probably contribute to decreased growth.

The floor type is a determinant factor of certain foot lesions in pigs (Gillman et al., 2009) and can affect their health, performance and behaviour. A higher oxidative stress in pig reared on totally slatted floor was also observed by Rossi et al. (2009). These stress elements create a negative effect on animal welfare and may impair productivity in growing pigs, reduce growth rates and generate significant cost savings, so the use of a specific dietary supplementations should support the pig's potential for lean growth at least cost.

### **1.3 Nutraceuticals and their preventive or potential therapeutical effect**

Food is an important stimulus for the growth and maintaining of gastrointestinal mucosa, and, in this respect, gut structure is influenced by the route of nutrient administration, dietary composition of the meal, and the availability in it of specific nutrients. The term nutraceutical was coined from “nutrition” and “pharmaceutical” in 1989 by Stephen Defelice (Brower 1998). According to him “a nutraceutical is any substance that is a food or a part of food and provides medical or health benefits, including the prevention and treatment of diseases”. There are in their meaning differences between the terms “functional foods” and “nutraceuticals”. When food is being cooked and prepared using scientific intelligence with or without knowledge of how or why it is being used, the food is called “functional food”. Thus, functional food provides the body with the

required amount of vitamins, fats, proteins, carbohydrates, etc. needed for its healthy survival. When functional food aids in the prevention and/or treatment of diseases and/or disorder, it is called “nutraceutical” (Pandey et al., 2010). However, very often the concepts of nutraceuticals, functional or medical foods, or dietary supplements are confusing and they can be frequently used interchangeably. According other Authors “functional food” is a more general term to emphasize foods with specific or strong purposes (Bagchi, 2006 and Koletzko et al., 1998). “Dietary supplements” have more defined health roles such as vitamins, minerals, herbs or other botanicals, amino acids, and other dietary substances intended to supplement the diet by increasing the total dietary intake of these ingredients (Halsted, 2003). Dietary supplements are not intended to treat or cure disease (Ross, 2000), whereas nutraceuticals emphasize the expected results of these products, such as prevention or treatment of diseases (Bagchi, 2006 and Zeisel 1999).

Nutraceuticals differ from dietary supplements in the following aspects: nutraceuticals must not only supplement the diet but should also aid in the prevention and/or treatment of disease and disorder, and nutraceuticals are used as conventional foods or as sole items of a meal or diet (Kalra 2003).

Nutraceuticals should be considered in two ways:

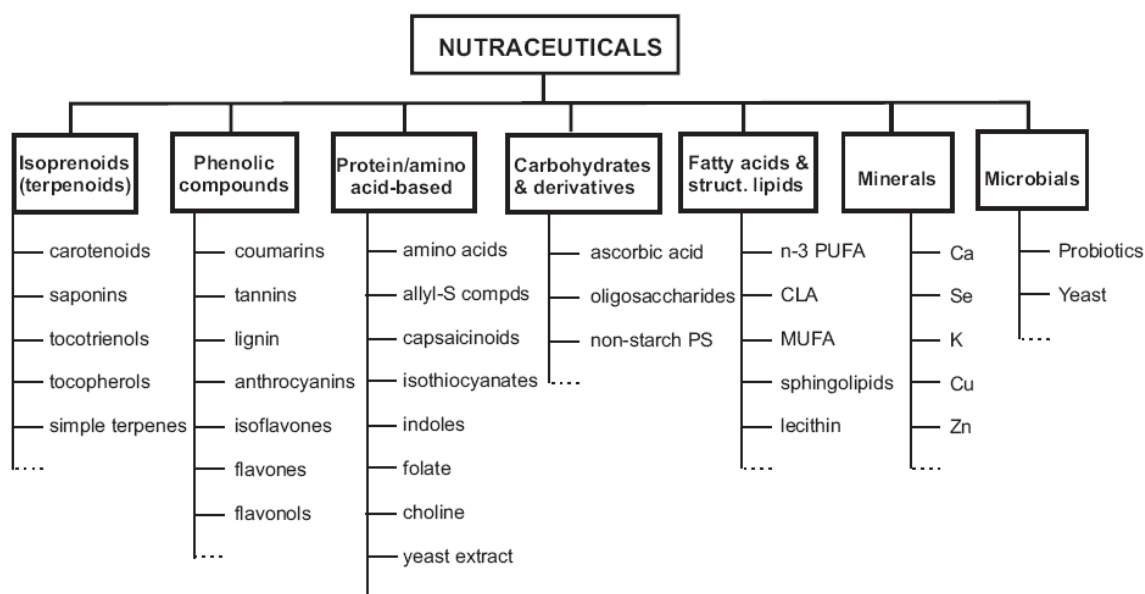
- Potential nutraceuticals;
- Established nutraceuticals.

A potential nutraceutical is a product that holds a promise of a particular health or medical benefit; such a potential nutraceutical becomes an established one only if there are sufficient clinical data to demonstrate such a benefit.

The food products used as nutraceuticals are categorized (proposed by Kokate et al., 2002) in:

- Probiotics
- Prebiotics
- Dietary fibers
- Omega 3 fatty acids
- Antioxidant molecules.

A more complex classification is that one presented by Wildman (2000) (Table 4).



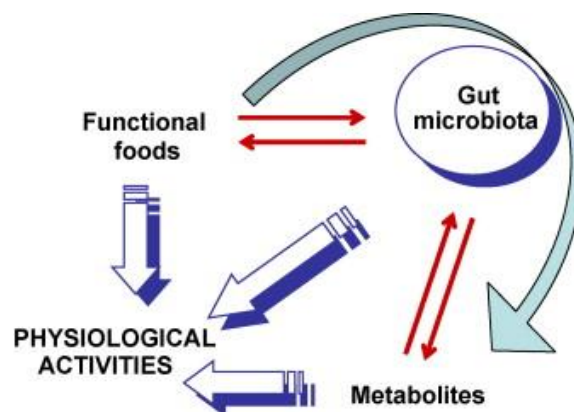
**Table 4.** Groups of nutraceuticals (Wildman 2000)

A rather updated description of them says that nutraceuticals are natural bioactive, chemical compounds that have health promoting, disease preventing or medicinal properties. Nutrients, herbal and dietary supplements are major constituents of nutraceuticals and collaborate each other in maintaining health, act against various disease conditions and thus promote the quality of life. Several studies have demonstrated the relationship between diet and health status, above all in humans. Nutraceuticals have preventive, therapeutic and physiological effects (Cockbill, 1994; Aarts 1998; Duggan et al., 2002).

Various studies attest the beneficial effects of these substances. The health benefits are described in several areas, including cancer, atherosclerosis and other cardiovascular disease (CVD), the aging processes and immune response-enhancing effect, diabetes and mental health. Their contributions in chemoprevention are several (Gholse and Yadav, 2012): they can decrease the side effects of conventional cancer treatment; they can protect normal cells from the indiscriminate damage done by cancer treatment; they can enhance the effects of cancer treatment; they can abrogate or delay the onset of cancer and they can destroy cancer after it appears, at least according the review by Gholse and Yadav (2012).

Numerous nutraceuticals have been found to target and attenuate the risk factors, including oxidative stress and mitochondrial dysfunction, typical of neurodegenerative diseases, thereby preventing or delaying the progression of Parkinson's disease (Chao et al., 2012). They have a role in cardiovascular disease in reduction symptoms and risks, and also in joint disease, like osteoarthritis and rheumatoid arthritis (Chaturvedi et al., 2011).

### 1.3.1 Nutraceutical's effect on gut



**Figure 8.** Interaction between gut microorganisms and “functional foods”

Food is an important stimulus for the growth and maintaining of gastrointestinal mucosa, and, in this respect, gut structure is influenced by the route of nutrient administration, dietary composition of meal and availability in it of specific nutrients. Nutrition is obviously a critical determinant in the functional development and growth of the gastrointestinal tract. Functional feed and nutraceuticals are alternative substances to the use of antibacterial agents and chemotherapeutics during weaning in the rearing farm, as they can adequately stimulate the local defensive responses, and favorably influence resident gastrointestinal microflora, but are also able to improve nutrient digestion and absorption (Domeneghini et al., 2006). They may act either alone or in a synergistic manner as classical nutrients to modulate the growth, repair and barrier function of the gastro-intestinal mucosa (Ziegler et al., 1999).

A long list of phytochemicals has been demonstrated to exert gastro protective effects in *in vivo* experimental trials (Romano et al., 2012).

In the stomach there is a massive production of reactive oxygen species (ROS), their concentration being 1000-fold higher than that in other tissues or plasma (Hiraishi et al., 1993). Generation of ROS contributes to exogenous injury to the gastric mucosa, including damage brought about by ethanol or nonsteroidal anti-inflammatory drugs (NSAIDs) (Mizui et al., 1987; Pihan et al., 1987). Moreover, ROS play a major role in the multistep process leading to the development of gastric cancer (Oliveira et al., 2003).

In the past few years much effort has been made to provide solid knowledge of the mechanisms underlying the beneficial effects of nutraceuticals in the gastrointestinal tract. In particular, a major area of interest is for the use of biologically active chemical components of plants, i.e. phytochemicals, in a

number of gastrointestinal disorders (Romano et al., 2012). While the major focus of phytochemical research has been on cancer prevention, several products of plant origin are being used and/or under study for a variety of other gastrointestinal problems in human and animals.

The health status of an animal has a marked effect on its performance. An understanding of gastro-intestinal anatomy, physiology and function is critical to achieving optimum nutritional efficiency. The interaction between the physical and chemical environment within the intestinal lumen and the bacterial population is very influential on the enteric health of the animal. Maintenance of a good symbiotic relationship between the host animal and its intestinal microflora is now recognized as a critical component in the development of good nutritional strategies. The metabolic activity of the gut microbiota on bioactive food components can modify the host exposure to these components and their potential healthy effects. Food bioactive compounds also exert significant effects on the intestinal environment, modulating the gut microbiota composition and probably its functional effects on mammalian tissue (Laparra and Sanz, 2010).

A great deal of literature have demonstrated that several nutrients are available that may play a role in the pig nutritional plan for their trophic and cytoprotective effects on the gastrointestinal apparatus (Vente-Spreuwwenberg and Beynen, 2003; Torrallardona et al., 2003, 2007; Domeneghini et al., 2004; Huang et al., 2004; Manzanilla et al., 2004; Bontempo et al., 2006; Kommera et al., 2006; Mroz et al., 2006; Pierce et al., 2006, 2007).

### *1.3.2 EU legislation*

Safe animal feed is important for the health of animals themselves, the environment and for the safety of foods of animal origin, as well. There are many examples of the close relationship between the safety of animal feed and the foods we eat. The most famous case was about mammalian meat and bone meal (MBM), which was banned from all farm animal feed in the EU since 2001 because of the spread of Bovine Spongiform Encephalopathy (BSE) in cattle: BSE infected meat was associated with the variant Creutzfeldt-Jakob Disease (vCJD) in humans.

Feed additives play an important role in modern livestock production and are a focus of the EU regulatory framework. According to Regulation (EC) No 1831/2003, only additives that have been thoroughly submitted to an official authorization procedure may be placed on the market. Authorization guidelines are for: specific animal species, specific conditions of use, and ten years periods. Additives may be classified into the following categories:

- **Technological additives** (e.g. preservatives, antioxidants, emulsifiers, stabilizing agents, acidity regulators, silage additives)

- **Sensory additives** (e.g. flavours, colorants)
- **Nutritional additives** (e.g. vitamins, minerals, aminoacids, trace elements)
- **Zootechnical additives** (e.g. digestibility enhancers, gut flora stabilizers)
- **Coccidiostats and histomonostats**

The European Food Safety Authority (EFSA) is responsible for conducting the evaluation of the data submitted requesting authorizations. EFSA evaluates the safety and efficacy of each additive and checks for adverse effects on human and animal health and on the environment. After a favorable opinion of the EFSA, the Commission prepares a draft Regulation to grant authorization, following the procedure involving Member States within the Standing Committee on the Food Chain and Animal Health - Animal Nutrition ([http://ec.europa.eu/food/food/animalnutrition/feedadditives/legisl\\_en.htm](http://ec.europa.eu/food/food/animalnutrition/feedadditives/legisl_en.htm)). EFSA has a key role in providing independent scientific advice to support the authorization process for feed additives. This work is carried out by EFSA's Panel on additives and products or substances used in animal feed (FEEDAP). Co-operating with the European Commission, the Panel carries out an evaluation of each new additive submitted for authorization. Companies wishing to market a specific additive in the EU need to submit an application with information on the identity of the additive, its conditions of use, control methods and data demonstrating its efficacy and safety.

The European legislation on animal feed provides a framework for ensuring that feedstuffs do not present any danger to human or animal health or to the environment. It includes rules on the circulation and use of feed materials, requirements for feed hygiene, rules on undesirable substances in animal feed, legislation on genetically modified food and feed and conditions for the use of additives in animal nutrition (<http://www.efsa.europa.eu/en/topics/topic/feed.htm>).

The Regulation contains also additional provisions. For example it lays down provisions phasing out the authorizations of antibiotic feed additives as from 1 January 2006. It also contains certain provisions regarding the labeling and packaging of feed additives and procedures for supervision, modification, suspension, revocation and renewal of authorizations, and about confidentiality and data protection. The Regulation also contains certain provisions regarding transitional measures and about the status of products placed on the market before the entry into force of Regulation (EC) No 1831/2003

In accordance with Article 17 of Regulation (EC) No 1831/2003 on additives for use in animal nutrition, the Commission has established a Community Register of feed additive (Revision 152 released 04 December 2012).

There are many nutraceutical substances which may affect healthy animal's status, i.e. prebiotic and probiotic, amino acids, organic acids, proteins, PUFA, plant extracts, micronutrients and antioxidants (phytochemicals). During my PhD thesis I have focused my attention on the latter two.

#### **1.4 Micronutrient use in animal nutrition**

Micronutrients include all vitamins and minerals. Vitamins and trace minerals are important antioxidant components of animal diets and their roles in maintaining animal health and correct immune functions are to be considered indispensable.

Vitamin A inhibits iNOS gene transcription in vascular smooth muscle cells, endothelial cells, cardiac myocytes, and mesangial cells. 1,25-Dihydroxyvitamin D<sub>3</sub>, vitamin K<sub>2</sub>, and niacin inhibit iNOS expression in inflammatory cells of the brain (macrophages, microglia, and astrocytes), the bleomycin-mouse model of lung fibrosis, and vascular smooth muscle cells, respectively. In addition, many carotenoids suppress iNOS expression and inducible NO synthesis in activated macrophages and promyelocytic HL-60 cells. By reducing NO generation by iNOS, these vitamins play an important role in preventing radical induced cytotoxicity, thus displaying antiatherogenic and antineuroinflammatory roles (Fang et al., 2002).

Vitamins also directly scavenge ROS and upregulate the activities of antioxidant enzymes. Among them, vitamin E has been recognized as one of the most important antioxidants. Vitamin E inhibits ROS-induced generation of lipid peroxyl radicals, thereby protecting cells from peroxidation of PUFA in membrane phospholipids, from oxidative damage of plasma very low-density lipoproteins, cellular proteins, DNA, and from membrane degeneration. A dietary deficiency of vitamin E reduces the activities of hepatic catalase, GSH peroxidases, and glutathione reductase (Chow et al., 1969), induces liver lipid peroxidation, and causes neurologic and cardiovascular disorders (Muller 1990; Carr et al., 2000), all of which can be reversed by dietary vitamin E supplementation. In support of the critical antioxidant role of vitamin E, Yokota et al. (2001) demonstrated increases in brain lipid peroxidation and neurodegeneration in mice with a deficiency of  $\alpha$ -tocopherol transfer protein.

The antioxidant role of physiologic concentrations of vitamin C has been well established in the literature. For example, Fischer-Nielsen et al. (1992) found that vitamin C exhibits a protective effect against free radical-induced oxidative damage. In addition, dietary supplementation of vitamin B<sub>12</sub> and folic acid reduces radical-induced radiation damage, improves blood leukocyte cell counts, and decreases the mortality of irradiated rats. Remarkably, a deficiency of dietary choline increases hepatic concentrations of 8-hydroxydeoxyguanosine (an indicator of DNA damage) and promotes the growth of hepatocellular

carcinomas in rats, which can be prevented by dietary supplementation of choline or vitamin C. Further, folate, vitamin B6, and vitamin B12 are required for homocysteine metabolism by serving as cofactors for methionine synthase (B12), cystathionine synthase (B6), and cystathionase (B6) and as a substrate (5-methyltetrahydrofolate) for methionine synthase. Because homocysteine contributes to oxidative stress in endothelial cells, these vitamins help reduce the risk of cardiovascular disease in humans and animals (Fang et al., 2002).

The importance of trace mineral nutrition relative to the maintenance of productivity and prevention of deficiency symptoms has been largely recognized (Miller, 1981; NRC, 2001). Zinc is widely distributed throughout the body as a component of metalloenzymes and metalloproteins (Vallee and Falchuk, 1993). Zinc finger proteins play an integrated role in regulating gene expression, consequently impacting a wide variety of body functions including cell division, growth, hormone production, metabolism, appetite control, and immune function (Predieri et al., 2003; Vallee and Falchuk, 1993). Zinc has a catalytic, coactive, or structural role in a wide variety of enzymes that regulate many metabolic and immune processes (Vallee and Falchuk, 1993). Copper and Manganese function as components of metalloenzymes that take part in reduction reactions. These metalloenzymes are involved in multiple physiological processes including respiration, carbohydrate and lipid metabolism, antioxidant activities, and collagen formation (Andrieu, 2008; NRC, 2001; Tomlinson *et al.*, 2004). In addition, ceruloplasmin, that binds up to 95% of circulating Cu, regulates iron availability, takes part in oxidation-reduction reactions, and may regulate immune function (Healy and Tipton, 2007). Like Zn, both Cu and Mn are important for keratin formation and are components of SOD (Tomlinson *et al.*, 2004).

Iron is an essential element in the diet. It is a component of body systems that are involved in the utilization of oxygen. It forms part of haemoglobin, the red pigment in erythrocytes, which allows oxygen to be carried from the lungs to the tissues.

Selenium functions as a component of at least 25 different selenoproteins (Andrieu, 2008). In these proteins, Se allows the proteins to donate hydrogen and take part in reduction reactions. Selenoproteins include the enzyme iodothyronine deiodinase, which is important in regulating general metabolism, and glutathione peroxidase and thioredoxin reductase, which are important components of antioxidant and immune systems (Andrieu, 2008; NRC, 2001).

Zinc, Cu, and Mn are integrated components in these systems due to their presence in SOD which reduces the superoxide free radical to hydrogen peroxide. Selenium is a component of glutathione peroxidase, which then converts hydrogen peroxide into water.



Due to the diversity of proteins and enzymes containing Zn, Cu, Mn, and Se, these trace minerals are essential for a wide variety of physiological processes regulating growth, production, reproduction, and health. Deficiencies in these nutrients consequently lead to reduced performance, and dairy cattle diets are formulated with trace mineral supplements to prevent deficiencies (Miller, 1981; NRC, 2001). However, chemical composition of trace mineral supplements varies among different species, and research is showing that some supplements are better available to support animal productivity and health than others.

I have with special attention analyzed the role of copper as dietary supplemented in pig.

#### 1.4.1 Copper (Cu)

*Copper* is an essential trace element that play a vital role in the physiology of animals: it is necessary for foetal growth and early post-natal development, for haemoglobin synthesis, for a correct connective tissue structure especially in the cardiovascular system and in bones, for a proper nerve function and bone development, and for an adequate development of inflammatory processes (EUROPEAN COMMISSION 2003). Copper is the third most abundant trace mineral in the body, but it is often deficient because food sources high in this mineral are not frequent.

Copper works together with iron to make red blood cells, and it is the major inorganic component of epinevrium and collagen. Copper is involved in maintenance of immunity, as well as in fertility, formation of melanin, and the promotion of correct pigmentation. It is believed to play a role in preventing high blood pressure, heart arhythmia, oxidation of the cells, and keeping cholesterol low (Caley 2012). Copper is used by the body to manufacture numerous enzymes, many of which work as antioxidants.

Several investigations have shown that the addition of copper to the diets of pigs increases their growth performances (Stahly et al., 1979; Cromwell et al., 1989, 1998; Hill et al., 2000). This positive effect on growth seems to be dependent on a simultaneous increase in feed intake. The addition of supranutritive levels of Cu to the diets of young growing pigs increases their average daily gain and gain efficiency (Zimmerman, 1986).

Cu has been recognized as having antibacterial and antimycotic activity and the ability to indirectly stimulate growth. Several observations revealed that levels of copper sulphate incorporated in the diet modified quantitatively some Gram positive bacterial intestinal populations as demonstrated for *Streptococcus* spp. (Dunning and Marquis, 1998). Cu may promote the processes of tissue repair in the small intestine and stimulate the synthesis of digestive enzymes, resulting in a better digestion and absorption of nutrients and potentially improving growth

performances. A considerable number of experiments have been performed on animals of different body weight, different ages and different levels with various results (Braude 1948; Barber et al., 1965). Just considering such a number of different results obtained in different pig trials, I have performed a study aimed at a special morpho-functional analysis of the pig small intestine.

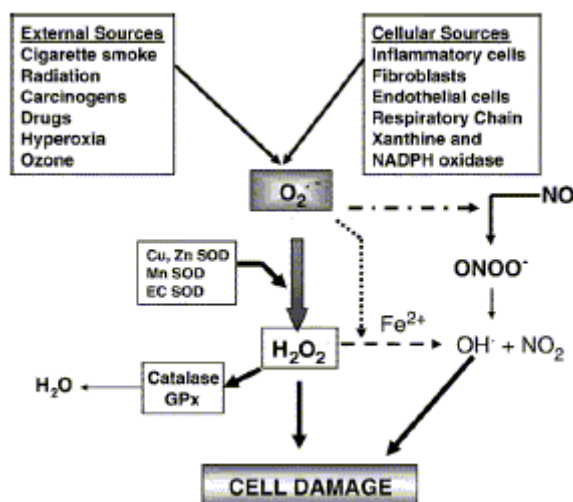
## **1.5 Antioxidant use in animal nutrition**

In the recent years, antioxidants have gained a high level of scientific importance because of their potential as prophylactic and therapeutic agents in many diseases. Exposure to free radicals from a variety of sources has led organisms to develop a series of defence mechanisms (Cadenas, 1997). Defence mechanisms against free radical-induced oxidative stress involve: i) preventative mechanisms, ii) repair mechanisms, iii) physical defences, and iv) antioxidant defences.

The discovery of the role of free radicals as possible causative agents in cancer, diabetes, cardiovascular diseases, autoimmune diseases, neurodegenerative disorders, aging, has led to a medical revolution that is promising a new paradigm of healthcare (Ratnam et al., 2006). In a biological system, an antioxidant can be defined as “any substance that when present at low concentrations compared to that of an oxidizable substrate would significantly delay or prevent oxidation of that substrate” (Halliwell et al., 1995). The oxidizable substrate may be any molecule that is found in either foods or biological substrates, including carbohydrates, DNA, lipids, and proteins. Concerning food that is composed, among others, of a variety of biomolecules, the US official regulatory bodies that overlook the food-supply simply categorize antioxidant substances as food additives, and define them as “substances used to preserve food by retarding deterioration, rancidity, or discoloration due to oxidation” (Code of Federal Regulations, Food and Drug Administration) (Wanasundara and Shahidi 2005). On the other hand, the definition proposed by the Panel on Dietary Antioxidant and Related Compounds of the Food and Nutrition Board is that “a dietary antioxidant is a substance in food that significantly decreases the adverse effects of reactive oxygen species (ROS), reactive nitrogen species (RNS), or both on normal physiological functions in human” (2002). ROS and RNS are generated at cellular level from physiological processes directed to produce energy and metabolites or to generate defenses against invasive microorganism.

Antioxidants (Fig.9) are substances that may protect cells from the possible damage caused by unstable molecules known as free radicals: antioxidants interact with and stabilize free radicals and may prevent some of the damages, which free radicals might otherwise cause. Free radical damage may lead to cancer. Examples of antioxidants include beta-carotene, lycopene, vitamins C, E,

A and other substances (Sies, 1997). An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals, which start chain reactions that may damage cells.



**Figure 9.** Schematic representation of the formation of ROS/RNS (GliSODin; May 2009. <http://mydrinkact.biz/pdf/sod.pdf>.)

Antioxidants terminate these chain reactions by removing free radical intermediates and inhibit other oxidation reactions by being oxidized themselves. As a result, antioxidants are often reducing agents such as thiols, ascorbic acid or polyphenols (Sies, 1997).

Although oxidation reactions are crucial for life, they can also be damaging; hence, plants and animals maintain complex systems of multiple types of antioxidants, such as glutathione, vitamin C and vitamin E as well as enzymes such as catalase, superoxide dismutase and various peroxidases. Low levels of antioxidants, or inhibition of the antioxidant enzymes, causes oxidative stress and may damage or kill cells. As oxidative stress might be an important part of many human diseases, the use of antioxidants in pharmacology is intensively studied, particularly as treatments for stroke and neurodegenerative diseases (review by Hamid et al., 2010). However, it is unknown whether oxidative stress is the cause or the consequence of disease. Antioxidants are also widely used as ingredients in human dietary supplements in the hope of maintaining health and preventing diseases such as cancer and coronary heart disease (Hamid et al., 2010).

Antioxidants are abundant in fruits and vegetables, as well as in other foods including nuts, grains and some meats, poultry and fish.

Antioxidants are grouped in two classes:

- (1) **Primary or natural antioxidants.**
- (2) **Secondary or synthetic antioxidants.**

**Primary** antioxidants are the chain breaking substances, which react with free radicals and convert them into more stable products (Hamid et al., 2010). Antioxidants of this group are mainly phenolic in structures and include the following ones (Hurrell, 2003):

- Antioxidants: minerals - These are co-factors of antioxidants enzymes. Their absence will definitely affect metabolism of many macromolecules such as carbohydrates. Examples include selenium, copper, iron, zinc and manganese.

- Antioxidants: vitamins – They are needed for most body metabolic functions. They include: vitamin C, vitamin E, vitamins B.

- Phytochemicals - These are complex chemicals found in plants, in which phenolic compounds are present that are neither vitamins nor minerals. These include: terpenoids, polyphenolics, glucosinolates, thiosulfonates, phytosterols, anthraquinones, capsaicin, piperine, chlorophyll, betaine, pectin, oxalic acid

**Secondary** antioxidants are synthetic phenolic compounds that perform the function of capturing free radicals and stopping the chain reactions, they include (Hurrell, 2003):

- i. Butylated hydroxyl anisole (BHA).
- ii. Butylated hydroxytoluene (BHT).
- iii. Propyl gallate (PG) and metal chelating agent (EDTA).
- iv. Tertiary butyl hydroquinone (TBHQ).
- v. Nordihydro guaretic acid (NDGA).

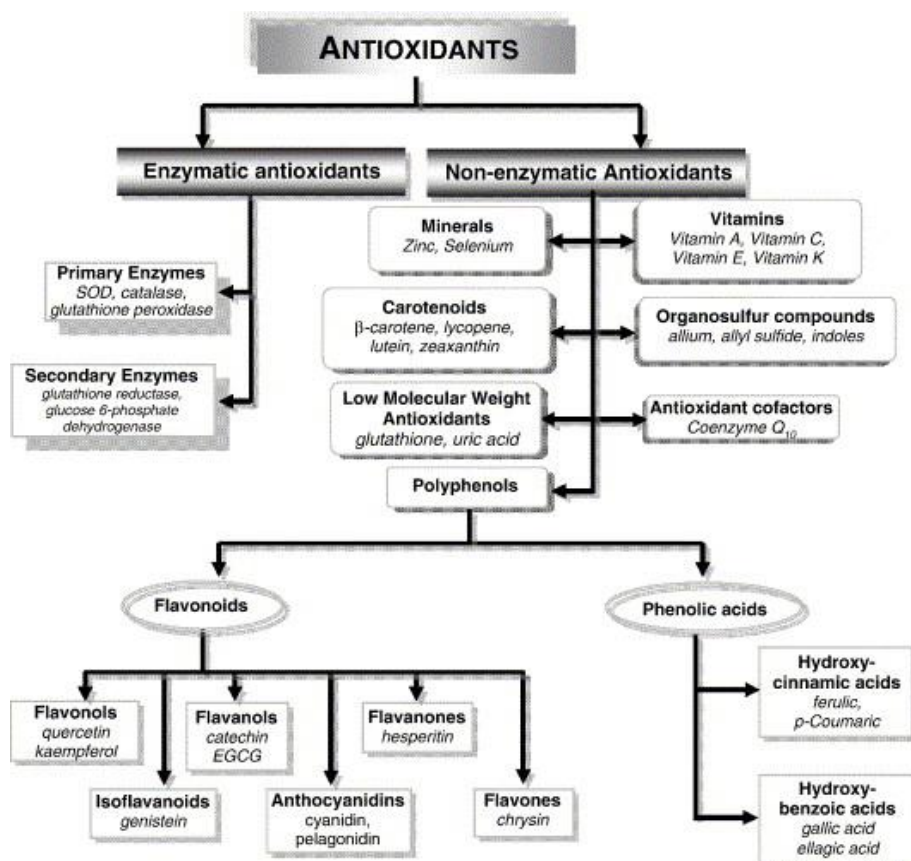
Two principle mechanisms of action have been proposed for antioxidant. The first is a chain-breaking mechanism by which the primary antioxidant donates electrons to the free radicals present in the system. The second mechanism involves removal of ROS and RNS initiator by quenching chain initiator catalyst (Hamed et al., 2010).

Generally, free radicals attack the nearest stable molecules abstracting its peripheral electron/s to attain stability. When the attacked molecule loses its peripheral electron/s, it becomes a free-radical itself, these formations of free-radicals continue on and on, and finally result in the disruption of the substance. Free-radical contribute to the occurrence of many different diseases above all when formed in excess. Chemically, a molecule is oxidized when electrons are removed and reduced when electrons are added. The body generates energy by gradually oxidizing the molecules and macromolecules contained in its food in a controlled manner and storing it in the form of chemical potential energy called

ATP (Adenosinetriphosphate). Free-radicals are generated largely during the production of ATP in the mitochondria. During this process, radicals coming out from the mitochondria from reactive oxygen species such as superoxide anion and hydroxyl radicals and other reactive oxygen species such as singlet oxygen, impair the body system especially at the site where the free radicals have been generated.

A healthy cell has its mortal enemies in free-radicals. Free-radicals constantly seek out healthy cells and attack their vulnerable membranes eventually causing cellular degeneration and death. Normal molecules in the body have two (a paired group) electrons in their outer shell. A molecule with a single electron (unpaired) in its outer shell is called a free radical. The destructive effects of free-radicals can be prevented with the addition of antioxidant in the diet or by antioxidant supplements. A good anti-oxidant complex supplement actually has advantages over diet sources in that the complex has many different specific types of anti-oxidants which seek out and destroy free radicals at many various cellular sites. A single antioxidant, for example Vitamin E, only protects the outer fatty layers of the cell. It will not stabilize DNA which, for example, is one the main effects of the anti-oxidant Vitamin C. The process by which different anti-oxidants disperse through the bloodstream to protect the cells at different sites is referred to in science as "anti-oxidant synergy." When a specific anti-oxidant meets a free radical in the bloodstream at its appropriate activity site, it naturally combines with it and converts the free radical to harmless water and oxygen. As a result, as anti-oxidant increases due to the supplementation of higher amounts of a greater variety of anti-oxidants, cellular damage lessens and performance and health improves (Hamid et al., 2010).

The body has developed several endogenous antioxidant systems to counteract the production of reactive oxygen intermediates (ROI). These systems can be divided into enzymatic and non-enzymatic groups. Ratnam et al. (2006) classified antioxidants as follows (Fig. 10):



**Figure 10.** Classification of antioxidants (from Ratnam, 2006)

Some of these antioxidants are endogenously produced: they include enzymes, low molecular weight molecules and enzyme cofactors. Among non-enzymatic antioxidant, many are obtained from dietary sources. Dietary antioxidant can be divided into various classes, of which polyphenols is the largest class (Ratman et al., 2006).

Antioxidants can cancel out the cell-damaging effects of free radicals. This statement is supported by studies performed in a variety of scientific areas, including physiology, pharmacology, nutrition and even food processing (Magalhaes et al., 2009).

Dietary antioxidants confer significant protection to intestinal epithelial cells from pro-apoptotic oxidant stress. The phytochemical mixtures found for example in the teas, cat's claw (*Uncaria guianensis*, obtained as a freeze dried concentrate from Rainforest Phytoceuticals, LLC, Delmar, NY) and green tea, appear to be more effective than vitamin C in reducing apoptosis in some cell lines, and this at concentrations that suggest that they may be acting at levels distinct from the mere scavenging of the oxidant signal (Miller et al., 2001). Diet supplementation with these or related antioxidants may prove valuable in limiting the pathophysiology of numerous disorders associated with gut inflammation. Different studies attest the efficacy of antioxidant dietary

supplementation in gut diseases (Burke et al., 1997; Kruidenier and Verspaget, 1998; Miller et al., 2001; Langmead et al., 2002; Calder, 2008).

It is now well recognized that reactive oxygen species (ROS) such as superoxide, the hydroxyl radical, hydrogen peroxide, hypochlorous acid and oxidant derivatives, such as N-chloramines, are produced in excess by the inflamed mucosa in inflammatory bowel disease (IBD) and may be pathogenic by their own. Oxidative damage is involved in the pathogenic process of idiopathic chronic inflammatory bowel disease. Although specific intervention in the oxidative cascade showed promising results in animal models and preliminary human patient trials, the clinical efficacy of antioxidants still has to be established (review by Kruidenier and Verspaget, 2002, Rezaie et al., 2007). Diet supplementation with antioxidants may prove valuable in limiting the pathophysiology of numerous disorders associated with gut inflammation (Miller et al., 2001). In recent years there is a growing evidence that reactive oxygen species contribute to organ injury in many systems including the heart, liver and central nervous system (Marubayashi et al., 1985). The literature in the last years focus on the antioxidant effect on liver damage (Cai et al., 2002; Khan et al., 2005; Olaleye and Rocha 2007; Stagos et al., 2012), in particular for their antioxidant, anticancer, antimutagenic and anti-inflammatory effects displayed by antioxidants.

Many studies demonstrate the beneficial effect of antioxidants in the prevention and protective therapy for liver injury, and there are a number of compounds classified as nutraceuticals that have proven effective in supporting the diseased or damaged liver (Taboada 2011). An oxidative stress status is characterized by depletion of endogenous antioxidant enzyme activities such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione S-transferase (GST). Different studies suggest that dietary antioxidants may protect the liver against oxidative stress-inducing effects of chemicals by modulating the antioxidant enzymes and oxidative status of hepatocytes (De Oliveira et al., 2012). Khan et al., (2006) demonstrated the protective effects of black tea extract on the levels of lipid peroxidation and antioxidant enzymes in liver of mice with pesticide-induced liver injury. De Oliveira et al., (2012) demonstrated in vivo and in vitro the antioxidant activity and hepatoprotective properties of polyphenols from *Halimeda opuntia* Lamouroux. Also Skrzydlewska et al., (2002), Frei and Higdon (2003), Yuan et al., (2006) and Abolfathi et al. (2012) demonstrated the beneficial effect of tea polyphenols on damaged liver. In spite of this proof of beneficial effects, many questions related to antioxidant effect of phytochemical extracts remain unanswered and much more work is clearly needed, above all comparing the trials conducted in different species.

### 1.5.1 Phytochemicals

Animal studies have shown that dietary phytochemical antioxidants are capable of removing free radicals. Among them, phenolic and polyphenolic compounds, such as the flavonoids and catechins contained in edible plants, exhibit potent antioxidant activities (Fang et al., 2002)

A large body of literature (Fang et al., 1998a, 1998b, Yang et al., 1998, Cui et al., 2000; Voudin et al., 2000; Fang et al., 2002) has documented the beneficial effects of tea polyphenolic compounds on scavenging free radicals and on their role in the prevention and therapy of disease. They enhance red blood cell resistance to oxidative stress *in vitro* and *in vivo*, effectively scavenge superoxide and hydroxyl radicals, and inhibit oxidative modification of low density lipoproteins. Dietary supplementation by tea polyphenols decreases serum concentrations of total cholesterol and malondialdehyde (an indicator of lipid peroxidation) and increases serum concentrations of high density lipoproteins in humans. Tea polyphenols are beneficial for the treatment of coronary heart disease, hypertension, and type 2 diabetes.

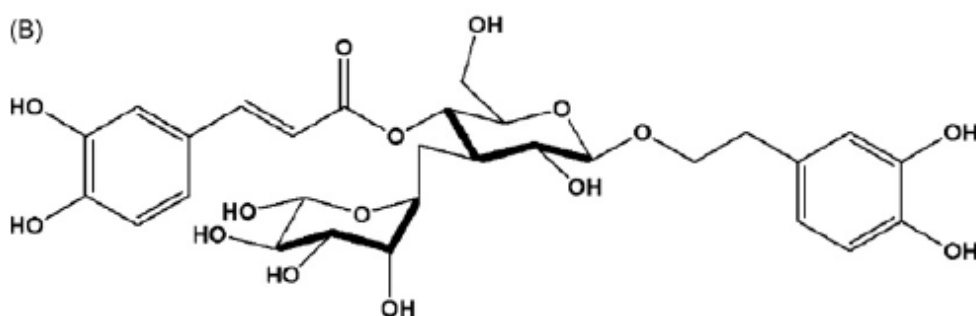
$\beta$ -carotene and other carotenoids, such as  $\alpha$ -carotene,  $\gamma$ -carotene, and  $\beta$ -cryptoxanthin, are potent antioxidants of plant origin,  $\beta$ -carotene reacts with a peroxy radical to form a resonance-stabilized carbon-centered radical within its conjugated alkyl structure, thereby inhibiting the chain propagation effect of ROS. Although lycopene, lutein, canthaxanthin, and zeaxanthin do not possess provitamin A activity, their antioxidant actions are similar to, or even greater than, those of  $\beta$ -carotene. The biochanin A phytoestrogen dose-dependently inhibits iNOS expression, inducible NO synthesis and the growth of the MCF-7 human breast cancer cell line. For a comparison also, glucosamine, which has been used to treat arthritis in humans and dogs, inhibits inducible NO synthesis by suppressing iNOS protein expression in activated macrophages of many tissues (e.g., liver and lung).

Research on flavonoids and other polyphenols, their antioxidant properties, and their effects in disease prevention truly began after 1995, and in the recent years polyphenols become the principal class of phytochemicals, that receive a large attention for their potential antioxidant effects and health beneficial.

My attention focalized one class of polyphenols: Phenylpropanoid derivates and in particular Verbascoside.



### 1.5.2 Verbascoside



**Fig 11** Structure of Verbascoside

Verbascoside (VB) is a phenylpropanoid glycoside (PPG), a water-soluble derivative of phenylpropanoids (PPs), a large group of polyphenols. There is a growing evidence that PPGs are powerful antioxidants by either direct scavenging reactive oxygen and nitrogen species, or acting as chain-breaking peroxy radical scavengers (Afanas'ev 2005). The biological properties of verbascoside, also known as acteoside, have been described in the literature and comprise a wide spectrum of activities, including antioxidant, anti-inflammatory, photo-protective and chelating actions. In particular, an important antioxidant effect of verbascoside has been recently documented by Aleo et al. (2005) in an experimental study that compared numerous natural antioxidant substances using various method of determination. Already in 1996 Wang et al., have studied the scavenging effect of verbascoside on superoxide anion and hydroxyl radical, and they demonstrated that verbascoside is an effective hydroxyl radical scavenger, and the number of phenolic hydroxyl group in the structure of the verbascoside is directly related to its scavenging activities (Wang et al., 1996).

Verbascoside possesses numerous biological properties, that include: antioxidant (Wong et al., 2001), antibacterial (Rigano et al., 2007) and anticancer activity (Ohno et al., 2002; Lee et al., 2007; Picerno et al., 2005). Verbascoside has also a significant neuroprotective effect acting against neurotoxicity induced by 1-methyl-4-phenylpyridinium ion, which induce either apoptosis or necrosis via mitochondria dysfunction. It may be useful for treatment of patients with Parkinson's disease (Sheng et al., 2002).

Very interesting is the anti-inflammatory activity of verbascoside. VB has been shown to modulate nitric oxide (NO) production and the expression of inducible nitric oxide synthase (iNOS) in activated macrophages (Xiong et al. 2000; Lee et al. 2005). It also inhibits histamine, arachidonic acid release, and prostaglandin E2 production in RBL-2H3 mast cells, suggesting a possible application of the compound as an anti-inflammatory remedy (Lee et al. 2006).

There is a large body of evidence showing that the production of reactive oxygen and nitrogen species plays key roles in inflammatory bowel disease (Grisham, 1994; Szabo, 2003). In addition, it has been shown that ROS causes DNA single-strand damage, leading to poly(ADP ribose) synthetase activation and cell death (Zingarelli et al., 1997). Some evidence exists to support the possible role of poly(ADP ribose) synthetase activation in inflammatory bowel disease (Szabo et al. 1997). Mazzon et al. (2009) described the anti-inflammatory effects of VB in a rodent model of colitis and showed that the activity of VB may be dependent upon a combination of the following pharmacological properties of this agent: i) VB scavenges ROS including hydroxyl radicals, superoxide anions and NO, which would prevent the formation of peroxynitrite. This, in turn, prevents the activation of PARP (peroxisome proliferator-activated receptor) and the associated tissue injury; ii) VB reduces the recruitment of PMNs (polymorphonuclear neutrophils) into the inflammatory site. This effect of VB is very likely secondary to the prevention exerted by VB of endothelial oxidant injury and, hence, a preservation of endothelial barrier function as well as to the inhibition of NF- $\kappa$ B p65 expressions and MMP-9 (metalloproteinase type 9) and MMP-2 (metalloproteinase type 2) expression. These results support the view that the overproduction of reactive oxygen or nitrogen-free radicals contributes to IBD. So VB, which permeates biological membranes and functions as an intracellular radical scavenger, may be useful in the therapy of conditions associated with local or systemic inflammation (Mazzon et al., 2009).

Also Esposito et al. (2010), correlated the anti-inflammatory activity of verbascoside in a dinitrobenzenesulfonic acid (DNBS)-induced inflammatory bowel disease model with an enhancing of peroxisome proliferator-activated receptor (PPAR)-*a* activity. These observations suggest that verbascoside could use the same pathway as PPAR-*a* agonists in inflammatory diseases.

More in general, beneficial effects of herbs or botanicals in farm animals may arise from activation of feed intake and secretion of digestive secretions, immune stimulation, anti-bacterial, coccidiostatic, antihelminthic, antiviral or anti-inflammatory activity and either inhibition of oxidation or antioxidant properties (Wenk, 2003). The current evidence for protective effects of polyphenols against diseases incidence has generate new expectations for improvement in health (Scalbert *et al.*, 2005). Verbascoside and teupolioside are two phenylpropanoides including two polyphenolic rings and two (verbascoside) or three (teupolioside) carbohydrates connected into an olisaccaride chain. Corino et al. (2004), evaluated the effects of verbascoside and teupolioside, produced by plant cell lines of *Syringa vulgaris* and *Ajuga reptans* respectively, in post-weaning piglets on growth performances and oxidative status measured as reactive oxygen metabolites (ROMs). The natural antioxidant extracted from *Syringa vulgaris* used as dietary supplement showed higher average daily gain ADG and final weight

than control animals; such as dROMs showed an improved oxidative stability in both integrations of verbascoside. Feeding natural integration with phenylpropanoides glucoside could be considered in order to improve growing performances in post-weaning piglets especially in view of prohibition of use of antibiotics as growing substances (Corino et al., 2007).

## 1.6 References

**Abolfathi A.A., Mohajeri D., Rezaie A., Nazeri M.** (2012). Protective effects of green tea extract against hepatic tissue injury in Streptozotocin-induced diabetic rats. *Evidence-Based Complementary and Alternative Medicine*, Article ID 740671, 10 pages doi:10.1155/2012/740671

**Afanas'ev I.B.** (2005). Superoxide and nitric oxide in pathological conditions associated with iron overload: the effects of antioxidants and chelators. *Current Medical Chemistry*, 12: 2731–2739.

**Akyol Ö., Arslanoglu R., Durak** (1995). Activities of free radical and DNA turn-over enzymes in cancerous and non-cancerous human brain tissues. *Redox Report* 1, 255- 2597.

**Aleffi S., Petrai I., Bertolani C., Parola M., Colombatto S., Novo E., Vizzutti F., Anania F.A., Milani S., Rombouts K., Laffi G., Pinzani M., Marra F.** (2005). Upregulation of proinflammatory and proangiogenic cytokines by leptin in human hepatic stellate cells. *Hepatology*, 42 (6): 1339–1348.

**Aleo E., Ricci R., Passi S., Cataudella S.** (2005). A novel cyt-H<sub>2</sub>O<sub>2</sub>-chemiluminescences assay for measuring the reducing/antioxidant capacity on hydrophilic and lipophilic antioxidants and biological samples. *Progressive Nutrition*, 3, 154-182.

**Andrieu, S.** (2008). Is there a role for organic trace element supplements in transition cow health? *Veterinary Journal*, 176:77-83.

**Atuma C., Strugala V., Allen A., Holm L.** (2001). The adherent gastrointestinal mucus gel layer: Thickness and physical state in vivo. *American Journal of Physiology Gastrointestinal and Liver Physiology* 280:G922–G929.

**Bach T.A. and Carey H.V.** (1994). Developmental changes in neutrally mediated ion transport in piglet distal colon. *Pediatric Research*, 36: 144-151.

**Bagchi D.** (2006). Nutraceuticals and functional foods regulations in the United States and around the world. *Toxicology*, 221:1- 3.

**Bailey M., Haverson K., Inman C., Harris C., Jones P., Corfield G., Miller B., Stokes C.** (2005). The development of the mucosal immune system pre- and

post-weaning: balancing regulatory and effector function. *Proceedings of The Nutrition Society*, 64: 451-457.

**Barnett J.L., Cronin G.M., McCallum T.H., Newman E.A.** (1993). Effects of 'chemical intervention' techniques on aggression and injuries when grouping unfamiliar adult pigs. *Applied Animal Behaviour Science*, 36: 135–148.

**Barber R.S., Braude R., Mitchell K.G.** (1965). Studies on various potential growth stimulants for growing pigs with particular reference to their activity in supplementing that of copper sulphate. *British Journal of Nutrition*, 19, 575-579.

**Baum B., Lieber-Tenorio E.M., Enss M.L., Pohlenz J.F. and Breves G.** (2002). *Saccharomyces boulardii* and *Bacillus cereus* var. *Toyo* influence the morphology and the mucins of the intestine of pigs. *Zeitschrift für Gastroenterologie*, 40: 277-284.

**Bontempo V., Di Giancamillo A., Savoini G., Dell'Orto V., Domeneghini C.** (2006). Live yeast dietary supplementation acts upon intestinal morpho-functional aspects and growth in weanling piglets. *Animal Feed Science and Technology*, 129: 224–236.

**Boudry G., Péron V., Le Huërou-Luron I, Lallès J.P., Sève B.** (2004). Weaning induces both transient and long-lasting modifications of absorptive, secretory, and barrier properties of piglet intestine. *Journal of Nutrition*, 134: 2256-2262.

**Bouwens L., Baekeland M., De Zartger R., Wisse E.,** (1986). Quantitation, tissue distribution and proliferation kinetics of Kupffer cells in normal rat liver. *Hepatology*, 6: 718-722

**Braude, R.** (1948). Some observations on the behaviour of pigs in an experimental piggery. *Bulletin of Animal Behaviour*, 6:17-25.

**Breinekova K., Svoboda M., Smutna M., Vorlova L.** (2006). Markers of acute stress in pigs. *Physiological Research* 56: 323–329.

**Brown P.J., Miller B.G., Stokes C.R., Blazquez N.B., Bourne F.J.** (1988). Histochemistry of mucins of pig intestinal secretory epithelial cells before and after weaning. *Journal of Comparative Pathology*, 98: 313-23.

**Brower V.** (1998). Nutraceuticals:poised for a healthy slice of the healthcare market? *Nature Biotechnology*, 16:728-731.

**Brumm M.C., Miller P.S.** (1996). Response of pigs to space allocation and diets varying in nutrient density. *Journal of Animal Science*, 74: 2730–2737.

**Brunsgaard G.** (1997). Morphological characteristics, epithelial cell proliferation, and crypt fission in cecum and colon of growing pigs. *Digestive Diseases and Science*, 42: 2384-2393.

**Burke A., Lichtenstein G.R., Rombeau J.L.** (1997). Nutrition and ulcerative colitis. *Baillieres Clinical Gastroenterology*, 11(1):153–174.

**Burrin D.G., Stoll B., Guan X.** (2003). Glucagon-like peptide 2 function in domestic animals. *Domestic Animal Endocrinology*, 23: 103-122.

**Burrin D.G., Stoll B., Jiang R., Chang X., Hartmann B., Holst J.J., Greeley G.H., Reeds P.J.** (2000). Minimal enteral nutrient requirements for intestinal growth in neonatal pigs: how much is enough? *The American Journal of Clinical Nutrition*, 71: 1603-1610.

**Burrin D.G., Dudley M.A., Reed P.J., Shulman R.J., Perkinson S., Rosenberger J.** (1994). Feeding colostrum rapidly alters enzymatic activity and the relative isoform abundance of jejunal lactase in neonatal pigs. *Journal of Nutrition*, 124:2350–2357.

**Cai Y.J., Ma L.P., Hou L.F., Zhou B., Yang L., Liu Z.L.** (2002). Antioxidant effects of green tea polyphenols on free radical initiated peroxidation of rat liver microsomes. *Chemics and Physics of Lipids*, 120(1-2):109-17.

**Cadenas, E.** (1997). Basic mechanisms of antioxidant activity. *Biofactors*, 6, 391–397.

**Calder P.C.** (2008). Polyunsaturated fatty acids, inflammatory processes and inflammatory bowel diseases *Molecular Nutrition & Food Research*, 52 (8): 885–897.

**Caley D.** (2012). Copper and Menkes disease. Internet files April 10, 2012

**Carr A.C., Zhu B.Z., Frei B.** (2000). Potential antiatherogenic mechanisms of ascorbate (vitamin C) and  $\alpha$ -tocopherol (vitamin E). *Circulation Research*, 87:349.

**Chao J., Leung Y., Wang M., Chuen-Chung Chang R.** (2012). Nutraceuticals and their preventive or potential therapeutic value in parkinson's disease. *Nutrition Reviews*, 70(7): 373-386.

**Chaturvedi S., Sharma P.K., Garg V.K., Bansal M.** (2011). Role of nutraceuticals in health promotion. *International Journal of Pharm Tech Research*, 3(1): 442-448.

**Chow C.K., Reddy K., Tappel A.L.** (1969). Effect of dietary vitamin E on the activity of glutathione peroxidase *in vitro* and *in vivo* studies. *Journal of Clinical Investigation*, 48:1957.

**Chrousos G.P., Gold P.W.** (1992). The concepts of stress and stress system disorders: overview of physical and behavioral homeostasis. *The Journal of American Medical Association*, 267: 1244–1252.

**Cockbill, C.A.** (1994). Food law and functional foods. *British Food Journal*, 96(3): 3-4.

**Cromwell G.L., Stahly T.S., Monegue H.J.** (1989). Effects of source and level of copper on performance and liver copper stores in weanling pigs. *Journal of Animal Science*, 67, 2996-3002.

**Cui X., Dai X.G., Li W.B., Zhang B.L., Fang Y.Z.** (2000). Effects of Lu-Duo-Wei capsules on superoxide dismutase activity and contents of malondialdehyde and lipofuscin in the brain of the housefly. *The American Journal of Chinese Medicine*, 28:259.

**Curtis S.E.** (1996). Effects of environmental design on the pig's voluntary feed intake. *In: Proceedings Pork Industry Conference. University Illinois*, pp.60–73

**De Oliveira e Silva A.M., Vidal-Novoa A., Batista-González A.E., Pinto J.R., Portari Mancini D..A, Reina-Urquijo W., Mancini-Filho J.** (2012) *In vivo* and *in vitro* antioxidant activity and hepatoprotective properties of polyphenols from *Halimeda opuntia* (Linnaeus) Lamouroux. *Redox Report*, 17(2):47-53.

**Deplanke, B., Gaskins, H.R.,** (2001). Microbial modulation of innate defense: goblet cells and the intestinal mucus layer. *The American Journal of Clinical Nutrition*, 73 (suppl), 1131S–1141S.

**Dharmani P., Srivastava V., Kissoon-Singh V., Chadee K.** (2009). Role of intestinal mucins in innate host defense mechanisms against pathogens. *Journal Innate Immunity*, 1(2):123-35.

**Domeneghini C., Di Giancamillo A., Arrighi S., Bosi G.** (2006). Gut-trophic feed additives and their effects upon the gut structure and intestinal metabolism. State of the art in the pig, and perspectives towards humans. *Histology and Histopathology*, 21: 273-283.

**Domeneghini C., Di Giancamillo A., Savoini G., Paratte R., Bontempo V., Dell'Orto V.** (2004). Structural patterns of swine ileal mucosa following L-glutamine and nucleotide administration during the weaning period. An histochemical and histometrical study. *Histology and Histopathology*, 19(1):49-58.

**Dréau D., Lallès J.P.** (1999). Contribution to the study of gut hypersensitivity reaction to soybean proteins in preruminant calves and early-weaned piglets. *Livestock Production Science*, 60: 209-218.

**Duggan, C., Gannon, J., Walker, W.A.,** (2002). Protective nutrients and functional foods for the gastrointestinal tract. *American Journal of Clinical Nutrition*, 75, 789–808

**Duhamel G.E., Mathiesen M.R.** (1999). A population approach to diagnosis of grow-finish diarrhes complex. *Nebraska Swine Report*, 9-12

**Dunning J.C., Marquis R.E.** (1998). Anaerobic killing of oral streptococci by reduced, transition metal cations. *Applied and Environmental Microbiology*, 164: 27-33.

**Dunsford B.R., Haensly W.E., Knabe D.A.** (1991). Effects of diet on acidic and neutral goblet cell populations in the small intestine of early-weaned pigs. *American Journal of Veterinary Research*, 52: 1743–1752.

**Dunsford B.R., Haensly W.E., Knabe D.A.** (1990). Neutral and acidic goblet cell concentrations in the small intestine of the unweaned pig. *Biology of the Neonate*, 57: 194-199.

**Esposito E., Mazzon E., Paterniti I., al Toso R., Pressi G., Caminiti R., and Cuzzozrea S.** (2010). PPAR-  $\alpha$  Contributes to the Anti-Inflammatory Activity of Verbascoside in a Model of Inflammatory Bowel Disease in Mice *PPAR Research* 2010, Article ID 917312, 10 pages doi:10.1155/2010/917312



**Fanaro, S., Chierici, R., Guerrini, P.,Vigi, V.** (2003). Intestinal microflora in early infancy: composition and development. *Acta Paediatrica Suppl* 91, 48–55.

**Fang Y.Z., Yang S., Wu G.** (2002). Free Radicals, Antioxidants, and Nutrition. *Nutrition*, 18(10):872-9.

**Fang Y.Z., You Y.G., Chen G.M., et al.** (1998). Clinical observation on health care effects of tea polyphenol compound (Lu-Duo-Wei): prevention of coronary heart disease, hypertension and type II diabetes. *Paper presented at the Symposium on Tea and Anticarcinogenesis, Shanghai*

**Fang Y.Z., Chen G.M., Chen A.M., et al.** (1998). Effect of tea polyphenol compound (Lu-Duo-WEI) on inhibition of tumor growth. *Paper presented at the Symposium on Tea and Anticarcinogenesis, Shanghai*

**Frei B., Higdon J.V.** (2003) Antioxidant activity of tea polyphenols *in vivo*: evidence from animal studies *Proceedings of the Third International Scientific Symposium on Tea and Human Health: Role of Flavonoids in the Diet*

**Friedman S.L., Wei S., Blaner W.S.** (1993). Retinol release by activated rat hepatic lipocytes: regulation by Kupffer cell-conditioned medium and PDGF. *American Journal of Physiology Gastrointestinal and Liver Physiology*, 264: G947–G952.

**Friend. D. W., Gorrill A.D.R., MacIntyre T. M.** (1970). Performance and proteolytic enzyme activity of the suckling piglet creep-fed at one or three weeks of age. *Canadian Journal of Animal Science* ,50:439.

**Fischer-Nielsen A., Poulsen H.E., Loft S.** (1992). 8-Hydroxydeoxyguanosine *in vitro*: effects of glutathione, ascorbate, and 5-aminosalicylic acid. *Free Radical Biology and Medicine*, 13:121.

**Gaskins H., Kelley K.W.** (1995). Immunology and neonatal mortality. In: *The neonatal pig: development and survival*, 39-58. [Varley M.A. editor]. Wallingford, Oxon: CAB International.

**Ghose Y.N., Yadav S.R.** (2012). Developments in nutraceuticals for chemoprevention: a review. *International Journal of Pharmacy and Technology*, 4(1):1950-1973.

**Gillman C. E., KilBride A. L., Ossent P., Green L. E.** (2009). A cross-sectional study of the prevalence of foot lesions in post-weaning pigs and risks

associated with floor type on commercial farms in England. *Preventive Veterinary Medicine*, 91:146–152.

**Grisham, M. B.** (1994). Oxidants and free radicals in inflammatory bowel disease *Lancet*, 344,859-861.

**Hampson D.J.** (1994) Postweaning *Escherichia coli* diarrhoea in pigs. In *Escherichia coli in Domestic Animals and Humans*, [CL Gyles, editor]. Wallingford: CAB International.

**Halliwell, B., Gutteridge, J.M.C.** (2001). Free Radicals in Biology and Medicine. *Oxford University Press*. Oxford, UK. 936 pp.

**Halsted C.H.** (2003) Dietary supplements and functional foods: 2 sides of a coin? *The American Journal of Clinical Nutrition*, 77(4 Suppl):1001S-1007S.

**Hamme W. P., Weiss N., Holzapfel W.** (1991). The genera *Lactobacillus* and *Carnobacterium*. In the *Prokaryotes: A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications*. Bolows A., Truper H.G., Dworkin M., Harder W., Schleife K.H. (eds) New York, USA: Springer-Verlag, pp. 1535-1594.

**Hamid A.A., Aiyelaagbe O.O., Usam L.A., Ameen O.M., Lawal A.** (2010). Antioxidants: Its medicinal and pharmacological applications. *African Journal of Pure and Applied Chemistry*, 4(8):142-151.

**Healy, J., Tipton K.** (2007). Ceruloplasmin and what it might do. *Journal of Neural Transmission*, 114:777-781.

**Hedemann M.S., Hojsgaard S., Jensen B.B.** (2003). Small intestinal morphology and activity of intestinal peptidase in piglets around weaning. *Journal of Animal Physiology And Animal Nutrition*, 87: 32-41.

**Hill, G. M., Cromwell G. L., Crenshaw T. D., Dove C. R., Ewan R. C., Knabe D. A., Lewis A. J., Libal G. W., Mahan D. C., Shurson G. C., Southern L. L., Veum T. L.** (2000). Growth promotion effects and plasma changes from feeding high dietary concentrations of zinc and copper to weanling pigs (regional study). *Journal of Animal Science*, 78:1010-1016.

**Hiraishi H. , Terano A., Sugimoto T., Harada T., Razandi M., Ivey K.J.** (1994) Protective role of intracellular superoxide dismutase against extracellular oxidants in cultured rat gastric cells. *Journal of Clinical Investigation* , 93:331–8.

**Holden, P.J.** (1991). Swine costs and production. *Agri-practice*, 12 (1), 46-48.

**Hopwood D.E., Hampson D.J.** (2003). Interaction between the intestinal microflora, diet and diarrhoea, and their influences on the piglet health in the immediate post-weaning period. In *Weaning the Pig*, 199-219 [Pluske J.R., Dividich J.L., Verstegen M.W.A., editors]. Wageningen, the Netherlands: Wageningen Academic Publishers.

**Huang C., Qiao S., Lifa D., Piao X., Ren J.** (2004). Effects of lactobacilli on the performance, diarrhea incidence, VFA concentration and gastrointestinal microbial flora of weaning pigs. *Asian-Australasian Journal of Animal Science*, 17, 401-409.

**Hurrell R.** (2003). Influence of vegetable protein sources on trace element and mineral bioavailability. *Journal of Nutrition*, 133(9):2973S-2977S.

**Inoue R., Tsukahara T., Nakanishi N., Ushida K.** (2005). Development of the intestinal microbiota in the piglet. *Journal of General and Applied Microbiology*, 51: 257-265.

**Jungermann K.** (1995). Zonation of metabolism and gene expression in liver. *Histochemistry and Cell Biology*, 103: 81-91.

**Karla E.K.** (2003). Nutraceutical-definition and introduction *AAPS PharmSci* 5, 25 online [www.aapsj.org/view.asp?art=ps050325](http://www.aapsj.org/view.asp?art=ps050325).

**Khan, S.M., Sobti R.C., Kataria L.** (2005). Pesticide-induced alteration in mice hepato-oxidative status and protective of black tea extract. *Clinica Chimica Acta*, 358: 131-138.

**Kiernan F.** (1833). The anatomy and physiology of the liver. *Philosophical Transaction of the Royal Society*, (London) 123: 711-770.

**Kmiec Z.** (2001). Cooperation of liver cells in health and disease. *Springer*

**Kokate C.K., Purohit A.P., Gokhale S.B.** (2002). Nutraceutical and Cosmeceutical. *Pharmacognosy*, 21st edition, Pune, India: Nirali Prakashan, p 542-549.

**Koletzko B., Aggett P.J., Bindels J.G., Bung P., Ferre P., Gil A., Lentze M.J., Roberfroid M., Strobel S.** (1998) Growth, development and differentiation: A functional food science approach. *British Journal of Nutrition*, 80 Suppl.1: 5-45

**Kommerer S.K., Mateo R.D., Neher F.J., Kim S.W.** (2006). Phytobiotics and organic acids as potential alternatives to the use of antibiotics in nursery pig diets. *Asian-Australasian Journal of Animal Science*, 19: 1784-1789.

**Konstantinov S.R., Awati A.A., Williams B.A., Miller B.G., Jones P., Stokes C.R., Akkermans A.D.L., Smidt H., de Vos W.M.** (2006). Post-natal development of the porcine microbiota composition and activities. *Environmental Microbiology*, 8: 1191-1199.

**Konstantinov S.R., Smidt H., Akkermans A.D.L.** (2005). Ecology and activity of clostridia in the intestine of mammals. In *Handbook of Clostridia*, 787-796. [Durre P. editor]. Boca Raton, FL, USA: CRC Press.

**Konstantinov S.R., Awati A., Smidt H., Williams B.A., Akkermans A.D.L., de Vos W.M.** (2004). Specific response of a novel and abundant *Lactobacillus amylovorus*-like phylotype to dietary prebiotics in the ileum and colon of weaning piglets. *Applied and Environmental Microbiology*, 70: 3821-3830.

**Kruidenier L., Verspaget H.W.** (2002). Review article: oxidative stress as a Pathogenic factor in inflammatory bowel disease--radicals or ridiculous? *Alimentary Pharmacology and Therapeutics*, 16(12):1997-2015.

**Lay D.C. Jr., Matteri R.L., Carroll J.A., Fangman T.J., Safranski T.J.** (2002). Prewaning survival in swine. *Journal of Animal Science*, 80: 74-86.

**Lallès J.P., Bosi P., Smidt H., Stokes C.R.** (2007). Nutritional management of gut health in pigs around weaning. *Proceedings of the Nutrition Society*, 66: 260-268.

**Lallès J.P., Boudry G., Favier C., Le Floc'h N., Luron I., Montagne L., Oswald I.P., Pié S., Piel C., Sève B.** (2004). Gut function and dysfunction in young pigs: physiology. *Animal Research*, 53: 301-316.

**Langmead L., Dawson C., Hawkins C., Banna N., Loo S., Rampton D.S.** (2002). Antioxidant effects of herbal therapies used by patients with inflammatory bowel disease: an in vitro study. *Alimentary Pharmacology Therapeutics*, 16(2):197-205.

**Lebret B., Meunier-Salaün M.C., Foury A., Mormède P., Dransfield E., Dourmad J.Y.** (2006). Influence of rearing conditions on performance, behavioral, and physiological responses of pigs to preslaughter handling, carcass traits, and meat quality. *Journal of Animal Science*, 84: 2436-2447.

**LeDividich, J., M. Vermorel, 1. Noblet, J. C. Bouvier, Aumaitre A.** (1980). Effects of environmental temperature on heat production energy retention, protein and fat gain in early weaned piglets. *British Journal of Nutrition*, 44: 313

**Lee, K.W., Kim, H., Lee, Y., Park, H.J., Choi, J.W., Ha, J., Lee, K.T.** (2007). Acteoside inhibits human promyelocytic HL-60 leukemia cell proliferation via inducing cell cycle arrest at G0/G1 phase and differentiation into monocyte. *Carcinogenesis*, 28: 1928–1936.

**Lee J. Y., Woo E.R., Kang K. W.** (2005). Inhibition of lipopolysaccharide inducible nitric oxide synthase expression by acteoside through blocking of ap-1 activation. *Journal of Ethnopharmacology*, 97: 561–566.

**Lievin-Le Moal V., Servin A.L.** (2006). The front line of enteric host defense against unwelcome intrusion of harmful microorganisms: mucins, antimicrobial peptides, and microbiota. *Clinical Microbiology Reviews*, 19:315–337.

Literature Review of Cantaloupe Melan Sod Extract/Wheat Gliadin Biopolymer (GliSODin) and Its Beneficial Health Aspects [technical publication]. Paris, France: GliSODin; May 2009. <http://mydrinkact.biz/pdf/sod.pdf>. Accessed July 22, 2009.

**Lopez-Pedrosa J.M., Torres M.I., Fernández M.I., Ríos A., Gil A.** (1998). Severe malnutrition alters lipid composition and fatty acid profile of small intestine in newborn piglets. *Journal of Nutrition*, 128: 224-233.

**Lykkesfeldt J., Svendsen O.** (2007). Oxidants and antioxidants in disease: Oxidative stress in farm animals. *The Veterinary Journal*, 173: 502–511.

**Magalhães L.M., Santos M., Segundo M.A., Reis S., Lima J.L.F.C.** (2009). Flow injection based methods for fast screening of antioxidant capacity. *Talanta*, 77: 1559–1566.

**Manzanilla E.G., Perez J.F., Martin M., Kamel C., Baucells F., Gasa J.** (2004). Effect of plant extracts and formic acid on the intestinal equilibrium of earlyweaned pigs. *Journal of Animal Science*, 82, 3210-3218.

**Marchant J.N., Mendl M.T., Rudd A.R., Broom D.M.** (1995). The effect of agonistic interactions on the heart rate of group-housed sows. *Applied Animal and Behavioural Science*, 46: 49–56.

**Matsuo K., Ota H., Akamatsu T., Sugiyama A., Katsuyama T.** (1997). Histochemistry of the surface mucous gel layer of the human colon. *Gut*, 40: 82-789

**Mazzon E, Esposito E, Di Paola R, Riccardi L, Caminiti R, Dal Toso R, Pressi G, Cuzzocrea S.** (2009). Effects of verbascoside biotechnologically produced by *Syringa vulgaris* plant cell cultures in a rodent model of colitis. *Naunyn Schmiedebergs Archives of Pharmacology*, 380(1):79-94.

**McCracken B.A., Spurlock M.E., Roos M.A., Zuckermann F.A., Gaskin H.R.** (1999). Weaning anorexia may contribute to local inflammation in the piglets small intestine. *Journal of Nutrition*, 129: 613-619.

**McCracken B.A., Gaskins H.R., Ruwe-Kaiser P.J., Klasing K.C., Jewell D.E.** (1995). Diet-dependent and diet-independent metabolic responses underlie growth stasis of pigs at weaning. *Journal of Nutrition*, 125:2838-2845.

**Miller M.J.S., Angeles M.S., Reuter B.K., Bobrowski P., Sandoval M.** (2001) Dietary antioxidants protect gut epithelial cells from oxidant-induced apoptosis *BMC Complementary and Alternative Medicine*, 1:11.

**Miller W. J.** (1981). Mineral and vitamin nutrition of dairy cattle. *Journal of Dairy Science*, 64:1196-1206.

**Mizui, T., Sato H., Hirose F., Doteuchi M.** (1987). Effect of antiperoxidative drugs on gastric damage induced by ethanol in rats. *Life Science*, 41: 755-763.

**Moncada D.M., Kammanadiminti S.J., Chadee K.** (2003). Mucin and Tolllike receptors in host defense against intestinal parasites. *Trends Parasitology* , 19:305–311.

**Montagne L., Boudry G. Favier C., Le Huërou-Luron I., Lallès J.P., Sève B.** (2007). Main intestinal markers associated with the changes in gut architecture and function in piglets after weaning. *British Journal of Nutrition*, 97: 45-57.

**Montagne L., Piel C., Lallès J.P.** (2004). Effect of diet on mucin kinetics and composition: nutrition and health implications. *Nutritional Reviews*, 62(3):105-14.

**Montagne L., Toullec R., Formal F., Lallès J.P.** (2000). Influence of dietary protein level and origin on the flow of mucin along the small intestine of the preruminant calf. *Journal of Dairy Science*, 83: 2820-2828.

**Motta P.M.** (1984). The three dimensional microanatomy of the liver. *Japanese Archives of Histology*, 47: 1-30

**Mroz Z., Koopmans S.J., Bannink A., Partanen A.K., Krasucki W., Overland M., Radcliffe S.** (2006). Carboxylic acids as bioregulators and gut growth promoters in non-ruminants. In: *Biology of Nutrition in Growing Animals* (Eds R Mosenthin, J. Zentek and T. Zebrowska) Volume 4, pp 81-133, Elsevier Limited

**Muller D.P.** (1990). Antioxidant therapy in neurological disorders. *Advances in Experimental Medicine and Biology*, 264:475.

**Neutra M.R. and Forstner J.F.** (1987). Gastrintestinal mucus: synthesis, secretion and function. In: *Physiology of the gastrointestinal tract*, 975-1009 [Johnson L.R. edition] Raven press, New York, NY.

**NRC.** (2001). Nutrient Requirements of Dairy Cattle. *7th revision edition National Academy Science*, Washington, DC.

**Nuñez M.C., Bueno J.D., Ayudarte M.V., Almendros A., Ríos A., Suárez M.D., Gil A.** (1996). Dietary restriction induces biochemical and morphometric changes in the small intestine of nursing piglets. *Journal of Nutrition*, 126: 933-944.

**Ohno T. Inoue A. M. Ogihara A. Y. Aracoglu I.** (2002). Antimetastatic activity of acteoside, a phenylethanoid glycoside. *Biological and Pharmaceutical Bulletin*, 25(5) 666—668.

**M. T. Olaleye, Rocha B.T.J.** (2008). Acetaminophen-induced liver damage in mice: Effects of some medicinal plants on the oxidative defense system *Experimental and Toxicologic Pathology*, 59 (5) 319–327.

**Oliveira C.P., Kassab P., Lopasso F.P., Souza H.P., Janiszewski M., Laurindo F.R., Iriya K., Laudanna A.A.** (2003). Protective effect of ascorbic acid in experimental gastric cancer: reduction of oxidative stress. *World Journal Gastroenterology*, 9(3):446-8.

**Pandey M., Verma R., Saraf S.A.** (2010). Nutraceuticals: new era of medicine and health. *Asian journal of Pharmaceutical and clinical research*, 3 (1): 11-15.

**Picerno P., Autore G., Marzocco S., Meloni M., Sanogo R., Aquino R. P.** (2005). Anti-inflammatory activity of verminoside from *kigelia africana* and evaluation of cutaneous irritation in cell cultures and reconstituted human epidermis. *Journal Natural Products*, 68:1610-1614.

**Pierce K.M., Sweeney T., Brophy P.O., Callan J.J., Fitzpatrick E., McCarthy P., O'Doherty J.V.** (2006). The effect of lactose and inulin on intestinal morphology, selected microbial populations and volatile fatty acid concentrations in the gastro-intestinal tract of the weaned pig. *Animal Science* 82, 311-318.

**Piguet P.F., Vesin C., Donati Y., Barazzone C.** (1999). TNF-induced enterocyte apoptosis and detachment in mice: induction of caspases and prevention by a caspase inhibitor ZVAD-fmk. *Laboratory Investigation*, 79: 495-500.

**Pihan G., Regillo C., Szabo S.** (1987). Free radicals and lipid peroxidation in ethanol- or aspirin-induced gastric mucosal injury. *Digestive Disease and Science*, 32(12):1395–1401.

**Pluske J.R., Hampson D.J. and Williams I.H.** (1997). Factors influencing the structure and function of the small intestine in the weaned pig: a review. *Livestock Production Science*, 51: 215-236.

**Potten C.S. and Loeffler M.** (1990). Stem cells: attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the crypt. *Development*, 110: 1001-1020.

**Predieri, G., Tegoni M., Cinti E., Leonardi G., Ferruzza S.** (2003). Metal chelates of 2-hydroxy-4-methylthiobutanoic acid in animal feeding: preliminary investigations on stability and bioavailability. *Journal of Inorganic Biochemistry*, 95:221-224.



**Racanelli V. and Rehermann B.**, (2006). The liver as an immunological organ. *Hepatology*, 43: S54-S62.

**Rafferty J.F, Nougush Y., Fisher J.E. Hasselgren P-O.** (1994). Sepsis in rats stimulates cellular proliferation in the mucosa of the small intestine. *Gastroenterology*, 107: 121-127.

**Rappaport A.M.**, (1976). The microcirculatory acinar concept of normal and pathological hepatic structure. *Beiträge zur Pathologie*, 157: 215-243.

**Rappaport A.M., Borowy Z.J., Loughheed W.M., Lotto W.N.** (1954). Subdivision of hexagonal liver lobules into a structural and functional unit. *Anatomical Record*, 119: 11-33.

**Ratnam D.V., Ankola D.D., Bhardwaj V., Sahana D.K., Ravi Kumar M.N.V.** (2006). Role of antioxidants in prophylaxis and therapy: a pharmaceutical perspective. *Journal of Controlled Release*, 113:189-207.

**Rezaie A., Parker R.D., Abdollahi M.** (2007). Oxidative stress and pathogenesis of inflammatory bowel disease: an epiphenomenon or the cause? *Digestive Diseases and Science*, 52(9): 2015-21.

**Rigano D. Formisano C. Basile A, Lavitola A. Senatore F. Rosselli S. Bruno M.** (2007). Antibacterial activity of flavonoids and phenylpropanoids from marrubium globosum ssp. Libanoticum. *Phytotherapy Research*, 21, 395–397.

**Romano M., Vitaglione P., Sellitto S., D'Argenio G.** (2012). Nutraceuticals for protection and healing of gastrointestinal mucosa. *Current Medical Chemistry*, 19(1):109-17. Review.

**Ross S.** (2000). Functional foods: the Food and Drug Administration perspective. *The American Journal of Clinical Nutrition*, (Suppl)71: 1735-8.

**Rossi R., Corino C., Pastorelli G., Durand P., Prost M.** (2009). Assessment of antioxidant activity of natural extracts. *Italian Journal of Animal Science*, 8(2): 655-657.

**Sapolsky R.M., Romero L.M., Munck A.U.** (2000). How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocrine Reviews*, 21: 55-89.

**Sasse D., Spornitz U.M. and Maly I.P.** (1992). Liver architecture. *Enzyme* 46:8-32.

**Scalbert A., Johnson I.T., Saltmarsh M.** (2005). Polyphenols: antioxidants and beyond. *The American Journal of Clinical Nutrition*, 81(1):215S-217S.

**Sheng G.Q., Zhang J.R., Pu X.P., Ma J., Li C.L.** (2002). Protective effect of verbascoside on 1-methyl-4-phenylpyridinium ion-induced neurotoxicity in pc12 cells. *European Journal of Pharmacology*, 451: 119-124.

**Shields, R. G., Eksuom Jr. K. E., Mahan D. C.** (1980). Effect of weaning age on digestive enzyme development in swine from birth to ten weeks. *Journal of Animal Science*, 50:257

**Shirkey T.W., Siggers R.H., Goldade B.G., Marshall J.K., Drew M.D., Laarveld B., Van Kessel A.G.** (2006). Effects of commensal bacteria on intestinal morphology and expression of proinflammatory cytokines in the gnotobiotic pig. *Experimental Biology of Medicine*, 231(8):1333-45.

**Sierro F., Pringault E., Assman P.S., Kraehenbuhl J.P., Debard N.** (2000). Transient expression of M-cell phenotype by enterocyte-like cells of the follicle-associated epithelium of mouse Peyer's patches. *Gastroenterology*, 119: 734-43.

**Sies H.** (1997). Oxidative stress: oxidants and antioxidants. *Experimental Physiology*, 82(2): 291-295.

**Somogyi A., Rosta K., Pusztai P., Tulassay Z., Nagy G.** (2007) Antioxidant measurements. *Physiological Measurement*, 28: 41–55.

**Spreeuwenberg M.A.M., Verdonk J.M.A.J., Gaskins H.R., Verstegen M.W.A.** (2001). Small intestine epithelial barrier function is compromised in pigs with low feed intake at weaning. *Journal of Nutrition*, 131: 1520-1527.

**St-Pierre, N. R., B. Cobanov, and G. Schmitkey.** (2003). Economic losses from heat stress by US livestock industries. *Journal of Dairy Science*, 86(E Suppl.):E52–E77

**Stagos D., Amoutzias G.D., Matakos A., Spyrou A., Tsatsakis A.M., Kouretas D.** (2012) Chemoprevention of liver cancer by plant polyphenols. *Food and Chemical Toxicology*, 50(6):2155-70.

**Stahly, T. S., Cromwell G. L.** (1979). Effects of environmental temperature and dietary fat supplementation on the performance and carcass characteristics of growing swine. *Journal of Animal Science*, 49:1476.

**Stoll B.X., Chang X., Fan M.Z., Reeds P.J., Burrin D.G.** (2000). Enteral nutrient intake determines the rate of intestinal protein synthesis and accretion in neonatal pigs. *The American Journal of Physiology*, 279: 49-57.

**Szabo C.** (2003). Multiple pathways of peroxynitrite cytotoxicity. *Toxicology Letters*, 140-141: 105-112.

**Szabo C., Lim L.H., Cuzzocrea S.** (1997). Inhibition of poly (ADP-ribose) synthetase attenuates neutrophil and exerts anti-inflammatory effects. *The Journal of Experimental Medicine*, 186: 1041-1049.

**Taboada C., Millán R., Míguez I.** (2011). Evaluation of the marine alga *Ulva rigida* as a food supplement: effect of intake on intestinal, hepatic, and renal enzyme activities in rats. *Journal of Medicinal Food*, 14(1-2):161-6.

**Tang M., Laarveld B., Van Kessel A.G., Hamilton D.L., Estrada A. and Patience J.F.** (1999). Effect of segregated early weaning on postweaning small intestinal development in pigs. *Journal of Animal Science*, 77: 3191-3200.

**Terabe M., Shimizu M., Mabuchi A., Matui S., Morikawa H., Kaneda K., Kakiuchi T., Yokomuro K.** (2000). Unresponsiveness of intrahepatic lymphocytes to bacterial superantigen: rapid development of suppressive Mac-1(high) cells in the mouse liver. *Hepatology*, 32: 507-513.

**Torrallardona D., Conde M.R., Badiola I., Polo J., Brufau J.** (2003). Effect of fishmeal replacement with spray-dried animal plasma and colistin on intestinal structure, intestinal microbiology, and performance of weanling pigs challenged with *Escherichia coli* K99. *Journal of Animal Science*, 81: 1220-1226.

**Torrallardona D., Conde R., Badiola J.I., Polo J.** (2007). Evaluation of spray dried animal plasma and calcium formate as alternatives to colistin in piglets experimentally infected with *Escherichia coli* K99. *Livestock Science*, 108, 303-306.

**Tomlinson, D.J., Socha M.T., DeFrain J.M.** (2008). Role of trace minerals in the immune system. Page 39-52. In: *Proceedings Penn State Dairy Cattle Nutrition Workshop*. Grantville, PA.

**Turck D., Feste A.S., Lifchitz C.H.** (1993). Age and diet affect the composition of porcine colonic mucins. *Pediatric Research*, 33: 564-567.

**Vallee, B.L., Falchuk K. H.** (1993). The biochemical basis of zinc physiology. *Physiological Review*, 73:79-118.

**Van Beers-Schreurs H.M.G., Nabuurs M.J.A., Vellenga L., Kalsbeekvandervalk H.J., Wensing T., Breukink H.J.** (1998). Weaning and the weanling diet influence the villous height and crypt depth in the small intestine of pigs and alter the concentration of short-chain fatty acids in the large intestine and blood. *Journal of Nutrition*, 128: 947-953.

**Van der Waaij D., Berghuis-de Vries J.M., Lekkerkerk-van der Wees J.E.C.** (1971). Colonisation resistance of the digestive tract in conventional and antibiotic-treated mice. *The Journal of Hygiene*, 69: 405-411.

**Vente-Spreuwenberg M.A.M., Verdonk J.M.A.J., Verstgen M.W.A., Beynen A.C.** (2003). Villus height and gut development in weaned piglets receiving diets containing either glucose, lactose, or starch. *British Journal of Nutrition*, 90: 907-913.

**Verdugo P.** (1990). Goblet cells secretion and mucogenesis. *Annual Review of Physiology*, 52: 1-18.

**Voudin K.A., Shukitt-Hale B., Mackonnons, Kalt W., Joseph J.A.** (2000). Polyphenols enhance red blood cell resistance to oxidative stress *in vitro* and *in vivo*. *Biochimica et Biophysica Acta*, 1523:117.

**Wanasundara P.K.J.P.D. and Shahidi F.** (2005). Antioxidants: Science, Technology, and Applications. *Bailey's Industrial Oil and Fat Products* DOI: 10.1002/047167849X.bio002

**Wang P. Kang J. Zheng R. Yang Z. Lu J. Gao J. Jia Z.** (1996). Scavenging effects of phenylpropanoid glycosides from pedicularis un superoxide anion and hydroxyl radical by the spin trapping method . *Biochemical Pharmacology*, 51: 687-691.

**Webel D.M., Finck B.N., Baker D.H., Johnson R.W.** (1997). Time course of increased plasma cytokines, cortisol, and urea nitrogen in pigs following intraperitoneal injection of lipopolysaccharide. *Journal of Animal Science*, 75: 1514–1520.

**Wenk C.** (2003) Herbs and botanicals as feed additives in monogastric animals. *Asian Australasian Journal of Animal Sciences* , 16: 282-289.

**Widdowson E.M., Colombo V.E., Artavanis C.A.** (1976). Changes in the organs of pigs in response to feeding for the first 24 h after birth. *Bioggy of the Neonate*, 28: 272-281.

**Wildman R.E.C.** (2000). Classifying nutraceuticals. In: *Handbook of Nutraceuticals and Functional Food* (R.E.C. Wildman ,ed). CRC Press, Boca Raton, Florida, p13-30.

**Williams B.A., Verstegen M.W.A., Tamminga S.** (2001). Fermentation in the monogastric large intestine: its relation to animal health. *Nutritional Research Reviews*, 4: 207-227.

**Winnock M., Garcia Barcina M., Lukomska B., Huet S., Saric J., Balabaud C., Bioulac Sage P.** (1995). Human liver-associated lymphocytes: an overview. *Journal of Gastroeneterology and Hepatology*,10 Suppl 1: S43-46.

**Winzer K., Becker W., Van Noorden C.J.F., Köhler, A.** (2000). Short-time induction of oxidative stress in hepatocytes of the European Flounder (*Platichthys flesus*). *Marine Environmental Research*, 50, 495-501.

**Wisse E.,** (1972). An ultrastructural characterization of the endothelial cell in the rat liver sinusoid under normal and various experimental conditions, as a contribution to the distinction between endothelial and Kupffer cells. *Journal of Ultrastructure Research*, 38:528-562.

**Wolter B.F., Ellis M., Curtis S.E., Parr E.N., Webel D.M.** (2000). Group size and floor-space allowance can affect weanling-pig performance. *Journal of Animal Science*, 78: 2062–2067.

**Wong I. Y. F. He Z. D. Huang Y. Chen Z. Y.** (2001). Antioxidative activities of phenylethanoid glycosides from ligustrum purpurascens. *Journal of Agricultural and Food Chemistry*, 49: 3113-3119.

**Yang G.Y., Liao J., Kim K., Yurkow E.J., Yang C.S.** (1998). Inhibition of growth and induction of apoptosis in human cancer cell lines by tea polyphenols. *Carcinogenesis*, 19:611.

**Yokota T., Igarashi K., Uchihara T., Jishage K., Tomita H., Inaba A., Li Y., Arita M., Suzuki H., Mizusawa H., Arai H.** (2001). Delayed-onset ataxia in mice lacking  $\alpha$ -tocopherol transfer protein: model for neuronal degeneration caused by chronic oxidative stress. *Proceedings of the National Academy of Sciences, USA*;98:15185.

**Yuan G.J., Gong Z.J., Sun X.M., Zheng S.H., Li X.** (2006). Tea polyphenols inhibit expressions of iNOS and TNF- $\alpha$  and prevent lipopolysaccharide-induced liver injury in rats. *Hepatobiliary and Pancreatic Disease International*, 5(2):262-7.

**Xiong Q., Tezuka Y., Kaneko T., Li H., Tran L.Q., Hase K., Namba T., Kadota S.** (2000). Inhibition of nitric oxide by phenylethanoids in activated macrophages. *European Journal of Pharmacology*, 400(1):137-44.

**Xu R.-J., Mellor D.J., Tungthanathanich P., Birtles M.J., Reynolds G.H., Simpson H.V.** (1992). Growth and morphological changes in the small and the large intestine in piglets during the first three days after birth. *Journal of Developmental Physiology*, 18:161–172.

**Zeisel SH.** (1999). Regulation of “Nutraceuticals”. *Science*, 285:185-86.

**Zhang H., Malo C., Boyle C.R., Buddington R.K.** (1998). Diet influences development of the pig (*Sus scrofa*) intestine during the first 6 hours after birth. *Journal of Nutrition*, 128: 1302-1310.

**Zhang H., Malo C., Buddington R.K.** (1997). Suckling induces rapid intestinal growth and changes in brush border digestive functions of newborn pigs. *Journal of Nutrition*, 127: 418-426.

**Zhu L.H., Zhao K.L., Chen X.L., Xu J.X.** (2012). Impact of weaning and an antioxidant blend on intestinal barrier function and antioxidant status in pigs *Journal of Animal Science*, 90(8): 2581-2589.

**Ziegler T.R., Fernandez-Estivariz C., Jonas C.R., Gu L.H., Jones D.P., Leader L.M.** (1999). Interactions between nutrients and peptide growth factors in intestinal growth, repair and function. *Journal of Parenteral and Enteral Nutrition*, 23: S174-S183.

**Zimmerman D.R.** (1986). Role of subtherapeutic levels of antimicrobials in pig production. *Journal of Animal Science*, 62(suppl.3):6.

**Zingarelli B., Cuzzocrea S., Zsengeller Z., Salzman A.L., Szabo C.** (1997). Protection against myocardial ischemia and reperfusion injury by 3-aminobenzamide, an inhibitor of poly (ADP-ribose) synthetase. *Cardiovascular Research*, 36:205–215.

**Zoetendal E.G., Collier C.T., Koike S., Mackie R.I., Gaskins H.R.** (2004). Molecular ecological analysis of the gastrointestinal microbiota: a review. *Journal of Nutrition*, 134: 465-472.





## **CHAPTER 2**

### **Objectives**



## 2. Objectives

The aim of my PhD thesis was to evaluate with a multidisciplinary approach the attempts to improve pig welfare in different crucial phases of the rearing period. This on the basis that: i) keeping farm animals healthy is necessary to assure their welfare and to obtain safe animal products; ii) pathologies and consequent difficulties in growth result in severe economic losses in farming; iii) a proper nutritional management may play a key role in animal production, above all when approached by more than one point of view.

I am aware that appropriate dietary composition and amount of nutrients are determinant for an optimal growth of animals, but the former are not unique determinants of the latter. After the banning (2006) from EU countries of the use of chemotherapeutics as growing substances, the interest of scientific research for alternative nutritional strategies is more and more increasing. Various nutritional approaches with different dietary integrations have been tested in the recent years for finding alternatives to the limited use of chemotherapeutics in animal rearing, as well as for enhancing the intrinsic quality of a food product of animal origin. In the last years a large body of scientific literature focuses on nutraceuticals.

Here I present three assays aimed at evaluating the effects of nutraceuticals in dietary supplementations applied to pigs in two different phases of the intensive farm industries: i) weaning, which is paradigmatic of a condition of acute stress (assay 2-chapter 4; assay 3-chapter 5), and ii) growing-finishing phase, which may be reputed an example of a condition of long term stress (assay 1-chapter 3).

In all these studies my approach was micro-anatomical, consistent with morpho-functional analyses of the pig gut. In addition with the description of important structural details identified in different target organs, sometimes differently quantified in treated animals in comparison with control ones (histometry), the used micro-anatomical approach let me to hypothesize in some instances mechanisms of action of the studied dietary interventions. Moreover I have worked to correlate the results obtained with the used micro-anatomical methods with those aimed at evaluating growth performance, in such a way respecting a multidisciplinary approach, which is useful when studying the swine that is important both as food animal species and a largely used animal model for biomedical problems.



## **CHAPTER 3- ASSAY 1**

# **Changes in nitrosative stress biomarkers in swine intestine following dietary intervention with verbascoside**

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### 3. Changes in nitrosative stress biomarkers in swine intestine following dietary intervention with verbascoside

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#### 3.1 Abstract

In farm animals, oxidative stress can be involved in several intestinal pathological disorders, and many antioxidant molecules, especially those ones of plant origin, can counteract free radicals, thus stabilizing the gut environment and enhancing health. The aim of the study was to investigate whether the use of verbascoside (VB), a polyphenolic plant compound, in pig feeding could modulate oxidative and/or nitrosative stress in the gut. Eighteen male piglets (Dalland) were assigned to two groups, which were fed with either a control diet (CON) or a diet supplemented with 5 mg/kg of verbascoside (VB) for 166 days. At slaughter, duodenum and jejunum specimens were collected. Immunohistochemistry and Western blot analyses were performed on the samples to evaluate free radical adducts, including acrolein (ACR), 8-hydroxydeoxyguanosine (8-OHdg) and nitrotyrosine (NT). A KRL test was also used to assess the total blood antioxidant activity, and no difference was observed. Immunohistochemistry and Western blot showed that dietary treatment decreased the levels of nitrotyrosine in enteroendocrine cell populations ( $P < 0.05$ ). Characterization of the enteroendocrine cell typology was then performed, and serotonin immunoreactive cells were revealed to be directly involved in decreasing the nitrosative stress status. This preliminary study demonstrates the important role of dietary VB in decreasing stress biomarkers in swine gut, thus highlighting a possible intervention aimed at building a large prospective for antioxidant dietary supplementation in food animal species.

**Key words:** natural extract, double immunofluorescence, histomorphometry, diffuse endocrine system.

## 3.2 Introduction

Free radicals are highly reactive molecules produced under normal biological conditions, mainly during oxygen consumption in redox reactions required to generate energy and eliminate xenobiotic and pathogenic organisms. Organisms have their own natural protection in the form of enzymatic and chemical detoxification systems against excessive free-radical generation, which in turn is responsible for various degrees of cellular damage. Thus, under normal physiological conditions, a balanced state is established between free-radical production and antiradical factor interventions (Lehucher-Michel et al., 2001). Nevertheless, various lifestyle, as well as nutritional and environmental factors are able to alter this balanced state, thus resulting in oxidative stress. This in turn may cause an impairment of the individual's overall defence capacity, thus leading to the development of many diseases, especially those ones chronic.

Many natural substances present in plants as secondary metabolites, such as phenolics and flavonoids, have the ability to directly neutralise free radicals. Really, some of these substances inhibit chain reactions that lead to the formation of additional radicals, thus preventing cellular damage, which is a frequent occurrence in both humans and other mammalian species. Some others activate antioxidant enzymes, such as glutathione peroxidase (Hu, 2011). At present, however, a limited knowledge exists about the translation between the large body of evidence supported by *in vitro* studies and the scarce quantity of studies concerning *in vivo* conditions (Fraga, 2007). By this point of view, the swine is an excellent candidate model to be examined, because it is either a largely reared food animal species or an extensively studied animal model for biomedical problems (Walters et al., 2011 a, b).

At present pigs have fast growth rates, not only due to precise feed formulations and genetic improvement, but also through better than previous management, which tends to respect animal welfare (Bezkorovainy, 2001). To sustain faster growth, sub-therapeutical levels of antibiotics have long been used by farmers as growth promoters. However, the long-term use of antibiotics as feed additives causes antibiotic resistance in pathogens (Bach Knudsen, 2001) and may select for antibiotic-resistant bacterial strains, which may then be transferred to other bacteria (Aarestrup, 1999). European Union (EU) laws against the use of antibiotics in animal feed have been in effect since 1 January 2006 (Regulation EC no 1831/2003). As a consequence, it is crucial to find alternative and healthier feed strategies, which are able to limit the microbial resistance (Daglia, 2011), as well as to stabilize the gut environment, enhance health, sustain food animal species performance, and improve the quality of the products derived.



The dietary use of a polyphenol such as verbascoside, a phenylpropanoid glycoside, has been suggested as being efficient in preventing or attenuating oxidative stress and hence improving animal health (Stevenson and Hurst, 2007; Virgili and Marino, 2008; Daglia, 2011). The antioxidant activity of verbascoside on cellular systems and several organs has been demonstrated in humans (Li et al., 1999; Chen et al., 2002; Vertuani et al., 2011). In animal species, the antioxidant properties of verbascoside have been demonstrated in rabbits (Liu et al., 2003), weaned piglets (Corino et al., 2007), and hares (Palazzo et al., 2011). In addition, Bruins et al. (2006) found that polyphenol tea extracts may inhibit net fluid and electrolyte losses involved in secretory diarrhoea from enterotoxigenic *Escherichia coli* in animal models. Sehm et al. (2007) reported that dietary polyphenol showed a positive effect on the structure of the gastrointestinal tract of post weaning piglets and can improve pig health. In addition Lien et al. (2007) found an improvement in average daily gain and immune response parameters in weaned piglets fed with plant polyphenols.

No data on the long-term nutrition with natural extracts, titrated in verbascoside, in pigs are available in the literature. We examined how a long-term dietary supplementation with verbascoside could influence the intestinal structure of pigs, and we investigated whether the use of verbascoside in pig nutrition could modulate oxidative and/or nitrosative stress biomarkers in the intestine.

### 3.3 Materials and methods

#### 3.3.1 Animals and diet

Eighteen male pigs (Dalland) weighing  $7.1 \pm 0.2$  kg and aged  $25 \pm 2$  days, were randomly selected and assigned to two dietary groups: a control group (CON=9) and an experimental group fed with a diet supplemented with 5mg/kg of verbascoside (VB=9). The amount of VB in the feed was chosen on the basis of our previous studies on pigs (Corino et al., 2007; Pastorelli et al., 2011). On arrival the piglets were clinically healthy. The diet compositions were fortified to meet or exceed nutrient requirements (NRC 1998) for all nutrients in the growth phases. The animals were housed in individual cages under environmentally controlled conditions and given *ad libitum* access to water and diet. The antioxidant supplement contained a water-soluble extract of Verbenaceae (*Lippia* spp.) leaves (Consorzio Powerfeed, Costa de Nobili, Pavia, Italy). To avoid oxidation in the complete feed, the supplement was microencapsulated within a protective matrix of hydrogenated vegetable lipids using spray-cooling technology (Sintal Zootechnica, Isola Vicentina, Vicenza, Italy).

The trial lasted 166 days and the pigs were slaughtered at an average live weight of  $109.6 \pm 2.2$  kg.

All animals were treated in accordance with both the policies and the principles of laboratory animal care in compliance with European Union guidelines (86/609/EEC), which were approved by the Italian Ministry of Health (Law 116/92).

### 3.3.2 Blood and tissue samples

At the beginning of the trial and at 150 days, blood samples were collected by vena cava puncture before the morning feeding. The blood samples (total number of specimens = 36) were collected in 10-mL vacutainer glass tubes containing EDTA (Venoject, Terumo Europe N.V., Leuven, Belgium), which were immediately transported to the laboratory pending analysis.

At the end of the trial (166 days), the pigs were slaughtered by approved procedures (Italian Ministry of Health; DL .333/1998), and samples of proximal duodenum (5 cm after the pylorus) and proximal jejunum (5 cm after the beginning of the jejunum) were immediately excised from each pig (total number of specimens = 36). The samples were vacuum-packed and stored at  $-80^{\circ}\text{C}$ . Western blot analyses were performed on the samples within two months of collection.

For micro-anatomical analyses (histology, histomorphometry, immunohistochemistry, and double immunofluorescence), additional full thickness (approximately  $1\text{ cm}^3$ ) samples were removed at the same above-mentioned localizations from each pig. These samples (total number = 36) were promptly fixed in 4% para-formaldehyde in 0.01 M phosphate-buffered saline (PBS) pH 7.4 for no longer than 24 h at  $4^{\circ}\text{C}$ , dehydrated in a graded series of ethanol, cleared with xylene, and embedded in paraffin (see below).

### 3.3.3 Blood analyses

The analyses on the total antioxidant activity of whole blood were performed within 24 hours of sample collection. The total antioxidant activity of the whole blood was evaluated using the KRL biological test based on free radical-induced haemolysis (Prost, 1992). The KRL test allows the *ex vivo* dynamic evaluation of the overall antioxidant defence potential of an individual. Whole blood was diluted (v:v 1:25) with KRL buffer (300 mosm/l), and 50  $\mu\text{l}$  of whole blood suspension were submitted to organic free radicals produced at  $37^{\circ}\text{C}$  under air atmosphere from the thermal decomposition of 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH). The kinetics of sample resistance to haemolysis were recorded using a 96-well microplate reader by monitoring the changes at 620 nm absorbance, at  $37^{\circ}\text{C}$  (Laboratoires Spiral, France). Results were expressed (in

minutes) as the time required to reach 50% of maximal haemolysis (half-haemolysis time, HT<sub>50</sub>).

### 3.3.4 Micro-anatomical analyses of the gut

#### *Histology and histomorphometry*

The full-thickness intestinal specimens were divided into two parts, oriented on a corkplate with the mucosa downwards, and fixed as already described. Following fixation, the specimens couples were embedded in paraffin: one for transversal and one for horizontal sectioning. The intestinal wall was transversally evaluated at three different levels (superficial, middle and lower) of the tissue blocks, each containing three groups of serial sections (Di Giancamillo et al., 2010). In the paraffin blocks used for the transversal sectioning, histomorphometrical analyses were performed. In addition, the horizontal sectioning of the middle layer enabled us to observe as much of the intestinal crypts as possible.

The serial and semi-serial microtome sections (4µm-thick) obtained were stained with Hematoxylin-Eosin (HE) sequential stain to ascertain structural details. Other sections from both the duodenum and jejunum were utilized as follows.

For the histomorphometry, on HE-stained sections the height of intestinal villi (V) (10 villi measured per section), the depth of intestinal crypts (C) (10 crypts measured per section), and the ratio of villi and crypts values (V:C ratio) were measured and calculated using image analysis software (Image pro Plus 6.3 Media Cybernetics Inc., Silver Springs, USA). Other sections from both the duodenum and jejunum were used for immunohistochemistry and double immunofluorescence.

#### *Immunohistochemical (IHC) analyses and cellular counts*

Immunostaining of the gut sections was performed as described above in order to detect the following markers of oxidative-nitrosative stress *in situ*. Acrolein (ACR) is produced by hyperoxidation of lipids, and it was identified as a secondary product resulting from lipid peroxidation *in vivo*. 8-hydroxydeoxyguanosine (8-OHdg), an oxidized nucleoside of DNA, is a sensitive marker of DNA damage caused by increased cellular production of reactive oxygen species (ROS), part of the cell aerobic metabolism. Reactive nitrogen species (RNS) such as peroxyxynitrite, can nitrate specific amino acids, such as tyrosine, thus altering the protein function. Nitrotyrosine (NT) is widely used as a sensitive marker of this reaction, indicating *in vivo* nitrosative stress, very closely linked to oxidative stress. All the primary antibodies used in this study for identifying stress biomarkers are detailed in Table 1.

**Table 1.** Primary antibodies used to identify oxidative/nitrosative stress biomarkers.

Target protein	Antibody	Species	Dilution	Characterization	Immunizing antigen/source
Nitrotyrosine (NT)	Anti-NT	Mouse monoclonal	1:1000 (IHC) 1:3000 (WB)	Immunohistochemistry Western blot	3-(4-hydroxy-3-nitrophenylacetamido) propionic acid conjugated to bovine serum albumin (BSA)/GeneTex, Inc
Acrolein (ACR)	Anti-ACR	Mouse monoclonal	1:500 (IHC) 1:1000 (WB)	Immunohistochemistry Western blot	Acrolein modified keyhole-lympet hemocyanine/Abcam
8-Hydroxy-2'-deoxyguanosine (8-OHdG)	Anti-8OHdG	Mouse monoclonal	1:1000 (IHC) 1:3000 (WB)	Immunohistochemistry Western blot	Chemical / Small Molecule: 8-Hydroxy-2'-deoxyguanosine conjugated Keyhole Limpet Hemocyanin/Abcam

Immunohistochemical staining was performed using the Elite ABC KIT system (Vector Laboratories, Inc., California, USA). Before applying the primary antibodies, endogenous peroxidase activity was blocked by incubating the sections in 3% H<sub>2</sub>O<sub>2</sub> in PBS. Non-specific binding sites were blocked by incubating the sections in normal goat serum (Dakocytomation, Italy). Sections were then incubated with the primary antibodies, overnight at 4 °C. After washing with PBS, sections were incubated with biotin-conjugated anti-mouse Ig antibodies (Dakocytomation), washed with PBS and reacted with peroxidase-labelled avidin-biotin complex (Vector Laboratories). The immunoreactive sites were visualized using a freshly-prepared solution of 10 mg of 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma, Italy) in 15ml of a 0.5M Tris buffer at pH 7.6, containing 1.5ml of 0.03% H<sub>2</sub>O<sub>2</sub>. To ascertain structural details, sections were counterstained with Mayer's haematoxylin.

The specificity of the immunostaining was verified by incubating sections with: (i) PBS instead of the specific primary antibodies ; (ii) preimmune sera instead of the primary antisera; (iii) PBS instead of the secondary antibodies; (iv) antisera which were pre-absorbed with an excess of respective synthetic peptides (3 µg/µl) before incubation with sections. The results of these controls were negative (i.e. staining was abolished).

Immunoreactive cells were counted by image analysis software in 10 fields using an Olympus BX51 light microscope (Olympus, Italy) equipped with a digital

camera (at X400 each field represented a tissue section area of about 0.036 mm<sup>2</sup>) (Di Giancamillo et al., 2008). The observer was not aware of the origin of the sections.

#### *Double immunofluorescence*

Because with cellular counting, only NT-immunoreactive mucosal cells were revealed to be influenced by VB (see below), we opted to characterize the cellular typology involved, using a double immunofluorescence procedure. The dewaxed and re-hydrated sections were incubated with the first-step primary antibody, 1:10 goat anti-mouse nitrotyrosine, for 24 h at 18–20 °C, then washed in TBS (Tris-buffered saline solution 0,05 M Tris/HCl, 0,15 M NaCl pH=7.6), and subsequently treated with the Avidin-Biotin blocking kit solution (Vector Laboratories Inc.). The sections were then washed in TBS for 10 min and incubated with a solution of goat biotinylated anti-rabbit IgG (Vector Laboratories Inc.) 10 µg/ml in TBS for 1 h at 18–20 °C. After rinsing twice in TBS, the sections were treated with Fluorescein-avidin D (Vector Laboratories Inc.), 10 µg/ml in NaHCO<sub>3</sub>, 0.1 M, pH 8.5, 0.15 M NaCl for 1 h at 18–20 °C. The sections were then washed in TBS and incubated with rabbit IgG (Vector Laboratories Inc.) for 2h to inhibit binding of the second primary antiserum to the goat anti-rabbit IgG used in the first sequence. For the second step of the double immunofluorescence procedure, the slides were treated with somatostatin, gastrin or serotonin antibodies (polyclonal rabbit anti-somatostatin-14, AB1976; polyclonal rabbit anti-gastrin, AB930; polyclonal rabbit anti-serotonin, AB938, all from Abcam, Italy). Sections were then rinsed in TBS for 10 min and incubated with 10 µg mL<sup>-1</sup> goat biotinylated anti-rabbit IgG (Vector Labs.) for 1 h at 18–20 °C. The sections were then washed twice in TBS, and treated with Rhodamine-Avidin D (Vector Laboratories Inc.), 10 µg mL<sup>-1</sup> in NaHCO<sub>3</sub>, 0.1 M, pH 8.5, with 0.15 M NaCl for 1 h at 18–20 °C. Finally, slides with tissue sections were embedded in Vectashield Mounting Medium (Vector Laboratories Inc.) and observed using a Confocal Laser Scanning Microscope (FluoView FV300, Olympus, Italy). The immunofluororeactive structures were excited using Argon/Helio-Neon-Green lasers, with excitation and barrier filters set for fluorescein and rhodamine. Images containing the superimposition of fluorescence were obtained by sequentially acquiring the image slice of each laser excitation or channel.

#### *3.3.5 Tissue homogenisation and Western blot analysis*

Tissue specimens were homogenised in 2 ml of an ice-cold lysis buffer (50 mM Tris–HCl, pH 7.5, 0.2% Triton X-100, and protease inhibitor mixture) per 200 mg of tissue using an Ultra-Turrax homogeniser (IKA-Werke, Staufen, Germany). The homogenate was then centrifuged at 20000 g for 20 min at 4°C

to remove all insoluble material. The supernatant was collected, and total protein content was measured using a commercial protein quantification kit (Pierce, Rockford, IL, USA) based on the bicinchoninic acid (BCA) colorimetric detection of the cuprous cation obtained by protein  $\text{Cu}^{2+}$  reduction in an alkaline medium. The optical densities were read at 562 nm against a calibration curve, using a bovine serum albumin (SIGMA, Italy) with a working range of 50–800  $\mu\text{g}/\text{ml}$ . Total proteins (35  $\mu\text{g}$ ) were resolved by 10% SDS-PAGE and electro-transferred onto nitrocellulose membranes. After blocking, membranes were incubated overnight at 4°C with primary antibodies (see Table 1) and subsequently with a horseradish peroxidase-conjugated secondary antibody (1:5000) at room temperature for 45 min. Immunoreactivity was detected by chemiluminescence autoradiography according to the manufacturer's instructions, and the images were scanned. The optical intensities of the protein bands of interest were determined densitometrically using Scion Image software. Each electrophoresis gel contained samples from both treatment groups in order to reduce between-blot effects. The values were normalized to GADPH levels.

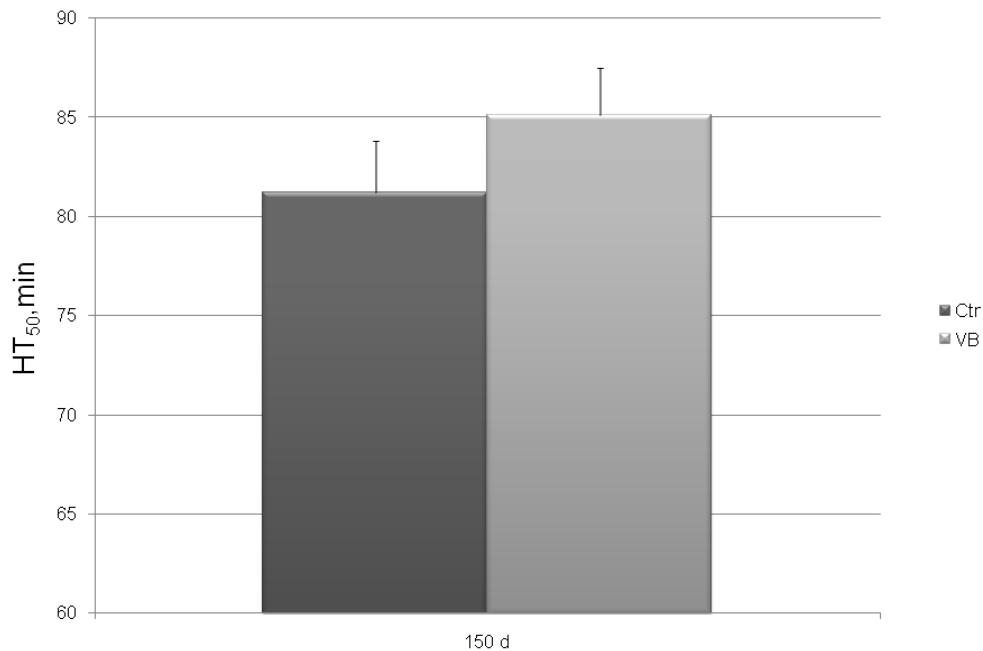
### *3.3.6 Statistical analyses*

Statistical analysis of the quantitative data was performed using the general linear model of the SAS package (version 8.1, Cary Inc., NC, USA). Blood analyses were submitted to one way ANOVA and the baseline measure of that variable (weaning values) was used as a covariate. Histomorphometrical analyses (cells counts) were conducted by ANOVA using the PROC MIXED procedure of the SAS package. The mixed model included the fixed effect of treatment and the random effect of the pig. The individual pig values were considered to be the experimental unit of all the response variables. The data were presented as least squared means  $\pm$  SEM. Differences between means were considered significant at  $P < 0.05$ .

## **3.4 Results**

### *3.4.1 Blood antioxidant activity*

Dietary supplementation with VB did not affect the blood total antioxidant activity in pigs (Fig. 1).



**Figure 1.** KRL test. Total antioxidant activity of whole blood in pigs fed control diet or diet supplemented with verbascoside. (Treatment effect P=0.287).

### 3.4.2 Micro-anatomical analyses

#### *Histology and histomorphometry*

The histological and structural aspects of both duodenum and jejunum in VB-supplemented animals was judged to be fully normal. Moreover, histomorphometrical analysis revealed no differences between the controls and treated animals (Table 2).

**Table 2.** Effects of dietary VB on villi height (V), crypts depth (C), V:C ratio in duodenum and jejunum.

Measure <sup>1</sup>	Ctrl	VB	P values
<u>Duodenum</u>			
Villi height (V), $\mu\text{m}$	380,15 $\pm$ 8,42	392.55 $\pm$ 26.31	0.385
Crypts depth (C), $\mu\text{m}$	502.44 $\pm$ 15.91	505.68 $\pm$ 26.31	0.885
V:C ratio	0.75 $\pm$ 0.06	0.78 $\pm$ 0.06	0.983
<u>Jejunum</u>			
Villi height (V), $\mu\text{m}$	406.27 $\pm$ 6.33	401.22 $\pm$ 8.72	0.560
Crypts depth (C), $\mu\text{m}$	461.18 $\pm$ 6.11	453.49 $\pm$ 14.79	0.713
V:C ratio	0.88 $\pm$ 0.05	0.88 $\pm$ 0.05	0.932

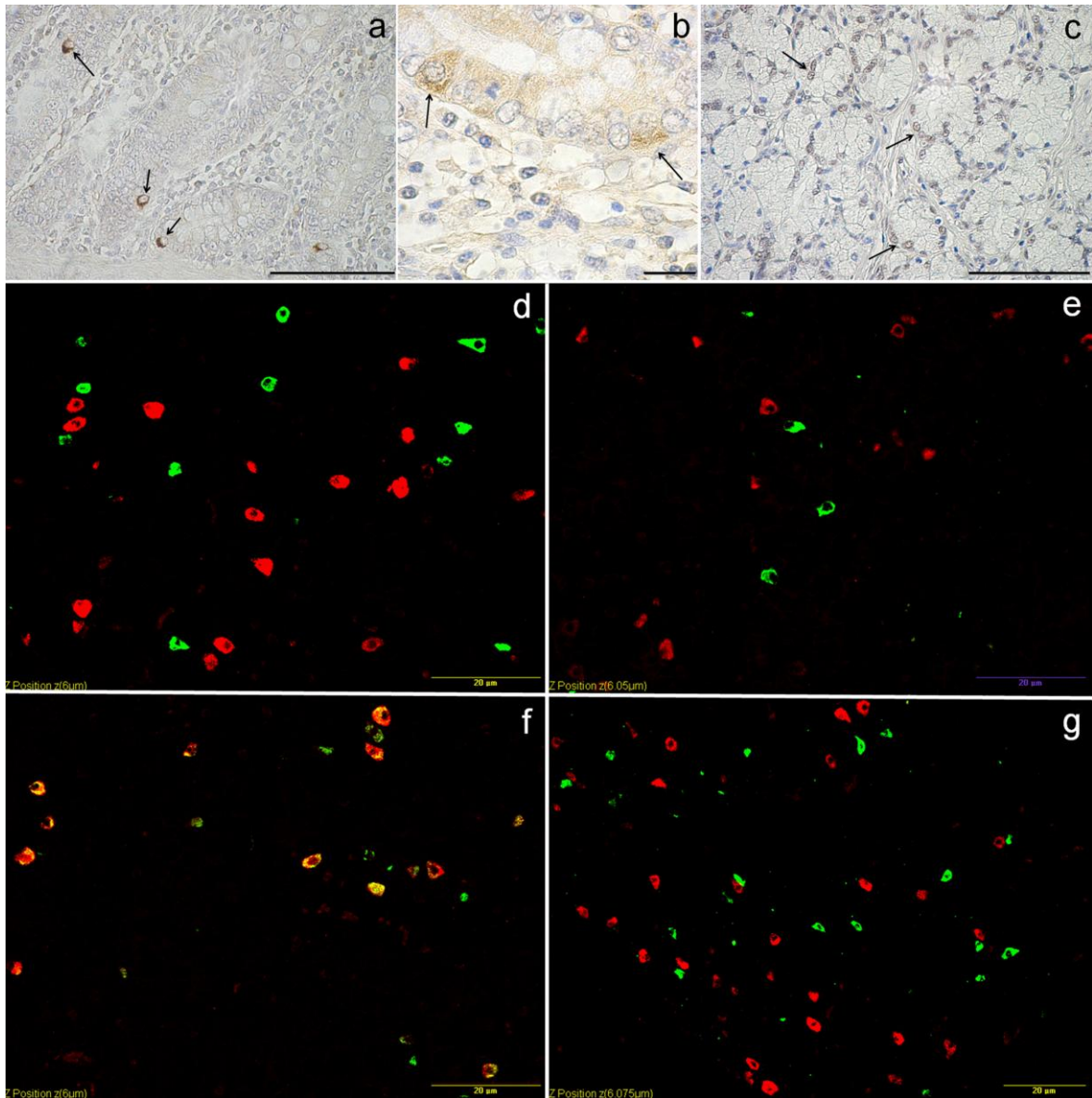
<sup>1</sup>Values are least squared means  $\pm$  SEM; n / treatment = 9.

### *Immunohistochemistry and cell counts*

Immunostaining of duodenum and jejunum with NT antibody revealed that in both control and treated animals, nitrotyrosine was present in small, roundish endocrine cells (Fig. 2a) and lymphocytes of the diffuse lymphatic tissue. We subsequently quantified NT immunoreactivity by cell counts, and found that NT was significantly lower in the duodenal endocrine cells of the treated animals, but not in the mucosal lymphocytes (Table 3). Jejunum on the other hand, showed no significant differences within the groups (Table 3). The immunoreactivity to ACR was present in small endocrine cells (Fig. 2b) and lymphocytes of the diffuse lymphatic tissue in both controls and treated animals. ACR quantification by cell counts revealed no differences within the groups in either the duodenum or jejunum (Table 3).

Finally, we evaluated the expression of 8-OHdg in VB pigs compared with controls. We noted that immunoreactivities were present in the nuclei of endocrine cells and mucosal lymphocytes of the diffuse lymphatic tissue in both duodenum and jejunum, but also in the mucous cell of the Brunner gland of the duodenum in both control and treated animals (Fig. 2c). Counts of the immunoreactive structures did not show any differences within the treatment groups in both duodenum and jejunum (Table 3).





**Figure 2.** Micro-anatomical analyses in VB supplemented animals. Immunohistochemistry for (a) nitrotyrosine in small roundish endocrine cells of the duodenal crypts (arrows; scale bar 100µm), (b) acrolein in endocrine cells of the duodenal crypts (arrows, scale bar 10µm), (c) 8OHdg in some nuclei of duodenal Brunner glands (arrows, scale bar 100µm). Double immunofluorescence for (d) nitrotyrosine and somatostatin in duodenum with no co-localization (green and red respectively; scale bar 20µm), (e) nitrotyrosine and gastrin in duodenum with no co-localization (green and red respectively; scale bar 20µm), (f) nitrotyrosine and serotonin in duodenum with some co-expression (green and red respectively, and yellow for co-expression; scale bar 20µm), but not in jejunum (g) (green and red respectively; scale bar 20µm).

**Table 3** Effects of dietary VB on histomorphometrical analyses related to immunohistochemistry (oxidative/nitrosative stress biomarkers) of duodenum and jejunum<sup>1</sup>.

Measure1	CON	VB	P values
<b><u>Anti-NT</u></b>			
<b><u>Duodenum</u></b>			
Endocrine cells	10.95 ± 0.29	8.95 ± 0.26	< 0.001
Lymphocytes	5.00 ± 0.25	4.90 ± 0.20	0.664
<b><u>Jejunum</u></b>			
Endocrine cells	14.70 ± 0.14	14.30 ± 0.20	0.123
Lymphocytes	4.95 ± 0.19	5.05 ± 0.21	0.628
<b><u>Anti-ACR</u></b>			
<b><u>Duodenum</u></b>			
Endocrine cells	16.75 ± 0.30	16.50 ± 0.33	0.586
Lymphocytes	4.75 ± 0.17	4.45 ± 0.19	0.109
<b><u>Jejunum</u></b>			
Endocrine cells	15.10 ± 0.36	14.90 ± 0.35	0.710
Lymphocytes	3.10 ± 0.09	3.05 ± 0.07	0.709
<b><u>Anti-8OH-dg</u></b>			
<b><u>Duodenum</u></b>			
Endocrine cells (nuclei)	17.10 ± 0.32	17.45 ± 0.30	0.587
Lymphocytes (nuclei)	73.89 ± 0.74	73.20 ± 0.88	0.514
Brunner glands (nuclei)	90.20 ± 1.24	89.10 ± 0.91	0.480
<b><u>Jejunum</u></b>			
Endocrine cells (nuclei)	19.79 ± 0.43	19.30 ± 0.30	0.367
Lymphocytes (nuclei)	91.47 ± 0.94	91.80 ± 1.15	0.829

<sup>1</sup>Values are least squared means ± SEM; n / treatment = 9

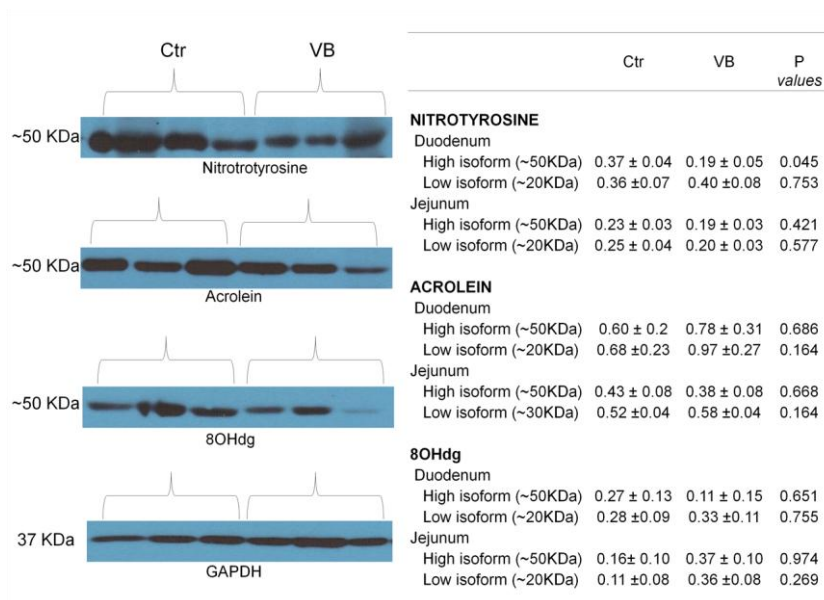
#### *Double immunofluorescence*

Because with cellular counting, only anti-NT immunoreactive endocrine cells were revealed to be influenced by VB, we decided to characterize the cellular typology involved. NT revealed no co-localization with somatostatin (Fig. 2d) and gastrin (immunostaining only for duodenum; Fig. 2e), whereas some co-expression was present with serotonin in the duodenum (Fig. 2f), but not in the jejunum (Fig. 2g) in VB animals.

#### *3.4.3 Western blot analyses*

The antibodies used in this study recognized adducts with two specific molecular weights: 50 and 20kDa in both duodenum and jejunum, with the exception of 50 and 30kDa for ACR jejunum. Comparison of the relative densities using the anti-ACR or anti-8OHdg revealed no statistical differences within the groups

both in duodenum and jejunum, whereas anti-NT' showed a statistically lower signal of both the isoforms in the duodenum of VB animals (Fig. 3).



**Figure 3.** Western blot. Nitrotyrosine protein adducts decreased in duodenum of VB animals ( $P < 0.05$ ), but not in jejunum; Acrolein and 8OHdg revealed no significant differences within the groups of treatment. Nitrotyrosine, acrolein and 8OHdg levels were normalized and quantitated to GAPDH.

### 3.5 Discussion and conclusion

In recent years there has been increased scientific interest in the application of antioxidants to dietary interventions due to findings linking oxidative stress with the development of chronic and degenerative diseases. Phenolic compounds that are commonly present as secondary metabolites in both edible and nonedible plants are above all in recent years studied because they are able to display several biological effects, including antioxidant activity (Hu, 2011). This is of a special importance considering that, when in excess, the reactive oxygen species (ROS) and the reactive nitrogen species (RNS), which are products of normal metabolic cellular pathways, can lead to the development of chronic pathological conditions (Finkel and Holbrook, 2000). As previously observed, acute and chronic stress can induce gastrointestinal oxidative stress and mucosal injury (Manashi et al., 2000; Chen et al., 2007). Quite recently, antioxidant therapies have been hypothesized as effective in contrasting some human degenerative diseases (Firuzi et al., 2011). Additionally, nutritional epigenetics is

now being debated against human age-related diseases (Park et al., 2012) and some types of malignancies (Shen et al., 2012).

Our results show that biomarkers of oxidative and nitrosative stress are detected in the mucosal components of both the duodenum and jejunum, especially in the endocrine cells that play a fundamental role in the control of the gut .

To our knowledge, this is the first time that epithelial cells belonging to the diffuse endocrine system have been correlated to oxido-redox balance. Less surprising is the presence of stress biomarkers in immunocompetent cells belonging to diffuse lymphatic tissue, as also observed in fish (Pascoli et al., 2011).

We focused our attention on the gut environment, because in farming systems, several stress factors (disease pressure, transportation, vaccination, feed transitions, long-term periods of farming, mixing and other environmental factors) play a major role in disturbing the gut. There are essentially two ways, by which stress negatively impacts on gut health: i) by causing disturbances in gastrointestinal motility and feed intake patterns, and ii) by leading to imbalances between resident microflora and microbial pathogens, thus increasing the risk of infections (Rostagno, 2009). These in turn result in systemic changes in the animal, such as oxidative stress and inflammation, leading to the loss of gut barrier integrity (Zhou and Zhong, 2012), and consequently diarrhoea and intestinal infections. The end result is increased use of drugs, higher veterinary costs, poorer efficiency and daily gain, higher mortality rate and consequent financial loss.

In this study, NT was found to be influenced by VB dietary intervention with a significant decrease, measured by NT-immunopositive endocrine cell count and Western blot in the duodenum, but not in the jejunum. This may be explained by considering that the duodenum is more exposed than jejunum to the chemical stress of the acidic contents of the stomach. Secondly, the duodenum is the first intestinal site in which the microencapsulated VB can affect the intestinal mucosa, and in which consequently the studied molecule may display (and possibly exhaust) its antioxidant properties. This considering, a gradual release in the active principle could be commercially performed in the encapsulation procedure in such a way to reach the intestinal tracts further and ensure a more extended antioxidant activity. We also performed double immunofluorescence tests for co-localization studies. Endocrine cells that were immunoreactive for serotonin were found to be the only ones directly involved in relieving nitrosative stress. In the gut, serotonin is synthesized by the enterochromaffin cells and intramural neurons (Gershon, 2003), and the gut is one of the major sites of serotonin synthesizing and releasing. Besides being a neurotransmitter in the central nervous system, serotonin has also emerged as a key mediator of various biological processes in peripheral tissues, such as the regulation of bowel

motility and secreting processes, cell proliferation and differentiation, as well as visceral sensitivity (Gershon and Tack, 2007). In addition, serotonin is a pivotal signalling molecule in the brain-gut axis, both in health and disease (Crowell and Wessinger, 2007). Because enterochromaffin cells were shown in this study to be less subject to nitrosative stress after VB supplementation, we would suppose that a reduction in the serotonin nitrosative stress status may indirectly influence the efficiency of the gut barrier, modulating the motility and the secretory processes of the mucosal cells.

Our data on total antioxidant activity of whole blood failed to reveal any difference between dietary treatments. An our recent study (Rossi et al., 2011) failed to reveal any differences in reactive oxygen molecules (d-ROM) production in post weaning piglets fed VB supplements. In contrast, other authors (Liu et al., 2003), have reported the antioxidant action of verbascoside on plasma TBARS values (malondialdehyde measure) of immobilized rabbits fed with 0.8 mg/kg. We suggest that the VB effect after long-term dietary supplementation may exert only a local (duodenum) and not a systemic antioxidant activity, due to the VB dosage used, which is possibly entirely metabolized by duodenal mucosa. To date, considerable evidence of oxidative stress has been obtained as causing a number of diseases in farm animals. Taking into account the great amount of oxidative stress-mediated conditions involved in all the farming phases (weaning, growing and fattening phases), there is a great potential for antioxidant supplementation in farm animals (Lykkesfeldt and Svendsen 2007; Lalles et al., 2011), although further studies to evaluate the optimal dose and length of the studied dietary supplementation and a better understanding of its mechanism of action are needed. Finally, considering the antioxidant activity of verbascoside, it would also be interesting to investigate its possible influence on meat quality and to assess its possible use as a substitute for synthetic vitamin E (alpha-tocopheryl acetate), which is usually present in feedstuff.

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### **3.6 References**

**Aarestrup F.M.** (1999). Association between the consumption of antimicrobial agents in animal husbandry and the occurrence of resistant bacteria among food animals. *International Journal of Antimicrobial Agents*, 2: 279-285.

**Bach Knudsen K.E.** (2001). Development of antibiotic resistance and options to replace antimicrobials in animal diets. *Proceedings of the Nutrition Society*, 60: 291-299.

**Bezkorovainy A.** (2001). Probiotics: determinants of survival and growth in the gut. *The American Journal of Clinical Nutrition*, 73: 399S-405S.

**Bruins M.J., Cermak R., Kiers J.L., van der Meulen J., van Amelsvoort J.M., van Klinken B.J.** (2006). *In vivo* and *in vitro* effects of tea extracts on enterotoxigenic *Escherichia coli*-induced intestinal fluid loss in animal models. *Journal of Pediatric Gastroenterology and Nutrition*, 43: 459-469.

**Chen R., Su J., Yang S., Li J., Wang T., Zhou H.** (2002). Effect of isoverbascoside, a phenylpropanoid glycoside antioxidant, on proliferation and differentiation of human gastric cancer cell. *Acta Pharmacological Sinica* 23: 997–1001.

**Chen Q., Le G.W., Shi Y.H., Zhang S.M., Jin X.** (2007). Effect of iron supplementation on intestinal function and oxidative stress in piglets with induced colitis. *Journal of Animal and Feed Sciences*, 16: 205-213.

**Corino C., Rossi R., Musella M., Cannata S. and Pastorelli G.** (2007). Growth performance and oxidative status in piglets supplemented with verbascoside and teupolioside. Proceedings of the ASPA XVII Congress, Alghero, 29 May - 1 June, 292-294.

**Crowell M.D. and Wessinger S.B.** (2007). 5-HT and the brain-gut axis: opportunities for pharmacologic intervention. *Expert Opin. Investg. Drugs* 16: 761-765.

**Daglia M.** (2011). Polyphenols as antimicrobial agents. *Current Opinion in Biotechnology*. (Epub ahead of print)

**Di Giancamillo A., Vitari F., Savoini G., Bontempo V., Bersani C., Dell'Orto V., Domeneghini C.** (2008). Effects of orally administered probiotic *Pediococcus acidilactici* on the small and large intestine of weaning piglets. A qualitative and quantitative micro-anatomical study. *Histology and Histopathology*, 23: 651-664.

**Di Giancamillo A., Vitari F., Bosi G., Savoini G., Domeneghini C.** (2010) The chemical code of the swine enteric neurons and the number of enteric glial

cells may be affected by dietary probiotics. *Neurogastroenterology and Motility*, 22: 271-278

**Finkel T., Holbrook N.J.** (2000). Oxidants, oxidative stress and the biology of ageing. *Nature*, 408: 239–247.

**Firuzi O., Miri R., Tavakkoli M., Saso L.** (2011). Antioxidant therapy: current status and future prospects. *Current Medicinal Chemistry*, 18: 3871-3888.

**Fraga C.G** (2007). Plant polyphenols: how to translate their in vitro antioxidant actions to in vivo conditions. *IUBMB Life* 59: 308-315.

**Gershon D.** (2003). Plasticity in serotonin control mechanisms in the gut, *Current Opinion in Pharmacology*, 3: 600–607.

**Gershon M.D. and Tack J** (2007). The serotonin signalling system: from basic understanding to drug development for functional GI disorders. *Gastroenterology*, 132: 397-414.

**Hu M.L.** (2011). Dietary polyphenols as antioxidants and anticancer agents: more questions than answers. *Chang Gung Medical Journal*, 34: 449-460.

**Lallès J.P., Lacan D., David J.C.** (2011). A melon pulp concentrate rich in superoxide dismutase reduces stress proteins along the gastrointestinal tracts of pigs. *Nutrition*, 27: 358-363.

**Lehucher-Michel M.P., Lesgards J.F., Delubac O., Stocker P., Durand P., Prost M.** (2001). Oxidant stress and human disease: current knowledge and perspectives for prevention. *Press Med*, 30:1017–1023.

**Li J.X., Xin D., Li H., Lu J.F., Tong C.W., Gao J.N., Chan K.M.** (1999). Effect of verbascoside on decreasing concentration of oxygen free radicals and lipid peroxidation in skeletal muscle. *Zhongguo Yao Li Xue Bao*, 20: 126-130.

**Lien T.F., Horng Y.M., Wu C.P.** (2007). Feasibility of replacing antibiotic feed promoters with the Chinese traditional herbal medicine Bazhen in weaned piglets. *Livestock Science*, 107: 97-102.

**Liu M.J., Li J.X., Guo H.Z., Lee K.M., Qin L., Chan K.M.** (2003). The effects of verbascoside on plasma lipid peroxidation level and erythrocyte

membrane fluidity during immobilization in rabbits: a time course study. *Life Science*, 73: 883-892.

**Lykkesfeldt J., Svendsen O.** (2006). Oxidants and antioxidants in disease: Oxidative stress in farm animals. *The Veterinary Journal*, 173: 502-511.

**Manashi B.D., Mark M.B.S., Case W.B.S.** (2000). The acute and chronic stress-induced oxidative gastrointestinal injury in rats, and the protective ability of a novel grape seed proanthocyanidin extract. *Nutrition Research*, 19: 1189-1199.

**NRC.** (1998). Nutrient Requirements of Swine, 10th rev. ed. Nat. Acad. Press, Washington, DC.

**Palazzo M., Vizzarri F., Cinone M., Corino C., Casamassima D.** (2011) Assessment of a natural dietary extract, titrated in phenylpropanoid glycosides, on blood parameters and plasma oxidative status in intensively reared Italian hares (*Lepus corsicanus*. *Animal*, doi:10.1017/S1751731110002569, 1-7.)

**Park L.K., Friso S., Choi S.W.** (2012). Nutritional influences on epigenetics and age-related disease. *Proceeding of the Nutrition Society*, 71: 75-83.

**Pascoli F., Negrato E., Di Giancamillo A., Bertotto D., Domeneghini C., Simontacchi C., Mutinelli F., Radaelli G.** (2011) Evaluation of oxidative stress biomarkers in *Zosterisessor ophiocephalus* from the Venice Lagoon, Italy. *Aquatic Toxicology*, 101: 512-20.

**Pastorelli G., Rossi R., Candotti P., Corino C.** (2011). Evaluation of stress due to mixing in piglet. Proceedings of the 19<sup>th</sup> ASPA Congress, Cremona, June 7-10, 2011. *Italian Journal of Animal Science*, 10 (suppl. 1): 6.

**Prost M.** (1992). Process for the determination by means of free radicals of the antioxidant properties of a living organism or a potentially aggressive agents. *In US Patent* 5,135,850.

Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. Available at: <http://irmm.jrc.ec.europa.eu/SiteCollectionDocuments/EC-1831-2003.pdf>

**Rossi R., Pastorelli G., Corino C.** (2011). Influence of dietary plant antioxidant on oxidative status and immune parameters in post weaning piglet. *Proceeding*



of the 19<sup>th</sup> ASPA Congress, Cremona, June 7-10, 2011. *Italian Journal of Animal Science*, 10(suppl. 1), 6-7.

**Rostagno** (2009). Can stress in farm animals increase food safety risk? *Foodborne Pathogens and Disease*, 6: 767-776.

**Sehm J., Linder Mayer H., Dummer C., Treutter D., Pfaffl M.W.** (2007). The influence of polyphenol rich apple pomace or red-wine pomace diet on the gut morphology in weaning piglets. *Journal of Animal Physiology and Animal Nutrition*, (Berl). 91, 289-96.

**Shen M., Chan T.H. and Dou Q.P.** (2012). Targeting tumor ubiquitin-proteasome pathway with polyphenols for chemosensitization. *Anticancer Agents in Medicinal Chemistry*, (Epub ahead of print).

**Stevenson D.E., Hurst R.D.** (2007). Polyphenolic phytochemicals--just antioxidants or much more?. *Cellular and Molecular Life Sciences*, 64, 2900-2916.

**Vertuani S., Beghelli E., Scalambra E., Malisardi, G., Copetti, S., Toso, R.D., Baldisserotto, A., Manfredini S.** (2011). Activity and stability studies of verbascoside, a novel antioxidant, in dermo-cosmetic and pharmaceutical topical formulations. *Molecules* 16, 7068-7080.

**Virgili F., Marino M.** (2008). Regulation of cellular signals from nutritional molecules: a specific role for phytochemicals, beyond antioxidant activity. *Free Radical Biology and Medicine*, 45, 1205-1216.

**Walters E.M., Agca Y., Ganjam V. And Evans T.** (2011 a). Animal models got you puzzled?: think pig. *Annals of the New York Academy of Sciences*, 1245: 63-64.

**Walters E.M., Samuel M.S., Wells K.D., Riley L.K. and Prather R.S.** (2011 b). The pig as a biomedical model: how the national Swine resource and research center can help you. *Reproduction, Fertility and Development*, 24: 284.

**Zhou Z and Zhong W.** (2012) Zinc and Hepatocyte Nuclear Factor-4 $\alpha$  in Alcohol-Induced Intestinal Barrier Dysfunction. *Journal of Epithelial Biology and Pharmacology*, 5 19-27.



## **CHAPTER 4- ASSAY 2**

# **Copper sulphate dietary intervention affects small intestine structure and large intestinal microbiota in post-weaning piglets**

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## 4. Copper sulphate dietary intervention affects small intestine structure and large intestinal microbiota in post-weaning piglets

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### 4.1 Abstract

The effects of dietary supplementation of different copper sulphate (CuSO<sub>4</sub>) forms in post-weaning piglets on growth performance, intestinal structure and microbiology, as well as on liver copper content were studied. At weaning 90 piglets were allotted to three dietary treatments for 18 d: control diet (with no copper sulphate) and two diets supplemented with 150 mg/kg of copper sulphate in either protected or unprotected form. Unprotected copper sulphate showed a numerical decrease in feed:gain ratio. Moreover, duodenum villi length and crypts depth revealed to be higher in the treated animals (unprotected form). Furthermore, the two copper supplements caused a decrease in Streptococci microbial populations, and a higher liver copper content. The obtained results revealed a modulation in the intestinal environment caused by addition of unprotected copper sulphate, as well as positive structural changes in the duodenum.

**Keywords:** piglets, copper sulphate, intestinal morphology, intestinal microbiology

## 4.2 Introduction

Copper is an essential trace element that plays a vital role in the physiology of animals: it is necessary for foetal growth and early post-natal development, for haemoglobin synthesis, for a correct connective tissue structure especially in the cardiovascular system and in bones, for proper nerve function and bone development, and for an adequate development of inflammatory processes. It is involved in different biochemical processes of the mammalian general metabolism, and a copper deficiency leads to several disturbances including depression of growth, anaemia, bowing of the legs, spontaneous fractures, ataxia of newborns, cardiac and vascular disorders, lack of pigmentation, decrease in some organs weight, depressed reproductive performance including diminished egg production (Opinion of the Scientific Committee for Animal Nutrition on the use of copper feedingstuff 2003).

Several investigations have shown that the addition of copper in the diets of pigs increases their growth performances (Stahly et al., 1979; Cromwell et al., 1989, 1998; Hill et al., 2000). This positive effect on growth seems to be dependent on a simultaneous increase in feed intake. The most common form of Cu used in feeds for growth promotion in pigs is the sulfate salt ( $\text{CuSO}_4$ ) (Cromwell et al., 1998). It is routinely supplemented to weanling pig diets at concentrations above the nutrition requirement of the animal because pharmacological concentrations of inorganic cupric sulphate ( $\text{CuSO}_4$ ) have been shown to have growth stimulatory properties in pigs (Barber et al., 1955; Bunch et al., 1961; Hawbaker et al., 1961; Armstrong et al., 2004). The addition of supranutritive levels of Cu to the diets of young growing pigs increases their average daily gain and gain efficiency (Zimmerman, 1986).

The mechanism by which this feed additive functions is not well understood. It is likely that possible toxic compounds produced by the resident microflora of the gastrointestinal tract of the pig are involved (Shurson et al., 1990). Reducing the production of these toxic compounds of microbial origin through the use of Cu may be one mechanism by which this feed additive is shown to be able to improve growth performance. The activity also of enzymes important in the transport of nutrients across the intestinal wall is decreased when bacteria are present in the gastrointestinal tract (Kawai and Morotomi, 1978).

Cu has been recognized as having antibacterial and antimycotic activity. Several observations revealed that different levels of copper sulphate incorporated in the diet modified quantitatively some Gram-positive intestinal bacterial populations as demonstrated for *Streptococcus* spp. (Dunning and Marquis, 1998). A considerable number of experiments have been performed on animals of different body weight, different ages and different levels of  $\text{CuSO}_4$  with various results. After weaning, it is well established that a villous atrophy is observed in

the pig small intestine (Hampson, 1986; Hedemann et al., 2003), and a decrease in the activity of digestive enzymes of pancreatic source has been reported (Hedemann and Jensen, 2004). Cu may promote the process of tissue repair in the small intestine and stimulate the synthesis of digestive enzymes, resulting in a better digestion and absorption of nutrients and potentially improving growth performances.

The current study was conducted to investigate the effects of two different forms of high dietary concentrations of  $\text{CuSO}_4$  on the growth performances, intestinal and liver microanatomy, and intestinal microbiota in piglets after weaning. The Cu dietary intervention was studied analyzing two forms of  $\text{CuSO}_4$ , one (the “protect” one) was microencapsulated for bypassing the acid environment of the stomach and proximal duodenum, the other was “unprotected”, for performing a proper comparison.

## **4.3 Materials and methods**

### *4.3.1 Animals and experimental design*

At weaning, ninety 26-d-old Landrace  $\times$  Large White piglets ( $8.4 \pm 0.2$  kg) were assigned to 1 of 3 dietary groups in a randomized complete-block design based on body weight and sex, with 3 pens/treatment (10 pigs/pen). One group of piglets was fed the basal diet (CTR) (Table 1), which contained no added  $\text{CuSO}_4$ .

**Table 1** *Composition of diets (as-fed basis)*

Item	BW 8-15 kg
Ingredient, %	
Steam-rolled corn	28.0
Corn, yellow	15.0
Barley	15.0
Wheat middings	8.0
Dried whey	5.0
Soy protein concentrate	4.0
Soybean meal 48	6.0
Fish meal, 70% CP	2.8
Rice protein meal, 65% CP	2.4
Dextrose	2.5
Wheat bran	3.0
Soy oil	3.0
Vitamin-mineral premix <sup>1</sup>	3.5
Dicalcium phosphate	1.0
L-Lysine·HCl	0.5
Preservative <sup>2</sup>	0.3
Calculated chemical composition <sup>3</sup>	
C.P., %	20.61
Ether extract, %	8.33
Crude fibre, %	3.09
Ash, %	6.13
Lysine, %	1.31
Methionine + cysteine, %	0.79
Threonine, %	0.85
Tryptophan, %	0.26

<sup>1</sup>Provided per kilogram of complete diet: Ca, 2.8 g; P, 0.14 g; Na, 1.33 g; vitamin A, 21,000 IU; vitamin D3, 2,000 IU; vitamin E, 175 IU; vitamin K (menadione sodium bisulphite), 3.8 mg; vitamin B1, 4.9 mg; vitamin B2, 9.8 mg; calcium D-pantothenate, 40 mg; niacin, 57.8 mg; vitamin B12, 0.09 mg; vitamin B6, 7.7 mg; folic acid, 3.4 mg; biotin, 0.33 mg; choline chloride, 1000.0 mg; Zn (ZnO), 150.0 mg; Cu (CuSO<sub>4</sub>), 0, 150 mg for control and treated groups; Mn (MnO), 108.0 mg; Fe (FeCO<sub>3</sub>), 470.0 mg; I (KI), 3.85 mg; Co (CoSO<sub>4</sub>), 1.40 mg; Se (as Na<sub>2</sub>SeO<sub>3</sub>), 490.0 µg. Premix containing Calcium formiate, *Saccharomyces cerevisiae*, sodium chloride, barley, butyric acid, dl-tryptophan, dl-methionine, l-treonine.

<sup>2</sup>Composition per kg of complete feed: formic acid, 0.3 g; lactic acid, 1.1 g; colloidal silica carrier 1.6 g.

<sup>3</sup>Calculated based on INRA 2004.

The other two diets differed in the administered form of CuSO<sub>4</sub>: i) diets with 150 mg/Kg of copper sulphate in protected form (150P), and ii) diets with 150 mg/Kg of copper sulphate in unprotected form (150UP). The “protected” form



of  $\text{CuSO}_4$  was microencapsulated in a protective matrix of hydrogenated vegetable lipids manufactured with a spray cooling technology (Sintal Zootecnica, Isola Vicentina, Vicenza, Italy). The diets were formulated to meet or exceeded National Research Council nutrient requirements for swine (NRC, 1998). The animals were housed in an environmentally controlled nursery and given *ad libitum* access to water and meal diet. Body weights (BW) and feed intake (FI) were recorded on days 0 and 18 for the determination of average daily gain (ADG), average daily feed intake (ADFI), and feed efficiency. After 18 days of experimental trial, 18 piglets (6 piglets/treatment) were randomly slaughtered. At slaughter body and liver weights were recorded. All procedures involving animals were in accordance with the European Community guidelines (n. 86/609/CEE) and approved by the Italian Ministry of Health (L. n. 116/92).

#### 4.3.2 Histology, Histochemistry and Histometry

##### *Histology*

At slaughter (6 piglets/treatment), small fragments of the small intestine (duodenum, jejunum, and ileum) and liver (similar lobes) were collected from each animal. The samples (total  $n=72$ ) were immediately fixed in 4% paraformaldehyde in 0.01M phosphate-buffered saline (PBS) pH 7.4 for 24h at 4°C, dehydrated in a graded series of ethanol, cleared with xylene and embedded in paraffin. Microtome sections (4 $\mu\text{m}$ -thick) were obtained from each sample and stained with Hematoxylin-Eosin (HE) sequential staining to ascertain structural details with an optical microscope Olympus BX51 (Olympus, Italy) equipped with a digital camera.

##### *Histochemistry*

Other sections of the small intestine (duodenum, jejunum, and ileum) were used to determine the mucin profile utilizing the following histochemical stains:

- (a) the Alcian blue 8GX pH 2.5-periodic acid Schiff (AB-PAS) sequence, which reveals neutral (PAS-reactive, purple stained) and acid (AB-reactive, azure stained) glycoconjugates, and
- (b) the high iron diamine-Alcian blue 8GX pH 2.5 (HID-AB) sequence, which demonstrates sulphated (diamine-positive, brown-black stained) and sialylated (AB-reactive, azure stained) glycoconjugates respectively.

These histochemical reactions selectively evidence the intestinal mucous cells.

##### *Histometry (performed on HE-stained sections):*

For gut histometry, the height of intestinal villi (V) (10 villi measured per section), the depth of intestinal crypts (C) (10 crypts measured per section), and the ratio of villi and crypts values (V:C ratio) were measured and calculated.

For liver histometry, hepatocytes were measured in their longest and shortest axes, and their area was calculated; in addition the number of hepatocytes nuclei was determined (per mm<sup>2</sup> of hepatic tissue) (Martins et al., 2005) (Table 3).

#### 4.3.3 Liver content of CuSO<sub>4</sub>

At slaughter, liver samples (1mg) from each of the 18 piglets were mineralized in the same microwave system using 1 ml nitric acid (65% SPA grade). The samples were then diluted to 20 ml using bi-distilled water. Cu determinations were carried out using an inductively coupled plasma atomic emission spectrophotometer Ultima 2 (Horiba Jobin Yvon, Milan, Italy). Cu determinations in liver samples were carried out by inductively coupled plasma optical emission spectrometry as described by Lopez *et al.* (2007). All analyses were performed in duplicate.

#### 4.3.4 Microbiological analyses

Intestinal content samples (around 10 g) from the caecum and the ascending colon were immediately transferred into sterile tubes containing 10 mL of sterile saline solution (9 g/Lt NaCl). At the laboratory, the samples were processed by seeding in *tryptic Soy Broth* (Oxoid, Italy) at 1:10 ratio and then they were incubated for 18 hours at 37 °C. After the incubation time, the total bacterial counts were performed using the dilution method onto *Tryptic Soy Agar* (Oxoid, Italy); for *Streptococci* culture *Bile Esculine Agar* (Oxoid, Italy) was used, while for *E. coli* and *Lactobacillus* spp. were used, respectively, *MacConkey Agar* and *Man-Rogosa-Sharpe Agar* (Oxoid, Italy). All the plates for total bacterial counts and for the *E. coli* enumeration were incubated at 37 °C for 24 hours; whereas for *Streptococci* and *Lactobacilli* counting the samples were incubated at 37 °C under 5% CO<sub>2</sub> atmosphere. The bacterial counts were expressed as Colony Forming Unit/ mL (CFU/mL). The bacterial identification was performed using selective media and biochemical tests (e.g., catalase, oxidase, urease, citrate, etc.). All the analyses were performed twice.

#### 4.3.5 Statistical analyses

Statistical analysis of the quantitative data was performed using the general linear model of the SAS (version 8.1, Cary Inc., NC). ANOVA was used to determine the effect of the copper on the growth performance values, on the copper content of the liver and on the intestinal microbiological analyses. Histometrical analyses were analyzed by ANOVA using the PROC MIXED of the SAS package. The mixed model included the fixed effects of treatment and the

random effect of the piglet. The individual piglet values were considered to be the experimental unit of all response variables. The data were presented as least squared means  $\pm$  SEM. Differences between means were considered significant at  $P < 0.05$ .

## 4.4 Results

Growth performance parameters are reported in Table 2.

**Table 2.** Growth Performance in piglets with different dietary treatments

	CTR	150P	150UP
Start Weight , kg	8.44 $\pm$ 0.01	8.42 $\pm$ 0.01	8.43 $\pm$ 0.21
Weight at 18 d, kg	12.26 $\pm$ 0.30	12.08 $\pm$ 0.30	12.57 $\pm$ 0.30
ADG <sup>1</sup> , g/d	212 $\pm$ 16.5	203 $\pm$ 17.1	229 $\pm$ 16.7
ADFI <sup>1</sup> , g/d	558 $\pm$ 86.2	441 $\pm$ 30.9	374 $\pm$ 14.4
F:C <sup>1</sup> , kg/kg	2.61 $\pm$ 0.27	2.20 $\pm$ 0.19	1.63 $\pm$ 0.22

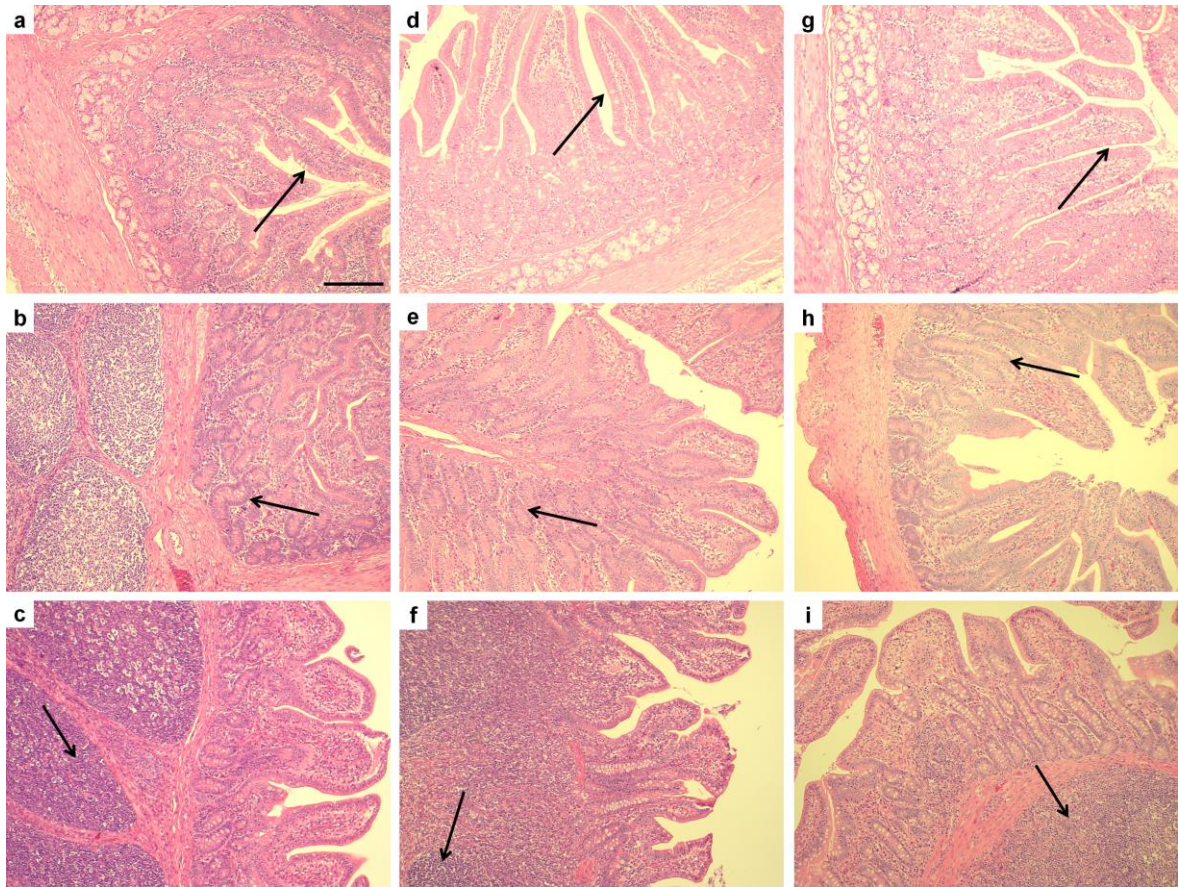
<sup>1</sup>ADG: average daily gain; ADFI: average daily feed intake; F:C: feed conversion rate

The mean weights of the piglets sacrificed at 18 d were  $12.0 \pm 0.2$  kg. Growth performance in the studied period of the trial (0-18 d) did not show any significant difference among the treatments. However, the results showed that 150UP form-fed piglets tended to improve the feed conversion rate(F:C).

### 4.4.1 Histology, Histochemistry and Histometry

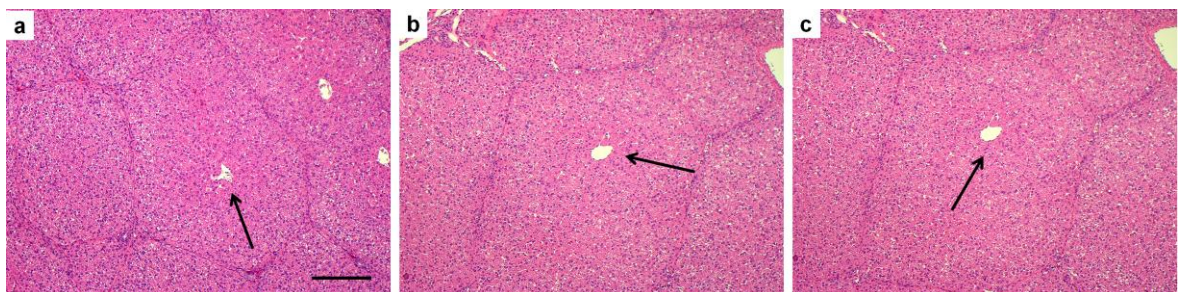
#### *Histology*

Histology showed that copper sulphate in both the studied experimental forms did not show detrimental effects upon the structure of the small intestine (duodenum, jejunum and ileum), which revealed to be anatomically normal in the three groups of treatments (Figs 1)



**Fig. 1.** Histological analyses of duodenum (a, d, g), jejunum (b, e, h) and ileum (c, f, i), HE. All figures have the same scale bar located in Fig 1A: scale bar: 200  $\mu$ m. **a,d,g)** Normal aspect of the small intestinal mucosa: note the numerous villi (arrows) which appear to be regularly arranged and shaped in all animal groups (a=CTR; d=150P, g=150UP); **b,e,h)** Normal aspect of the small intestinal mucosa: intestinal crypts (arrows) are evident in all the piglets groups (b=CTR; e=150P, h=150UP); **c, f, i)** Gut-associated lymphoid tissue is evident (arrows).

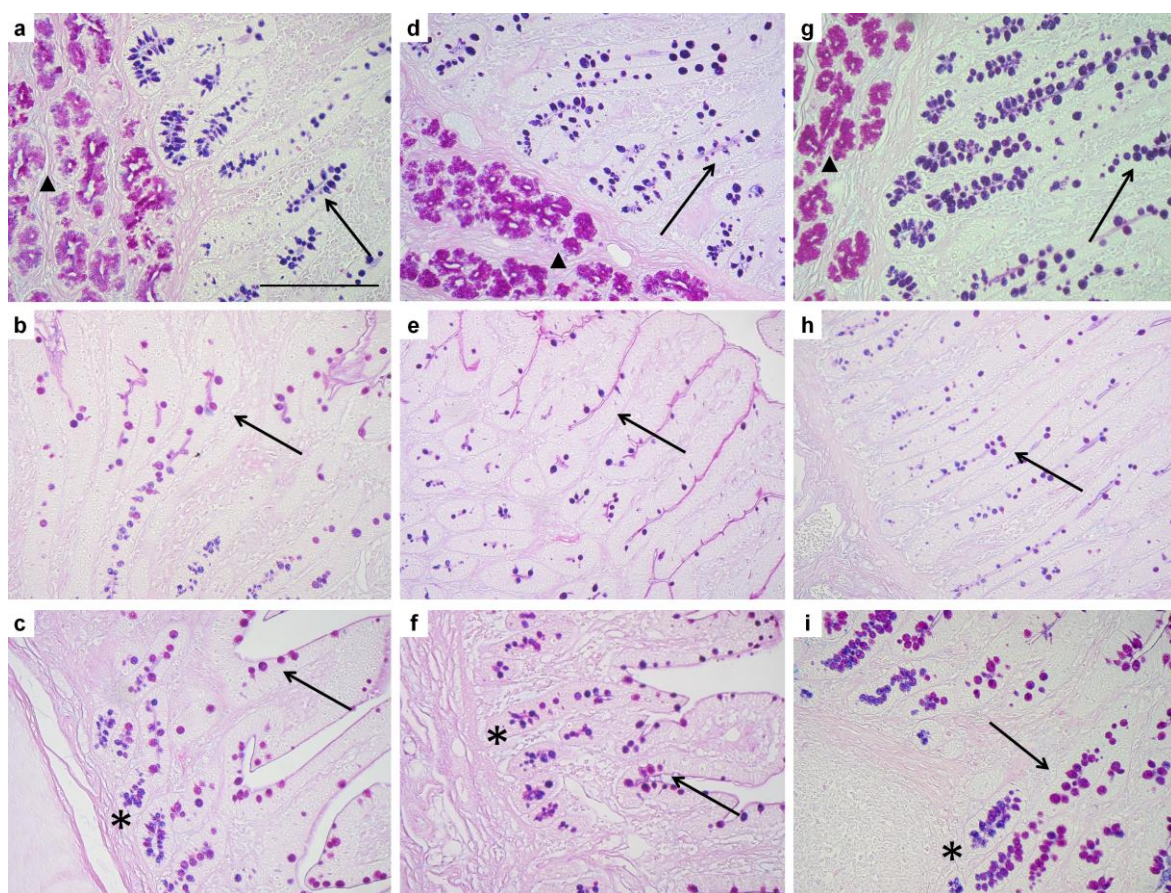
Liver histology showed normal structural aspects in all the studied animals, too (Figs. 2).



**Fig. 2.** Histology of liver, HE. All figures have the same scale bar located in Fig 2a: scale bar: 200  $\mu$ m. The normal architecture of liver lobules is evident: note the presence of centrolubular vein (arrows) (a=CTR; b=150P, c=150UP).

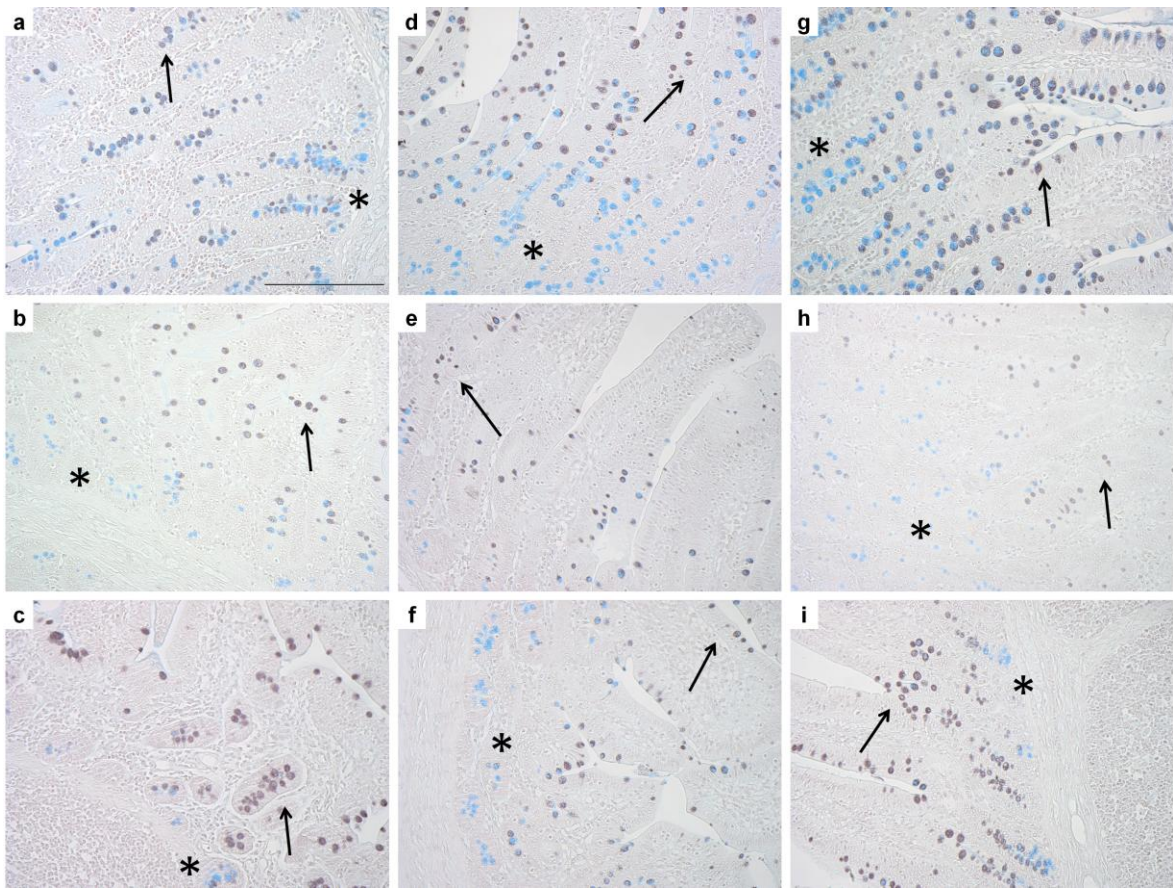
### Histochemistry

The AB/PAS sequential staining showed that intestinal mucous cells contained mixtures of neutral and acidic glycoconjugates in all the small intestinal samples examined (Figs. 3) in either control or treated piglets, so that the large majority of mucous cells appeared violet (arrows). In the ileum however, a compartmentalization was evident, because villi mucous cells prevalently contained neutral (purple-stained) glycoconjugates and intestinal crypts mucous cells contained acid (blue-stained) glycoconjugates (Fig. 3, c,f,i, asterisks). In addition, duodenal Brünner glands were always PAS-reactive, irrespective of the treatments.



**Fig. 3.** Glycoconjugate-histochemistry of duodenum (a, d, g), jejunum (b, e, h) and ileum (c, f, i), AB pH 2.5/PAS sequence. All figures have the same scale bar located in Fig 3A: scale bar: 200  $\mu$ m. There is a high prevalence of mixed (violet colour) glycoconjugates in the mucous cells of duodenum and jejunum (arrows). In ileum (c=CTR; f=150P, i=150UP), the intestinal crypts mucous cells contained prevalently acid (blue) glycoconjugates (asterisks). The duodenum Brünner glands contained always neutral glycoconjugates (a=CTR, d=150P, g=150UP, arrowheads)

In treated and control piglets, sulphated glycoconjugates containing mucous cells predominated in the villi, and sialo-glycoconjugates containing mucous cells occurred mainly at the bases of the villi and along crypts (Figs. 4).



**Fig. 4.** Glycoconjugate-histochemistry of duodenum (a, d, g), jejunum (b, e, h) and ileum (c, f, i), HID AB pH 2.5 sequence. All figures have the same scale bar located in Fig 4A: scale bar: 200  $\mu$ m. The intestinal mucous cells contained prevalently sulphate (brown-black) glycoconjugates in the villi (arrows) and sialoglycoconjugates (azure) in the crypts (asterisks).

### *Histometry*

Histometrical analyses are reported in Table 3. Oral feeding with 150 mg/Kg of Copper sulphate in unprotected form resulted in an increase in villi (V) height and crypt depth ( $P=0.010$ ) of the duodenum. No differences between were observed in jejunum and ileum. Moreover, no quantitative differences have been observed in the liver of the three experimental groups.

**Table 3.** Effect of Copper sulphate (CTR= control, 150P=protected, 150UP= unprotected) on histometrical analyses of the small intestine (duodenum, jejunum and ileum) and liver of piglets.

Misure <sup>1</sup>	CTR	150P	150UP
<b>SMALL INTESTINE</b>			
<u>DUODENUM</u> (µm)			
Villi height (µm)	357.35 <sup>A</sup> ± 6.84	351.55 <sup>A</sup> ± 11.06	392.04 <sup>B</sup> ± 7.98
Crypts depth (µm)	436.54 <sup>A</sup> ± 9.19	439.80 <sup>A</sup> ± 7.92514	473.37 <sup>B</sup> ± 9.25
V:C ratio	0.82 ± 0.011	0.79 ± 0.02	0.83 ± 0.02
<u>JEJUNUM</u>			
Villi height (µm)	317.04 ± 14.87	345.83 ± 9.19	346.02 ± 10.05
Crypts depth (µm)	394.67 ± 8.06	380.19 ± 8.41	374.34 ± 6.66
V:C ratio	0.81 ± 0.04	0.91 ± 0.03	0.92 ± 0.02
<u>ILEUM</u>			
Villi height (µm)	364.93 ± 12.02	350.48 ± 7.39	343.65 ± 6.72
Crypts depth (µm)	367.90 ± 9.79	332.54 ± 8.39	327.27 ± 6.14
V:C ratio	1.00 ± 0.04	1.06 ± 0.03	1.05 ± 0.02
<b>LIVER</b>			
Nuclei (nr/mm <sup>2</sup> )	30.25 ± 1.02	30.00 ± 0.96	30.17 ± 0.84
Hepatocyte (area/mm <sup>2</sup> )	25.10 ± 0.22	26.00 ± 0.25	25.69 ± 0.31
<i>Major axis</i>	6.30 ± 0.03	6.29 ± 0.03	6.25 ± 0.05
<i>Minor axis</i>	5.05 ± 0.03	5.08 ± 0.03	5.10 ± 0.04

Values are means ± SEM, *n* =18; <sup>A, B, C</sup> P<0.01

#### 4.4.2 Liver content of CuSO<sub>4</sub>

Neither its weight nor its weight related to BW were influenced by the studied dietary treatments. The highest Cu concentration in the liver was found in the Cu 150 UP group, that was significantly higher in comparison to that found in control group and 150 P group (Table 4).

**Table 4.** Effect of Copper sulphate (CTR= control, 150P=protected, 150UP= unprotected) on liver weight and Cu content.

	CTR	150P	150UP
Piglet weight (at slaughter), kg	12.18 ± 0.85	12.06 ± 1.14	12.73 ± 0.63
Liver weight, g	253 ± 11.76	254 ± 9.91	273 ± 9.21
Liver incidence, %	2.07 ± 0.07	2.16 ± 0.06	2.28 ± 0.11
Cu, µg/g tissue	23.71 <sup>A</sup> ± 3.16	36.01 <sup>A</sup> ± 4.49	52.83 <sup>B</sup> ± 7.76

Values are means ± SEM, *n* =18; <sup>A, B, C</sup> P<0.0

#### 4.4.3 Microbiological analyses

Microbiological analyses are reported in Table 5. Total bacterial count revealed to be significantly higher in 150UP *vs* Control *vs* 150P (P<0.01) in caecum, whereas in colon the total bacterial count was higher in 150UP than 150 P. Different dietary treatments did not show any differences on the number of coliforms in the colon, but they were significantly lower in the caecum of the 150P piglets, in comparison with the other two groups (P<0.05). The number of Streptococci of the treated animals were significantly lower in the colon when compared to control group (P<0.01), whereas no statistical differences were observed in the caecum. No differences were observed on the number of Lactobacilli in the all examined intestinal tracts of the three groups of animals.

**Tabella 5.** Effect of Copper sulphate (CTR= control, 150P=protected, 150UP= unprotected) on microbiological analyses related to the caecum and colon of the piglets.<sup>1</sup>

	CTR	150P	150UP
<u>CAECUM</u>			
Total bacterial count	6.71 <sup>A</sup> ± 0.40	4.70 <sup>B</sup> ± 0.22	7.00 <sup>C</sup> ± 0.01
Coliforms	6.56 <sup>a</sup> ± 0.39	4.43 <sup>b</sup> ± 0.23	6.30 <sup>a</sup> ± 0.60
<i>Streptococcus spp</i>	6.37 ± 0.25	5.72 ± 0.24	5.77 ± 0.73
<i>Lactobacillus spp</i>	6.54 ± 0.65	6.22 ± 0.60	6.34 ± 1.11
<u>COLON</u>			
Total bacterial count	6.84 <sup>A</sup> ± 0.21	5.60 <sup>B</sup> ± 0.26	7.00 <sup>A</sup> ± 0.01
Coliforms	6.58 ± 0.30	6.22 ± 0.47	6.90 ± 0.72
<i>Streptococcus spp</i>	6.92 <sup>A</sup> ± 0.50	5.79 <sup>B</sup> ± 0.23	5.77 <sup>B</sup> ± 0.73
<i>Lactobacillus spp</i>	6.54 ± 0.66	5.70 ± 0.71	6.34 ± 0.87

<sup>1</sup>Expressed as log<sub>10</sub> CFU/ml; n=18; <sup>a, b</sup> P<0.5; <sup>A, B, C</sup> P<0.01

## 4.5 Discussion

The present study has been focused on the effects of two different forms of high dietary concentrations of CuSO<sub>4</sub> on the growth performances, intestinal and liver morphology, as well as the intestinal microbiological ecosystem in piglets after weaning.

Controversial data about the effect of dietary copper on growth performance has been reported: Cromwell *et al.* (1998) observed that 200 ppm of Cu from Cu chloride or sulphate improved ADG in weaning piglets. Similarly, Armstrong *et al.* (2004) showed that lower dietary Cu concentrations (125 ppm from either CuCit cupric citrate or CuSO<sub>4</sub> cupric sulfate) were as effective as 250 ppm of Cu from CuSO<sub>4</sub> for stimulating the growth of weanling pigs over a 45-d nursery



period. Also Zaho *et al.* (2007) found that Cu, 200 ppm from a commercial Cu-protein complex was an effective growth promoter in swine in the first 10 days of the post-weaning period, but the same Authors observed that it was not effective if considered during the overall post-weaning period (35 days) of the trial. Smith *et al.*, (1997) found that 28-d growth performance of weaning piglets fed 250 ppm Cu from CuSO<sub>4</sub> on a commercial farm did not differ from that of controls. Stansbury *et al.*, (1990) conducted a trial to evaluate growth promoter levels of CuSO<sub>4</sub> for weaning piglets and found no increases in ADFI or ADG for pigs fed 125 or 250 ppm Cu from CuSO<sub>4</sub> in any of the experiments. We only found a tendency to improving the feed conversion rate in piglets fed with the unprotected form of CuSO<sub>4</sub>.

Regarding histological analyses, all examined intestinal tracts showed a quite normal structure. Moreover, the liver structure appeared well conserved and the architecture of hepatic lobules was always well visible, thus suggesting that the studied dietary interventions did not cause detrimental effects. Histometrical analyses revealed that the dietary treatment with the CuSO<sub>4</sub> in unprotected form affected only duodenum, increasing villi length and crypt depth. Actually, this result may be considered together with the better numerical result obtained in feed conversion rate in the same animal group. Data presented in literature are quite controversial about intestinal histometry. In a work with high level of dietary Cu integrations diets fed to weaning piglets, Shurson *et al.* (1990) observed an increase in villus height and crypt depth. Changes in morphology due to addition of 250 ppm of Cu to the diet were not significant in a study by Radecki *et al.* (1992), but tendencies did exist that were similar to the observation of Shurson *et al.* (1990). Zhao *et al.* (2007) observed decreased crypts depth in the small intestine of weaning piglets treated with dietary 200ppm Copper sulphate. In the above mentioned study the intestinal morphology results suggest that growth stimulation with elevated dietary Cu may, in part, be related to its impact on the intestine.

The histochemical evaluation of mucous cells glycoconjugate content did not show differences among the experimental groups, thus evidencing that the studied dietary interventions did not modify the small intestinal mucin profile. Similar results were obtained in the same species by Hedemann *et al.* (2003), who observed that the staining area of neutral, acidic or sialic mucins of the intestinal mucous cells was not affected by treatments.

The most important organ involved in Cu metabolism is probably the liver. The liver receives Cu from serum albumin and is the major organ for Cu storage. At least three important Cu-related functions are associated with the liver: storage, ceruloplasmin synthesis, and preparation of the metal for biliary excretion (Roberts and Sarkar, 2008). In the present work, Cu in protected form failed to increase liver Cu content, a further indication that the Cu in this form is less

available. Only the unprotected form showed a significant increase of copper liver content compared to the other experimental groups. Our results are similar to those obtained by other Authors. Also Lauridensen et al. (1999) showed an increased level of Cu in the liver of piglets with dietary supplement of different dosages of Copper sulphate.

Finally, data related with coliform microorganisms are similar to the results obtained by Jensen (1998), whereas Højberg et al. (2005) found a decreased of coliforms in the colon of animals treated with 175 ppm of Copper sulphate. Varel et al. (1987), in accord with our results on the colon, viewed a decreased number of intestinal populations of Streptococci in piglets treated with 125 ppm of Copper sulphate.

In conclusion, micro-anatomical changes observed in the present study revealed that the dietary 150UP treatment increased villi length and crypt depth in the duodenum and this result may be correlated with the numerically better feed conversion rate in the 150UP group. Moreover, microbiological data showed an improvement in the intestinal ecosystem. In addition, our data suggest that the protected form may limit the nutrient absorption itself, compared to the unprotected form.

## 4.6 References

**Armstrong T.A., Cook D.R., Ward M.M., Williams C.M., Spears J.W.** (2004). Effect of dietary copper source (cupric citrate and cupric sulfate) and concentration on growth performance and fecal copper excretion in weanling pigs. *Journal of Animal Science*, 82:1234-1240.

**Bunch R.J., Speer V.C., Hays V.W., Hawbaker J.H., Catron D.** (1961). Effects of copper sulfate, copper oxide and chlortetracycline on baby pig performance. *Journal of Animal Science*, 20: 723-726.

**Barber R.S., Braude R., Mitchell K.G.** (1955). Antibiotics and copper supplements for fattening pigs. *British Journal of Nutrition*, 9: 378-381

**Cromwell G.L., Lindemann M.D., Monegue H.J., Hall D.D., Orr Jr D. E.** (1998). Tribasic copper chloride and copper sulfate as copper sources for weanling pigs. *Journal of Animal Science*, 76:118-123

**Cromwell G.L., Stahly T.S., Monegue H.J.** (1989). Effects of source and level of copper on performance and liver copper stores in weanling pigs. *Journal of Animal Science*, 67, 2996-3002.

- Dunning J.C., Marquis R.E.** (1998). Anaerobic killing of oral streptococci by reduced, transition metal cations. *Applied and Environmental Microbiology* 164: 27-33.
- Hampson D.** (1986). Alterations in piglet small intestine structure at weaning. *Research in Veterinary Science* 40:32-40.
- Hawbaker J.H., Speer V.C., Hays V.W., Catron D.V.** (1961). Effects of copper sulphate and other chemotherapeutics in growing swine rations. *Journal of Animal Science* 20: 163-167.
- Hedemann M.S., Højsgaard S., Jensen B.B.** (2003). Small intestinal morphology and activity of intestinal peptidases in piglets around weaning. *Journal of Animal Physiology and Animal Nutrition*, 7:32-41.
- Hedemann M.S., Jensen B.B.** (2004). Variations in enzyme activity in stomach and pancreatic tissue and digesta in piglets around weaning. *Archives of Animal Nutrition* 58: 47-59.
- Hill, G. M., Cromwell G. L., Crenshaw T. D., Dove C. R., Ewan R. C., Knabe D. A., Lewis A. J., Libal G. W., Mahan D. C., Shurson G. C., Southern L. L., Veum T. L.** (2000). Growth promotion effects and plasma changes from feeding high dietary concentrations of zinc and copper to weanling pigs (regional study). *Journal of Animal Sciences* 78:1010–1018.
- Højberg O., Canibe N., Poulsen H.D., Hedemann M.S., Jensen B.B.** (2005). Influence of dietary zinc oxide and copper sulphate on the gastrointestinal ecosystem in newly weaned piglets. *Applied and Environmental Microbiology* 71: 2267-2277
- Jensen, B. B.** (1998). The impact of feed additives on the microbial ecology of the gut in young pigs. *Journal of Animal and Feed Sciences* 7:45-64.
- Kawai Y., Morotomi M.** (1978). Intestinal enzyme activities in germfree, conventional, and gnotobiotic rats associated with indigenous microorganism. *Infection and Immunity* 19: 771.
- Radecki S.V., Ku P.K., Bennink M.R., Yokoyama M.T., Miller E.R.** (1992). Effect of dietary copper on intestinal mucosa enzyme activity, morphology and turnover rates in weanling pigs. *Journal of Animal Science*, 70: 1424-1431

**Roberts E.A., Sarkar B.** (2008). Liver as a key organ in the supply, storage, and excretion of Copper. *American Journal of Clinical Nutrition*, 88: 851S–8544S.

**Shurson G.C., Ku P.K., Waxler G.L., Yokoyama M.T., Miller E.R.** (1990). Physiological relationships between microbiological status and dietary copper levels in the pig. *Journal of Animal Science*, 68:1061

**Smith J.W., Tokach M.D., Goodband R.D., Nelssen J.L., Richert B.T.** (1997). Effects of the interrelationship between zinc oxide and copper sulfate on growth performance of early weaned pigs. *Journal of Animal Science*, 75: 1861–1866.

**Stahly, T. S., Cromwell G. L.** (1979). Effects of environmental temperature and dietary fat supplementation on the performance and carcass characteristics of growing swine. *Journal of Animal Science*, 49:1476.

**Stansbury W.F., Tribble L.F., Orr DE.** (1990). Effect of chelated copper sources on performance of nursery and growing pigs. *Journal of Animal Science*, 68:1318-1322.

**Varel V.H., Robinson I.M., Pond W.G.** (1987). Effect of dietary copper sulfate, aureo SP250, or clinoptilolite on ureolytic bacteria found in the pig large intestine. *Applied and Environmental Microbiology* 53:2009-2012

**Zhao J., Harper A.F., Estienne M.J., Webb K.E., McElroy A.P., Denbow D.M.** (2007). Growth performance and intestinal morphology responses in early weaned pigs to supplementation of antibiotic-free diets with an organic copper complex and spray-dried plasma protein in sanitary and non sanitary environments. *Journal of Animal Science* , 85: 1302-1310.

**Zimmerman D.R.** (1986). Role of subtherapeutic levels of antimicrobials in pig production. *Journal of Animal Science*, 62(suppl.3):6.

## **CHAPTER 5-ASSAY 3**

# **Verbascoside dietary supplementation in weaning piglets: a systemic or local (liver) antioxidant effect?**

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## 5. Verbascoside dietary supplementation in weaning piglets: a systemic or local (liver) antioxidant effect?

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### 5.1 Abstract

The aim of the study was to investigate whether the use of verbascoside, an antioxidant of vegetal origin, in weaning pig feeding could modulate oxidative stress in the liver. Twenty four weaned female piglets were assigned to three experimental groups: the first group were fed a diet with 9% of sunflower oil (T1), the second one were fed with the same diet of the T1 but with an integration of antioxidant mix (T2), in particular with a 5 mg/kg of verbascoside (VB), and the third group was fed a control diet (CTR), where the oil was substituted by starch. The trial went on thirty days, and at the end the animals were slaughtered and the liver specimens were collected. Immunohistochemistry and Western blot analyses were performed on the samples to evaluate free radical adducts, including Heat Shock Protein (Hsp) 70, Hsp 90, lysozym and desmin. These analyses showed an increase of the level of Hsp 70 in the liver treated with sunflower oil. A KRL test was also used to assess the total blood and plasma antioxidant activity, and showed a significant decrease in T1 and T2 groups than control. We evaluated also the impact of the use of antioxidant dietary integrations upon some plasma and liver enzymes related to oxidative stress: superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX). In the plasma compartment all enzymes were lower in the treated animals in comparison with control group. In the liver only SOD had significant results: T1 group presented a higher level of this enzyme in comparison with the other groups.

**Keywords:** pig; weaning; verbascoside; oxidative stress

## 5.2 Introduction

In recent years there is a growing evidence that reactive oxygen species (ROS) contribute to organ injury in many localizations including the heart, liver and central nervous system (Marubayashi et al., 1985). ROS are constantly formed as by-products of normal metabolic reactions and their formation is accelerated by stressful agents. In healthy animals, the generations of ROS is well balanced by the counterbalancing action of antioxidant defences (Khan et al., 2005). Under normal circumstances, abundant antioxidant enzymes (e.g., superoxide dismutases and glutathione peroxidases) metabolize these highly reactive derivatives of normal oxidative metabolism. If ROS are not removed in a timely manner by an antioxidant system, an imbalance between free radical generation and their removal could lead to oxidative stress (Zheng et al., 2010). Oxidative stress occurs when the antioxidant system is overwhelmed by the production of ROS in excess, which can lead to increased prevalence of inflammation and infectious disease via an impaired immune cell function.

Many natural substances in plants have ability to neutralize free radicals. Katiyar (2002) carried out a study on laboratory animals and have shown that many natural compounds (such as polyphenols, carotenoids, anthocyanidins and vitamin C) display interesting and significant antioxidant activities. Moreover, they are able to integrate the endogenous antioxidant defenses, which can be reduced in particular periods of stress. In the lifespan of the pig, the weaning phases occupy critical periods, above all in intensive rearing conditions. Weaning marks the end of the preferential relationships between the sow and the piglets. The rupture of the maternal link for milk supply has consequences on gut local immune status of the piglet and on the gut microflora (Hampson et al., 1985; Barnett et al., 1989). When pigs are weaned, they suffer of many stressors, including the sudden separation from their mother, mixing with unfamiliar piglets, and changing from a liquid diet (the highly-digestible milk) to a solid feed. So this crucial period is a complex step involving dietary, environmental, social and psychological stresses, which interferes deeply with feed intake that suddenly decreases, gut development and complex adaptation to the weaning diet (reviews by Pluske et al, 1997; Lallès et al. 2004). In addition, in European intensive rearing systems, the weaning takes place at an early age (21–28 d), probably exacerbating the level of general stress in these immature animals. The first organ suffering from nutrient shortage immediately post-weaning is the gut and this has dramatic consequences on its anatomy and functions, including barrier function against harmful antigens and pathogens. As a consequence, since the liver stands as an effective filter of bacterial products arising in the gastrointestinal tract, the possibility that an impairment of the ability to perform this function might initiate or perpetuate hepatic injury (Nolan 1979).



By a more general point of view, in the weaning period applied in commercial farms the young piglet is subjected to sudden and intense changes in metabolic balance and endocrine and immune status. Sauerwein et al. (2005) reported an increase of reactive oxygen metabolites and oxidative stress biomarkers in post-weaning piglets. Zhu et al. (2012) showed that the weaning period is characterized by a decrease in endogenous antioxidant cellular system in piglets, with a down regulation of digestive enzymes and antioxidant enzymes.

Phenylpropanoid glycosides (PPGs) are water soluble derivatives of phenylpropanoids (PPGs), a large group of natural phenols (Korkina, 2007). There is a growing evidence that verbascoside, like other plant phenols in general and PPGs in particular, is a powerful antioxidant by either directly scavenging reactive oxygen and nitrogen species or acting as chain-breaking peroxy radical scavenging (Afanas'ev, 2005). Verbascoside exhibits a number of biological activities including anti-oxidative, anti-bacterial, and anti-tumor actions, as recently reported by Santoro et al., 2008. Verbascoside demonstrated also important anti-inflammatory activities (Hausmann et al., 2007). Rossi et al. (2009) found that this substance has a greater antioxidant power compared to other phenolic compounds. In addition, Di Giancamillo et al. (2012) have quite recently shown that dietary verbascoside was able to decrease the nitrosative stress status in swine small intestine.

The aim of this study was to assess the effects of dietary administration of Verbascoside on growth performances, plasma and blood oxidative status, and liver oxidative status in piglets with an induction of oxidative stress with sunflower oil.

## 5.3 Material and methods

### 5.3.1 *Animals and diets*

Twenty-four female pigs (Hypor) weighing 10 kg, aged 21 days, were randomly selected and assigned to three dietary experimental groups: the first group were fed a diet with 9% of sunflower oil (T1), the second one were fed with the same diet of the T1 group but with an integration of antioxidant mix (T2), in particular with a 5 mg/kg of verbascoside (VB), and the third group was fed a control diet (C), where the oil was substituted by starch. The three experimental diets were isoenergetic. The trial lasted 30 days and at the end the piglets were slaughtered at an average live weight of  $22.02 \pm 0.5$  Kg.

All animals were treated in accordance with both the policies and the principles of laboratory animal care consistent with the European Union guidelines

(86/609/EEC), which were approved by the Italian Ministry of Health (Law 116/92).

### 5.3.2 Blood and tissue samples

At the beginning and at the end of the trial (0 and 30 days) blood samples were collected by vena cava puncture before the morning feeding (total number of specimens=48). The blood samples were collected in 10-mL vacutainer glass tubes containing EDTA (Venoject, Terumo Europe N.V., Leuven, Belgium) that were immediately transported to the laboratory pending analysis. Plasma samples were stored at -80°C for the determination of the activity of enzymes related to oxidative stress.

At the end of the trial, the pigs were slaughtered by approved procedures (Italian Ministry of Health; DL n.333/1998) and samples of liver were immediately excised from each pig (total number of specimens=24). The samples were vacuum-packed and stored at -80°C. Western blot analyses and ELISA assays were performed upon the samples within 2 weeks from collection.

For micro-anatomical analyses (histology and immunohistochemistry), additional liver samples (1 x 1 cm) were removed at the same localization from each pig. These samples (total number =24) were promptly fixed in 4% para-formaldehyde in 0.01 M phosphate-buffered saline (PBS) pH 7.4 for 24 h at 4 °C, dehydrated in a graded series of ethanol, cleared with xylene and embedded in paraffin.

### 5.3.3 Blood analyses

#### *Blood: KRL test*

The analyses on the total antioxidant activity of whole blood and of red blood cells were performed within 24 hours from samples collection. The total antioxidant activity of the whole blood was evaluated using the KRL biological test based on free radical-induced haemolysis (Prost 1990; 1992). The KRL Test allows the *ex vivo* dynamic evaluation of the overall antioxidant defence potential of an individual. Whole blood were diluted (v:v 1:25) with KRL buffer (300 mosm/l), and 50 µl of whole blood suspension were submitted to organic free radicals produced at 37 °C under air atmosphere from the thermal decomposition of 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH). The kinetics of sample resistance to haemolysis was recorded using a 96-well microplate reader by monitoring the changes at 620 nm absorbance, at 37°C (Laboratoires Spiral, France). Results are expressed as the time that is required to reach 50% of maximal haemolysis (half-haemolysis time, HT<sub>50</sub>), and are expressed in minutes.

#### *Plasma: ELISA analysis*

Plasma samples were analyzed for the evaluation of the activity of enzymes related to oxidative stress: superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX). The activity of superoxide dismutase (SOD) was measured by the Cayman's Superoxide Dismutase Assay Kit (Cayman Chemical Company, USA). Briefly, the kit utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. The SOD assay measures all three types of SOD (Cu\Zn, Mn, and FeSOD). The activity of catalase (CAT) was measured by the Cayman's Catalase Assay Kit (Cayman Chemical Company). The method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H<sub>2</sub>O<sub>2</sub>. The formaldehyde produced is measured colorimetrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as the chromogen. Purpald specifically forms a bicyclic heterocycle with aldehydes, which upon oxidation changes from colorless to a purple color. The activity of glutathione peroxidase (GPx) was measured by the Cayman's Glutathione peroxidase Assay Kit (Cayman Chemical Company). The kit measures GPx activity indirectly by a coupled reaction with glutathione reductase (GR). Oxidized glutathione (GSSG), produced upon reduction of hydroperoxide by GPx, is recycled to its reductase state by GR and NADPH. The oxidation of NADPH to NADP<sup>+</sup> is accompanied by a decrease in absorbance at 340nm. Under conditions in which the GPx activity is rate limiting, the rate of decrease is directly proportional to the GPx activity in the sample.

#### *5.3.4 Liver analyses*

##### *Liver: histology*

Serial microtome sections (4µm-thick) were obtained from each liver samples, and were stained with Hematoxylin-Eosin (HE) sequential staining to ascertain structural details.

Other sections were utilized for immunohistochemistry

##### *Liver: Immunohistochemistry*

Immunostaining of the liver sections was performed to detect the following markers of oxidative-nitrosative stress in situ, including Heat Shock Protein (Hsp) 70 and Heat Shock Protein 90, Lysozyme and Desmin. Hsps 70 and 90 are induced in response to various kinds of environmental and physiological stresses. The roles of Hsp upregulation in tissue and cell protection have been described in a variety of stress conditions by Kalmar and Greensmith (2009). Lysozyme is a marker of inducible macrophage activation and it identifies the

resident liver macrophages population, the Kupffer cells. Desmin, even if with some specie-specific differences, may be considered a marker of activated hepatic stellate cells, or Ito cells (HSCs) (Poli, 2000).

The antigen retrieval was performed either by heat, with a microwave treatment (for 5 minutes at 450W in citrate buffer pH 6) or by proteinase K treatment in PBS pH 7.4 at room temperature for 5 minutes.

Immunohistochemical stainings were performed using the Elite ABC KIT system (Vector Laboratories, Inc., California, USA). Before applying the primary antibodies, endogenous peroxidase activity was blocked by incubating the sections in 3% H<sub>2</sub>O<sub>2</sub> in PBS. Non-specific binding sites were blocked by incubating the sections in normal goat serum (Dakocytomation, Italy). Sections were then incubated with the primary antibodies (see Table 1) overnight at 4 °C.

**Table 1.** Primary antisera used

	<b>Code/source</b>	<b>Dilution</b>	<b>Incubation</b>	<b>Antigen retrieval</b>
<b>Heat shock protein 70 (Hsp 70), monoclonal</b>	N27F3-4/ Enzo LifeSciences	1:100 IHC 1:1000 WB	Overnight at RT	Heat
<b>Heat shock protein 90 (Hsp 90), monoclonal</b>	AC88 /Enzo LifeSciences	1:100 IHC 1:1000 WB	Overnight at RT	Heat
<b>Desmin clone D33, monoclonal</b>	H7094 Dakocytomation	1:50 IHC 1:500 WB	Overnight at RT	Heat
<b>Lysozyme EC3.2.1.17, polyclonal</b>	A0099 Dakocytomation	1:400 IHC 1:1500 WB	Overnight at RT	Proteinase K

After washing with PBS, sections were incubated with biotin-conjugated either anti-mouse or anti-rabbit Ig antibodies (Dakocytomation), washed with PBS and reacted with peroxidase-labeled avidin-biotin complex (Vector Laboratories). The immunoreactive sites were visualized using a freshly prepared solution of 10 mg 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma, Italy) in 15ml of a 0.5M Tris buffer at pH 7.6, containing 1.5ml of 0.03% H<sub>2</sub>O<sub>2</sub>. To ascertain structural details, sections were counterstained with Mayer's haematoxylin.

The specificity of the immunostaining was verified by incubating in parallel other sections with: (i) PBS instead of the specific primary antibodies (see Table 1); (ii) preimmune sera instead of the primary antisera; (iii) PBS instead of the secondary antibodies. The results of these controls were negative (i.e. staining was abolished).

Immunoreactive cells were valued using an Olympus BX51 light microscope (Olympus, Italy) equipped with a digital camera.

#### *Liver: tissue homogenisation and Western blot analysis*

Liver samples were homogenised in 2 ml of ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 0.2% Triton X-100, and protease inhibitor mixture) per 200 mg of tissue using an Ultra-Turrax homogeniser (IKA-Werke, Staufen, Germany). The homogenate was then centrifuged at 20000  $g$  for 20 min at 4°C to remove all insoluble material. The supernatant was collected, and total protein content was measured using a commercial protein quantification kit (Pierce, Rockford, IL, USA) based on bicinchoninic acid (BCA) colorimetric detection of the cuprous cation obtained by protein  $\text{Cu}^{2+}$  reduction in an alkaline medium. The optical densities were read at 562 nm against a calibration curve using bovine serum albumin (SIGMA, Italy) working range of 50–800  $\mu\text{g}/\text{ml}$ . Total proteins (35  $\mu\text{g}$ ) were resolved by 10% SDS-PAGE and electro-transferred onto nitrocellulose membranes. After blocking, membranes were incubated overnight at 4°C with primary antibodies (see Table 1) and subsequently with a horseradish peroxidase-conjugated secondary antibody (1:5000) at room temperature for 45 min. Immunoreactivity was detected by chemiluminescence autoradiography according to the manufacturer's instructions, and the images were scanned. The optical intensities of the protein bands of interest were determined densitometrically using the Scion Image software. Each electrophoresis gel contained samples from both treatment groups to reduce between-blot effects. The values were normalized to GADPH levels.

#### *Liver: tissue homogenisation and ELISA analysis*

Liver samples were homogenised in 1 ml of homogenization buffer (50 mM tris-HCl, pH 7.5, 5 mM EDTA and 1mM Dtt) per 100 milligrams of tissue, using an Ultra-Turrax homogeniser (IKA-Werke, Staufen, Germany). The homogenate was then centrifuged at 10,000  $\times g$  for 15 min at 4°C . After collecting the supernatant, the assay according to the kit booklet protocol (Cayman Chemical Company) was applied.

Liver samples were analyzed for the evaluation of the activity of enzymes related to oxidative stress: superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX), using the same kits utilized for plasma analyses, according manufacturer's instructions.

### 5.3.5 Statistical analyses

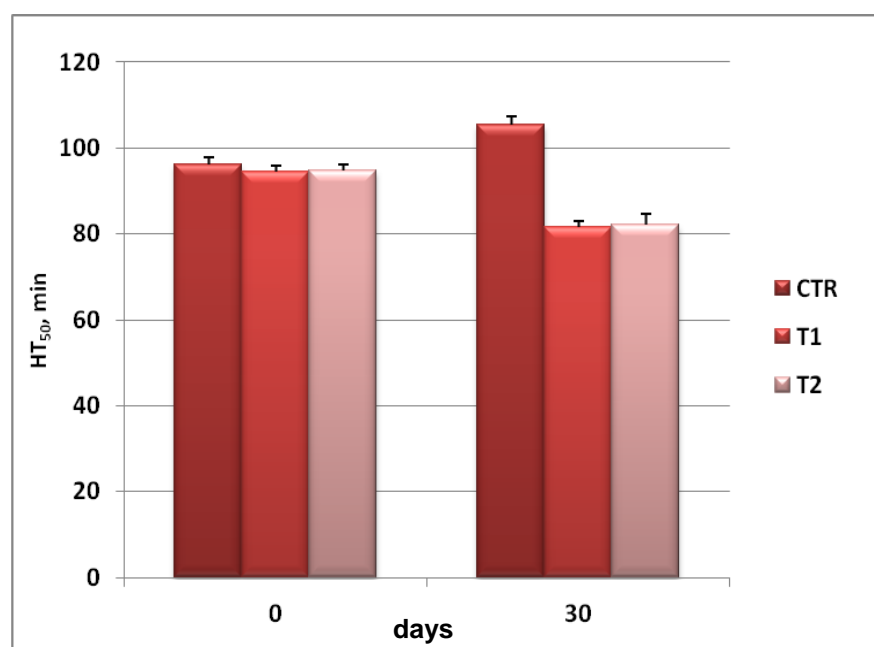
Statistical analysis of the quantitative data was performed using the general linear model of the SAS (version 8.1, Cary Inc., NC). Blood analyses were submitted to one way ANOVA and the baseline measure of that variable (weaning values) was used as a covariate. The individual pig values were considered to be the experimental unit of all response variables. The data were presented as least squared means  $\pm$  SEM. Differences between means were considered significant at  $P < 0.05$ .

## 5.4 Results

### 5.4.1 Blood analyses

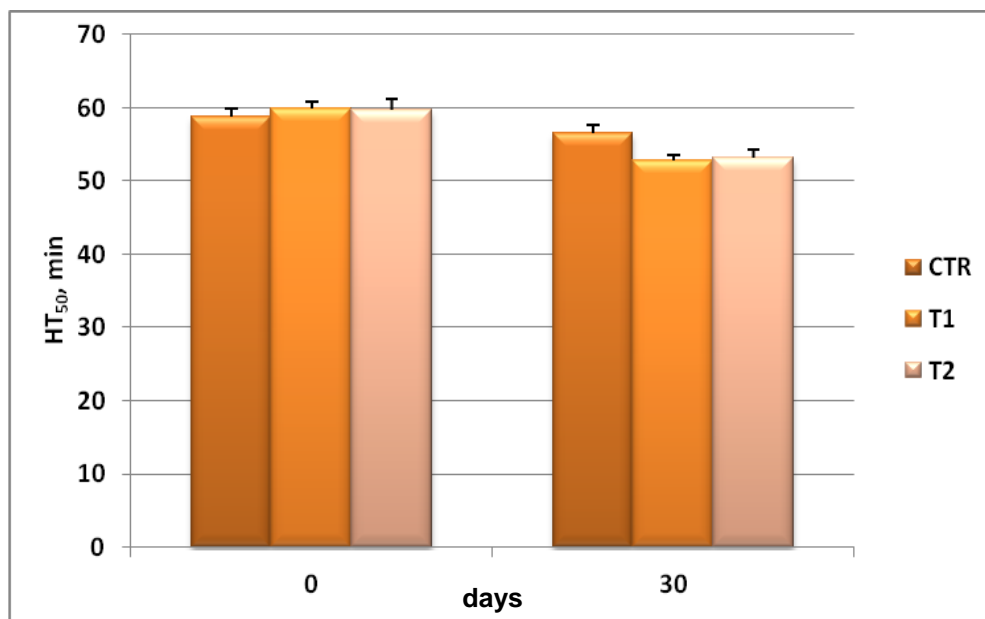
#### *KRL test*

The total antioxidant activity of the whole blood was evaluated using the KRL biological test based on free radical-induced haemolysis. There were significant results in relationship with time, with treatment ( $P < 0.001$ ) and with the interaction between time and treatment ( $P < 0.001$ ): at the end of the trial CTR group presented higher values compared with T1 and T2 groups (Fig.1).



**Fig. 1.** The total antioxidant activity of the whole blood in piglets in relationship with nutritional stress, time of sampling, and the interaction between the two factors

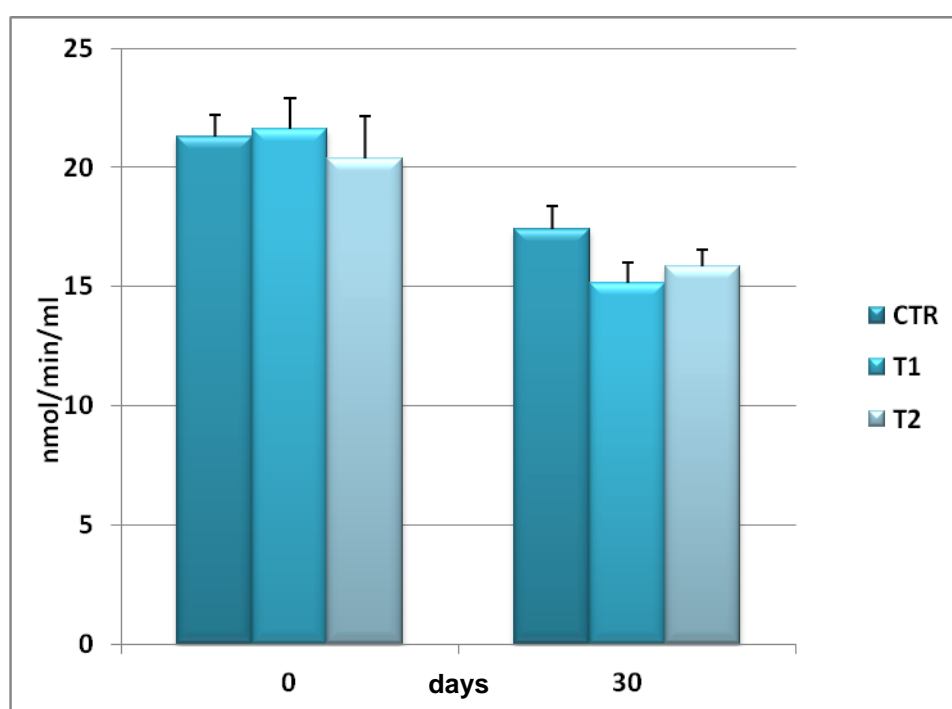
Moreover, a similar significant result was observed for the the total antioxidant activity of red blood cells; the CTR group showed higher value in comparison with T1 and T2 groups limited to the time of sampling ( $P < 0.001$ ), whereas the treatment ( $P = 0.236$ ) and the interaction between these two factors ( $P = 0.106$ ) were not significant (Fig.2).



**Fig. 2** The total antioxidant activity of red blood cells in piglets in relationship with nutritional stress and time of sampling

### *ELISA analyses*

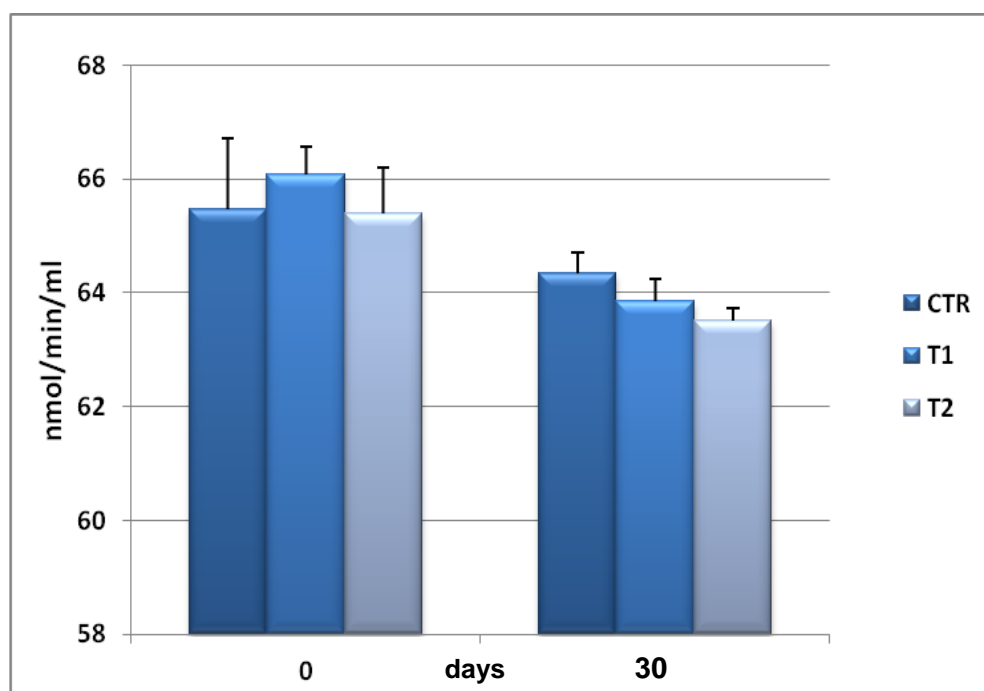
Plasma samples were analyzed for the evaluation of the activity of enzymes related to oxidative stress: superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX). The activity of catalase (CAT) showed a significant decrease of enzyme activity in relation with time of sampling ( $P < 0.001$ ) (Fig.3). No significant effects of treatment ( $P = 0.373$ ) and of the interaction between time and treatment ( $P = 0.622$ ) were observed. Orthogonal contrast made up on the enzyme activity after 30 days of dietary supplementation, showed that CAT activity was numerically lower in T1 and T2 groups in comparison with CTR ( $P = 0.088$ ). No differences between the two treated groups were observed.



**Fig. 3.** Plasma CAT activity in piglets in relationship with nutritional stress and time of sampling

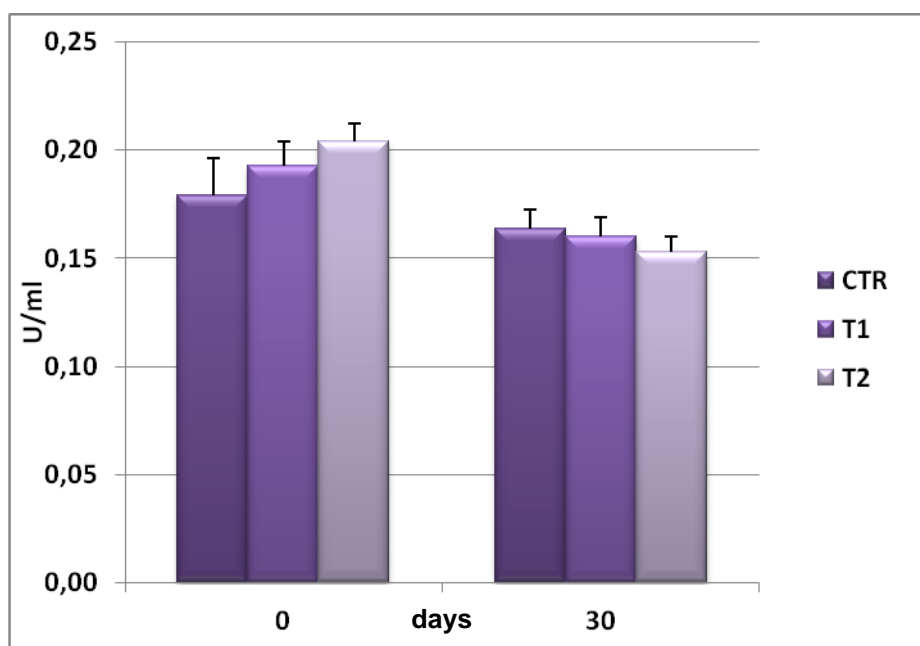


The activity of Glutathione Peroxidase (GPX) showed no significant results (Fig.4). Orthogonal contrast made up on Gpx activity after 30 days of dietary supplementation, showed a significant decrease of its value in T1 and T2 groups ( $P<0.001$ ) in comparison with CTR. No significant difference between the two treated groups was observed.



**Fig. 4.** Plasma GPX activity in piglets in relationship with nutritional stress and time of sampling

The activity of Superoxide dismutase (SOD) showed a significant result in relation with time of sampling ( $P < 0.001$ ) (Fig.5). No significant effects of treatment ( $P = 0.781$ ) and of the interaction between time and treatment ( $P = 0.287$ ) were observed. Orthogonal contrast made up on the SOD activity after 30 days of dietary supplementation showed no significant differences between experimental groups.



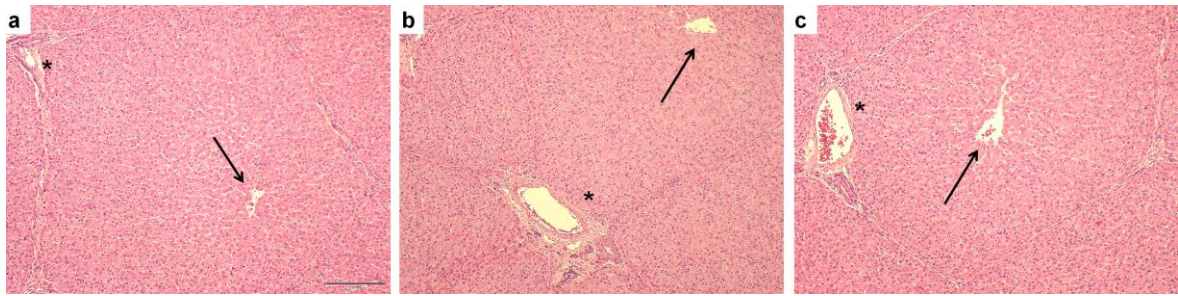
**Fig. 5.** Plasma SOD activity in piglets in relationship with nutritional stress and time of sampling

All plasma enzyme activities decreased in treatment groups in comparison to control group.

#### 5.4.2 Liver analyses

##### *Liver: histology*

The liver of all animal presented a normal structure and architecture of parenchyma, the lobules appeared regular with a normal aspect of central vein and portal area (Fig.6).



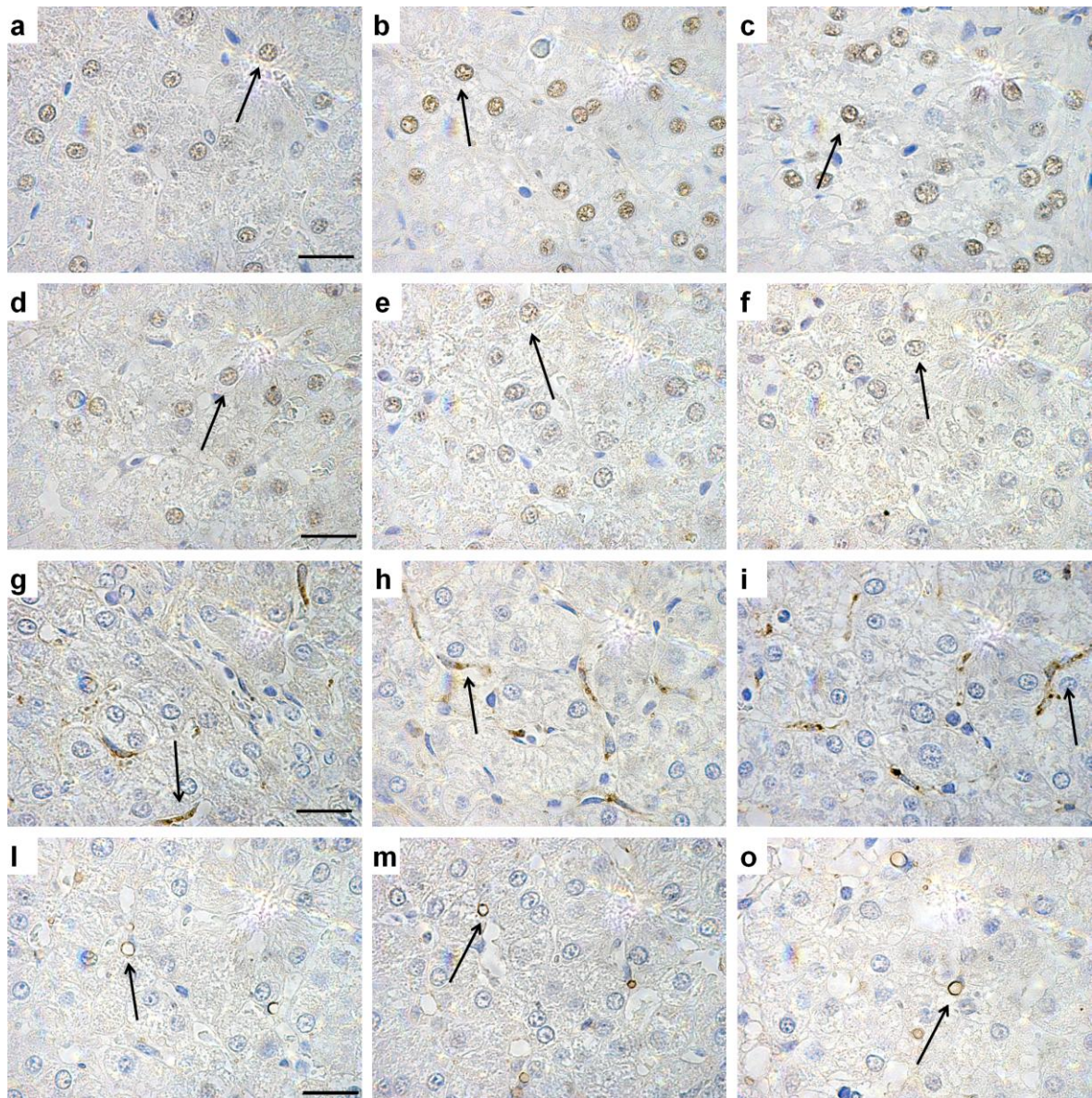
**Fig.6** Histology of liver , HE. All figures have the same scale bar located in fig. 6a: scale bar: 200  $\mu$ m. The normal architecture of liver lobules is evident: note the presence of central veins (arrows) and portal ares (asterisks) (a=CTR; b=T1, c=T2).

#### *Liver: immunohistochemistry*

Hepatocyte nuclei were abundantly immunoreactive for either Hsp 70 ( Fig.7 a,b,c) or Hsp 90 (Fig.7 d,e,f). Almost all the hepatocyte nuclei were immunopositive, irrespective of the treatment.

Lysozyme-immunoreactive, irregularly-shaped cells were present in peri-sinusoidal localizations. They have been interpreted as Kupffer cells and were frequently observed in the liver parenchyma of all the experimental piglets (Fig.7 g,h,i).

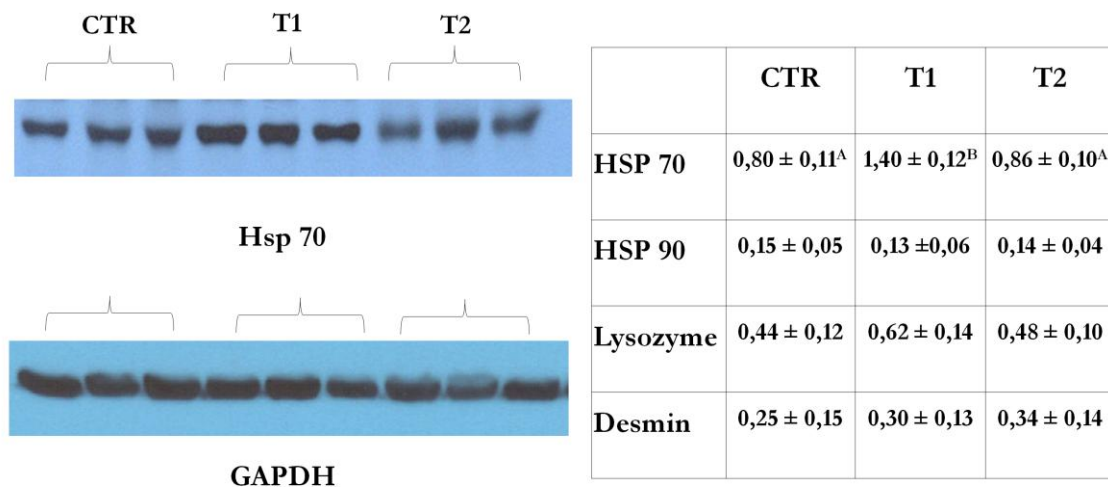
Desmin-immunoreactive, roundish cells were sparsely identified in the liver parenchyma of all the studied piglets (Fig.7 l,m,n). These cells were interpreted as hepatic stellate cells (or Ito cells), and their numbers did not apparently varied in relation to the treatment.



**Fig.7** Immunohistochemistry of the liver. All figures have the same scale bar located in fig.7 a: scale bar: 20  $\mu\text{m}$ . Hepatocyte nuclei were immunoreactive for either Hsp 70 ( Fig.7 a,b,c) (arrows) or Hsp 90 (Fig.7 d,e,f) (arrows). Perisinusoidal irregularly-shaped cells (Kupffer cells) were immunoreactive for Lysozyme (Fig.7 g,h,i) (arrows). Desmin-immunoreactive Ito cells in the liver parenchyma of all the studied piglets (Fig.7 l,m,n) (arrows) (a,d,g,l=CTR; b,e,h,m=T1, c,f,i,o=T2).

*Liver: Western blot*

This was performed for a proper immunochemical quantification of the immunohistochemical results. Hsp 70 only was shown to be higher in T1 than in T2 and Control groups (Fig. 8).



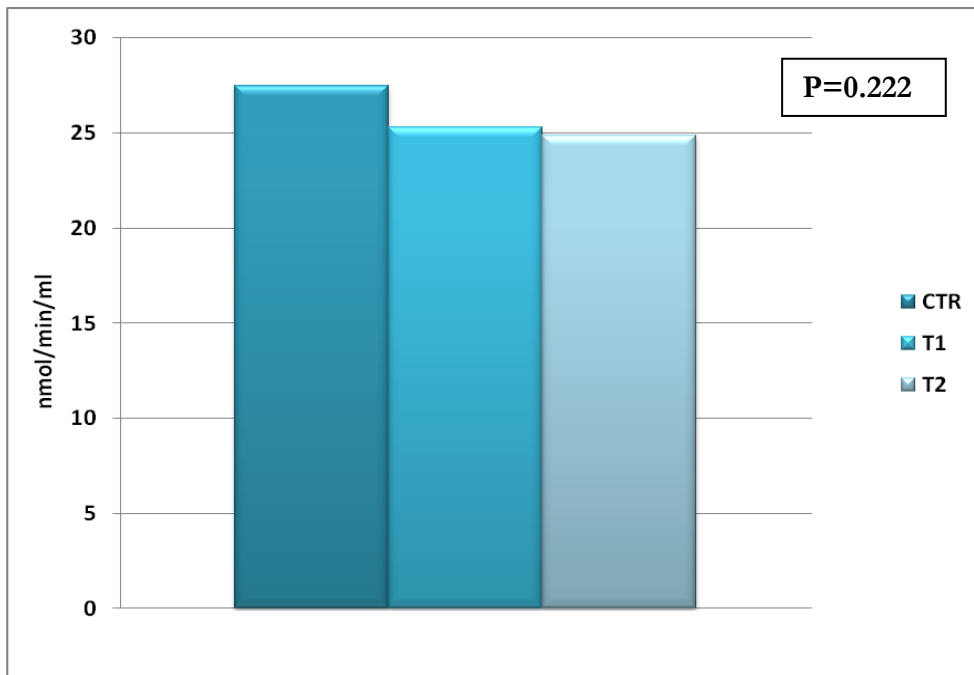
Values are means ± SEM, *n* =18; <sup>A, B</sup> P<0.01

**Fig.8** Western Blot analysis Hsp 70 protein adducts increased in liver of T1 animals (P<0.01). Hsp 70 level was normalized and quantitated to GAPDH.

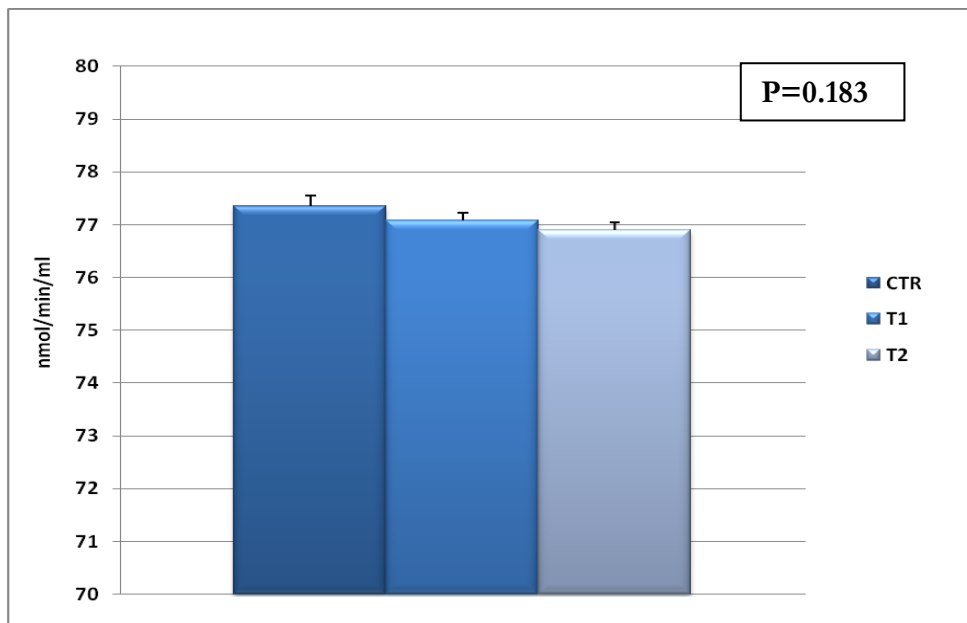
#### *Liver: ELISA analyses*

Liver samples were analyzed for the evaluation of the activity of enzymes related to oxidative stress: superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX), using the same Cayman kits utilized for performing plasma analyses.

Liver CAT (Fig.9) and GPX (Fig.10) enzyme activities were not shown to be different among groups. Liver SOD enzyme activity was shown to be higher in T2 and CTR than in T1 group (Fig.11).



**Fig. 9.** Liver CAT activity in piglets



**Fig. 10.** Liver GPX activity in piglets

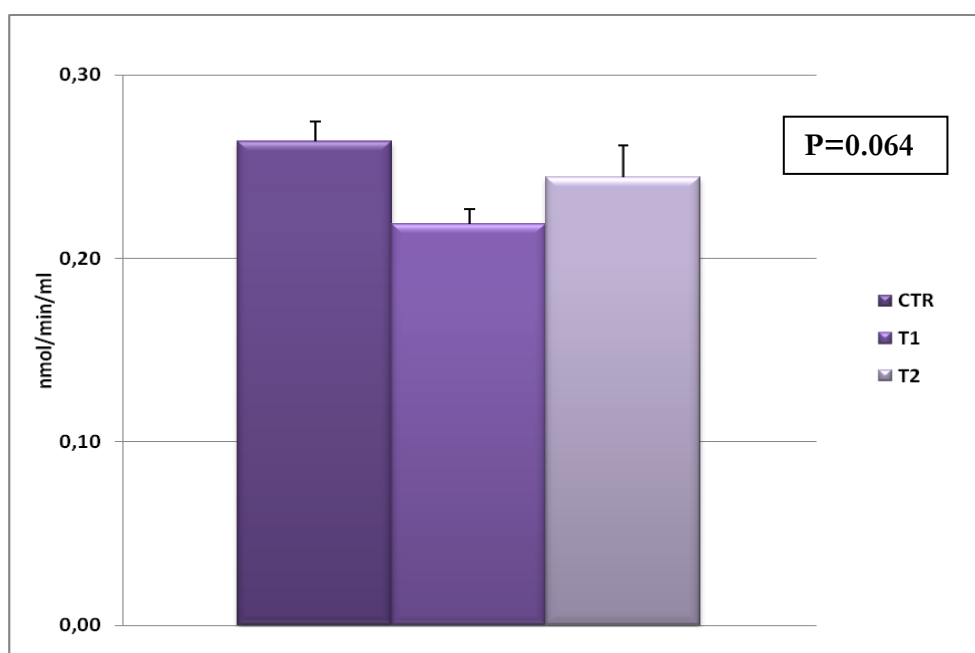


Fig. 11. Liver SOD activity in piglets

## 5.5 Discussion

In commercial pig farms the weaning period is complex by more than one point of view, because piglets have to adapt rapidly to important changes in their nutrition and environment. Weaning at an early age, as generally applied in intensive production systems in Europe, probably exacerbates the level of general stress in these immature animals (Lalles et al., 2004). There is a growing interest in the role of complementary and alternative medicine in maintaining health and in fronting disease in either humans or other mammalian species. Phytogetic feed additives as antioxidant substances are a subject of an intense debate. Special attention has been paid to verbascoside that has been repeatedly shown as a potent antioxidant in several scientific works (Li et al., 1999; Chen et al., 2002; Corino et al., 2007; Casamassima et al., 2009; Esposito et al., 2009; Casamassima et al., 2012; Di Giancamillo et al., 2012).

In the present work we aimed at evaluating the effects of an antioxidant dietary integration in weaning piglets, to which a “nutritional” stress was applied. KRL test showed a significant decrease of the total antiradical activity of blood and red blood cells ( $P < 0.001$ ) in relation with time of sampling. This result is in accord with Rossi et al. (2009), who similarly evidenced a decreased antiradical activity of blood and red blood cells in weaning piglets. We have also demonstrated a decrease of total antiradical activity in blood in relation with the

alimentary treatment. These data suggest that the nutritional stress caused by 9% sunflower oil in the diet was able to decrease antiradical activity of blood. This appears in contrast with the data obtained by Frankic and Salobir (2011), in which the alimentary stress obtained with the somministration of n3 PUFA (linseed oil) and integration with antioxidant in piglets didn't influence antioxidant status.

The evaluation of plasma enzyme activity of SOD, CAT and GPx showed different results. There is a decrease of SOD after weaning, in according with Petrovič et al. (2009), but in the same study there was an increase of GPx activity in post weaning (a result that we did not obtained). After 30 days of integration with 9% sunflower oil, CAT activity show a numerically tendency of decrease, like in Varma et al. (2004). Also GPx activity shows a numerically decrease in two treated group with oil in contrast to control group, like in Varma et al. (2004) study. Also Jiang et al. (2011), illustrated a significant decrease of this enzyme with high dosage of Zearalenone as a factor able of inducing oxidative stress.

Other Authors reported that an integration with high dosage of linseed oil and antioxidant mix didn't influence GPx activity (Frankic and Salobir, 2011; Frankic et al., 2009; Frankic et al., 2008).

Plasma SOD activity is not influenced by sunflower oil, in accord with Lauridsen et al. (1999), who used rapeseed oil, but in disaccord with Jiang et al. (2011), who demonstrated a significant decrease of SOD activity with dietary Zearalenone.

In this study we have in addition analyzed the effects of verbascoside antioxidant supplementation on the liver of piglets in order to evaluate if the possible antioxidant effects may be reputed systemic or "local", referred to the digestive system, or both. All liver samples presented a normal architecture of parenchyma and the anatomy of hepatic lobule appeared normal. With immunohistochemical and Western blot analyses we analyzed oxidative stress status of this organ utilizing Heat shock proteins as markers. The Hsps are a family of stress-responsive proteins present in every organism, from bacteria to humans. According to their molecular sizes, Hsp are classified into 4 major families, that is: small Hsps, Hsp 60, Hsp 70, and Hsp 90 (Watnabe et al., 2004). In the Hsp family, Hsp 70 is one of the most abundant and best characterized proteins. Many studies have shown that Hsp 70 protects the cell from various stresses (Oyake et al., 2006; Zhong et al., 2010).

In our study there is an increase of Hsp 70 (but not Hsp 90) in T1 group in comparison with control and T2 groups ( $P < 0.001$ ), that is piglets that underwent a nutritional stress (via the dietary administration of 9% sunflower oil) showed a hepatic higher level of Hsp 70 in comparison with control ones and piglets which underwent the same nutritional stress plus verbascoside. This results



could be linked to the increasing of this Heat shock protein in stressful conditions, as reported by other Authors (Jolly and Morimoto, 2000; Creagh et al., 2000). Since Hsp70 is a universal cytoprotection protein, it may enhance the tolerance to environmental changes or pathogenic conditions, increase the survival rate of stressed cells, and may also play critical role in preventing the activation of inflammatory cells. ROS have been implicated in the activation process as many oxidizing agents have been shown to result in Hsp induction, and treatment of cells with antioxidants prior to stresses such as heat shock attenuates the response (Gorman et al., 1999).

Moreover, we evaluated Lysozyme and Desmin with the aim of identifying the hepatic resident macrophages and, respectively, the hepatic stellate cells during their possible activation concomitant with lipid peroxidation and oxidative stress. During the present trial we did not find any quali- and quantitative modifications within the liver macrophages and hepatic stellate cells.

Biological antioxidants are natural compounds which can prevent the uncontrolled formation of free radicals and activated oxygen species, or inhibit their reaction with biological structures. These compounds include antioxidant enzymes existing in all oxygen-metabolizing cells, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX). CAT and SOD are the two major enzymes that remove ROS in vivo. SOD catalyzes the dismutation of superoxide anion ( $O_2^{\cdot-}$ ) into hydrogen peroxide ( $H_2O_2$ ), which is then degraded to  $H_2O$  by CAT or by glutathione peroxidase. A decrease in the activity of these antioxidants may lead to an excess of availability of  $O_2^{\cdot-}$  and  $H_2O_2$ , which in turn generates hydroxyl radicals, resulting in initiation and propagation of lipid peroxidation.

In the present study SOD activity revealed to be modified by the applied nutritional stress: its value is lower in T1 group in comparison to Control and T2 animals ( $P < 0.01$ ). We can hypothesize that the nutritional stress could determine a decrease of this antioxidant enzyme and the use of Verbascoside in T2 group re-increase the value similar to control group. This result is in accord with Schmatz et al. 2012, who demonstrated that the treatment with resveratrol was able to prevent the decrease of the activities of catalase (CAT), superoxide dismutase (SOD) improving the antioxidant defense. Based on these findings, we can suggest that verbascoside dietary supplementation in weaning piglets may provide a liver protection against oxidative damage, which is frequently occurring in intensive rearing condition, thus possibly protecting the individuals and the herd against the occurrence of inflammatory and infectious diseases..

## 5.6 References

**Afanas'ev I.B.** (2005). Superoxide and nitric oxide in pathological conditions associated with iron overload: the effects of antioxidants and chelators. *Current Medical Chemistry*, 12: 2731–2739.

**Barnett K.L., Kornegay E.T., Risley C.R., Lindemann M.D., Schurig G.G.** (1989). Characterization of creep feed consumption and its subsequent effects on immune response, scouring index and performance of weanling pigs. *Journal of Animal Science*, 67:2698–2708.

**Casamassima D., Palazzo M., Martemucci G., Vizzarri F., Corino C.** (2012) Effects of verbascoside on plasma oxidative status and blood and milk production parameters during the peripartum period in Lacaune ewes. *Small Ruminant Research*, 105 : 1-8.

**Casamassima D., Palazzo M., Presutti T., Colella G.E.** (2009). Productive performances, plasmatic oxidative status and some blood parameters in suckling lambs supplemented with verbascoside. *Italian Journal of Animal Science*, 8 (suppl.): 668.

**Chen R., Su J., Yang S., Li J., Wang T., Zhou H.** (2002). Effect of isoverbascoside, a phenylpropanoid glycoside antioxidant, on proliferation and differentiation of human gastric cancer cell. *Acta Pharmacol Sinica*, 23 (11), 997-1001.

**Corino C., Rossi R., Musella M., Cannata S., Pastorelli G.** (2007). Growth performance and oxidative status in piglets supplemented with verbascoside and teupolioside. *Italian Journal of Animal Science*, 6 (Suppl. 1): 292-294

**Creagh E.M., Sheehan D., Cotter T.G.** (2000). Heat shock proteins modulators of apoptosis in tumour cells. *Leukemia*, 14:1161–1173.

**Di Giancamillo A., Rossi R., Vitari F., Carollo V., Deponti D., Corino C., Domeneghini C.** (2012) Changes in nitrosative stress biomarkers in swine intestine following dietary intervention with verbascoside. *Histology and Histopathology*, 2012 Dec 17. [Epub ahead of print]

**Esposito E., Dal Toso R., Pressi G., Bramanti P., Meli R., Cuzzocrea S.** (2009). Protective effect of verbascoside in activated C6 glioma cells: possible

molecular mechanisms. *Naunyn Schmiedeberg's Archives of Pharmacology*, 381(1):93-105.

**Frankič T., Salobir J.** (2011). In vivo antioxidant potential of Sweet chestnut (*Castanea sativa* Mill.) wood extract in young growing pigs exposed to n-3 PUFA-induced oxidative stress. *Science of Food and Agriculture*, 91:1432–1439.

**Frankič T., Salobir K. and Salobir J.** (2009). The comparison of in vivo antigenotoxic and antioxidative capacity of two propylene glycol extracts of *Calendula officinalis* (marigold) and vitamin E in young growing pigs. *Journal of Animal Physiology and Animal Nutrition*, 93:688–694.

**Frankič, T., J. Salobir, Rezar V.** (2008). The effect of vitamin E supplementation on reduction of lymphocyte DNA damage induced by T-2 toxin and deoxynivalenol in weaned pigs. *Animal Feed Science and Technology*, 141:274–286.

**Gorman A.M., Heavey B., Creagh E., Cotter T.G., Samali A.** (1999). Antioxidant-mediated inhibition of the heat shock response leads to apoptosis. *FEBS Letters*, 445:98–102.

**Hampson D. J., Hinton M., Kidder D.E.** (1985). Coliform numbers in the stomach and small intestine of healthy pigs weaning at three weeks of age. *Journal of Comparative Pathology*, 95:353.

**Hausmann M., Obermeier F., Paper D.H., Balan K., Dunger N., Menzel K., Falk W., Schoelmerich J., Herfarth H, Rogler G.** (2007). *In vivo* treatment with the herbal phenylethanoid acteoside ameliorates intestinal inflammation in dextran sulphate sodium-induced colitis. *Clinical & Experimental Immunology*, 148 (2): 373-381.

**Isacchi B., Iacopi R., Bergonzi M.C., Ghelardini C., Galeotti N., Norcini M., Vivoli E., Vincieri F.F., Bilia A.R.** (2011). Antihyperalgesic activity of verbascoside in two models of neuropathic pain. *Journal of Pharmacy and Pharmacology*, 63(4):594-601.

**Katiyar S.K.** (2002). Treatment of silymarin, a plant flavonoid, prevents ultraviolet light-induced immune suppression and oxidative stress in mouse skin. *International Journal of Oncology*, 21: 1213-1222.

**Khan S.M., Sobti R.C., Kataria L.** (2005). Pesticide-induced alteration in mice hepato-oxidative status and protective effects of black tea extract. *Clinica Chimica Acta*, 358:131-138.

**Jiang S. Z., Yang Z. B., Yang W. R., Gao J., Liu F. X., Broomhead J., Chi F.** (2011). Effects of purified zearalenone on growth performance, organ size, serum metabolites, and oxidative stress in post-weaning gilts. *Journal of Animal Science*, published online April 29, 2011.

**Jolly C., Morimoto R.I.** (2000). Role of the heat shock response and molecular chaperones in oncogenesis and cell death. *Journal of National Cancer Institute*, 92:1564–1572.

**Lallès J.P., Boudry G., Favier C., Le Floc’h N., Luron I, Montagne L., Oswald I.P, Pié S., Piel C., Sève B.** (2004). Gut function and dysfunction in young pigs: physiology. *Animal Research*, 53: 301-316.

**Lauridsen C., Nielsen J.H., Henckel P., Sørensen M. T.** (1999). Antioxidative and oxidative status in muscles of pigs fed rapeseed oil, vitamin E, and copper. *Journal of Animal Science*, 77:105-115.

**Li J.X., Xin D., Li H., Lu J.F., Tong C.W., Gao J.N., Chan K.M.** (1999). Effect of verbascoside on decreasing concentration of oxygen free radicals and lipid peroxidation in skeletal muscle. *Chung-Kuo Yao Li Hsueh Pao-Acta Pharmacologica Sinica* 20 (2): 126-130.

**Marubayashi S., Dohi K., Ochi K., Kawasaki T.** (1985). Role of free radicals in ischemic rat liver cell injury: prevention of damage by alpha-tocopherol administration. *Surgery*, 99: 184-91.

**Nolan J.P.** (1979). The contribution of Gut-derived endotoxins to liver injury. *Yale Journal of Biology and Medicine* ,52 (1): 127-133.

**Oyake J., Otaka M., Matsushashi T., Jin M., Odashima M., Komatsu K., Wada I., Horikawa Y., Ohba R., Hatakeyama N., Itoh H., Watanabe S.** (2006). Over-expression of 70-kDa heat shock protein confers protection against monochloramine-induced gastric mucosal cell injury. *Life sciences* ,79: 300-305.

**Petrovič V., Novotný J., Hisira V., Link R., Leng L., Kováč G.** (2009). The Impact of Suckling and Post-weaning Period on Blood Chemistry of Piglets. *Acta Veterinaria Brno*, 78:365-371.

**Pluske J.R., Hampson D.J., Williams I.H.** (1997). Factors influencing the structure and function of the small intestine in the weaned pig: A review. *Livestock Production Science*, 51: 215-236.

**Rossi R., Corino C., Pastorelli G., Durand P., Prost M.** (2009). Assessment of antioxidant activity of natural extracts. *Italian Journal of Animal Science*, 8, 655–657.

**Santoro A., Bianco G., Picerno P., Aquino R.P., Autore G., Marzocco S., Gazzero P., Lioi M.B., Bifulco M.** (2008). Verminoside and verbascoside-induced genotoxicity on human lymphocytes: Involvement of PARP-1 and p53 proteins. *Toxicology Letters*, 178, 71–76.

**Sauerwein H., Schmitz S., Hiss S.** (2005). The acute phase protein haptoglobin and its relation to oxidative status in piglets undergoing weaning-induced stress. *Redox Report*, 10: 295-302.

**Schmatz R., Perreira L.B., Stefanello N., Mazzanti C., Spanevello R., Gutierrez J., Bagatini M., Martins C.C., Abdalla F.H., Daci da Silva Serres J., Zanini D., Vieira J.M., Cardoso A.M., Schetinger M.R., Morsch V.M.** (2012). Effects of resveratrol on biomarkers of oxidative stress and on the activity of delta aminolevulinic acid dehydratase in liver and kidney of streptozotocin-induced diabetic rats. *Biochimie*, 94(2):374-83.

**Varma P.S., Aruna K., Rukkumani R., Menon V.P.** (2004). Alcohol and thermally oxidized pufa induced oxidative stress: role of N-acetyl cysteine. *Italian Journal of Biochemistry*, 53(1):10-5.

**Watanabe D., Otaka M., Mikami K., Yoneyama K., Goto T., Miura K., Ohshima S., Lin J.G., Shibuya T.** (2004). Expression of a 72-kDa heat shock protein, and its cytoprotective function, in gastric mucosa in cirrhotic rats. *Journal of Gastroenterology*, 39: 724-733.

**Zheng P., Yu B., Lv M., Chen D.** (2010). Effects of oxidative stress induced by diquat on arginine metabolism of postweaning pigs. *Asian-Australasian Journal of Animal Science*, 23, 1 : 98 – 105.

**Zhu L.H., Zhao K.L., Chen X.L., Xu J.X.** (2012) . Impact of weaning and an antioxidant blend on intestinal barrier function and antioxidant status in pigs. *Journal of Animal Science*, 90 (8): 2581-2589.

**Zhong X., Wang T., Zhang X., Li W.** (2009). Heat shock protein 70 is upregulated in the intestine of intrauterine growth retardation piglets. *Cell stress and chaperones*, 15: 335-342.

# **CHAPTER 6**

## **General Discussion**





## 6. General discussion

About 2000 years ago, Hippocrates correctly in advance emphasized “Let food be your medicine and medicine be your food”.

In the last 20 years the perception of animal nutrition changed immensely in both scientists and human consumers. The importance of animal nutrition for animal health, animal welfare and quality of animal products, which is to be high from the point of view of nutritional value for humans, came to the forefront. Perception of animal nutrition not only has to consider nutritional requirements and lifestyle of human individuals and populations, but also sensory characteristics and hygienic quality and safety of animal products, as well as the impact of rearing farms on environment and sustainability of agriculture. Animal nutrition has pronounced direct impact in animal health and, even if indirectly through animal products, on human health and, through the impact of excreta of food animal species, on the environment.

In the recent years, new knowledge and improved research possibilities enabled us to pay a greater regard to the effect of nutrition on animal health and benefits from it. This type of scientific research was additionally promoted when Europe banned the dietary use of antibiotics as growing substances. After this banning (2006) the interest of scientific researchers for alternative nutritional strategies has dramatically increased and various nutritional approaches with different dietary integrations in different food animal species have been tested for finding alternatives to the limited use of chemotherapeutics in animal rearing, as well as for enhancing the intrinsic quality of a food product of animal origin. The focus now stands on the importance of the effect of nutrition on feed consumption, health status of the gastro-intestinal tract, function of the immune system, regulation of metabolism, prevention of negative effects of oxidative stress, removal and/or inhibition of non-nutritive feed substances.

It is proved that nutraceuticals are now receiving a large body of recognition as being beneficial for humans in preventing or treating coronary heart disease, obesity, diabetes, cancer, osteoporosis and other chronic and degenerative diseases such as Parkinson's and Alzheimer's diseases. Evidences indicate that the functional roles of natural (vegetal origin) compounds involve a wide array of beneficial biological processes, including activation of antioxidant defenses, signal transduction pathways, cell survival-associated gene expression, cell proliferation and differentiation and preservation of mitochondrial integrity that is necessary for correctly performing the respiratory processes at cell level. It appears that these properties play a crucial role in the protection against the pathologies of numerous stress-or age-related or chronic diseases. For all these reasons functional feed and nutraceuticals are considerable as alternative substances to the use of antibacterial agents and chemotherapeutics during

weaning in the rearing farms, as they can adequately stimulate the local defensive responses, and favorably influence resident gastrointestinal microflora, but are also able to improve nutrient digestion and absorption.

Keeping farm animals in healthy condition is necessary to obtain healthy animal products. Healthy problems often result in bad growth performances and as a consequence in severe economic losses, and consequently nutrition, in this conception, became more and more important. A feeding diet formulated to meet target nutrient requirements is necessary to overcome with success the rearing conditions, above all in some critical phases of the lifespan of swine intensive rearing, like weaning and growth-finishing phases.

The three works presented in my PhD thesis support the theory that appropriate dietary integrations are able to improve pig welfare in commercial farms, enhancing healthy status of the animals and influencing positively the production.

Copper as an essential trace element plays a fundamental role in the physiology of animals; but it has been also recognized as having antibacterial and antimycotic activity and the ability to stimulate growth. Controversial data about the effect of dietary copper has been reported but in my experimental trial I observed a numerical improving in the gain:feed ratio. The micro-anatomical changes observed revealed that an elevated dietary integration of  $\text{CuSO}_4$  in unprotected form increased villi length and crypt depth in the duodenum. The small intestinal micro-anatomical results suggest that growth stimulation with elevated dietary Cu may, in part, be related to its beneficial impact on the small intestinal mucosa.

In the other two works I evaluated the impact of a natural (vegetal origin) antioxidant in animal diet. Nutrition has a great impact on animal health also through its possible effect on oxidative stress, which through the oxidation of important biological molecules possibly leads to the damage and dysfunction of tissues and organ systems, as well as, a consequence, to a decreased productivity. Thus a good antioxidant protection may play a key role for insuring the health and productivity of farm animals.

I have focused my attention on the use of a natural antioxidant substance to manage oxidative stress in two stressful periods of the lifespan of reared pig. The discovery of the role of free radicals as co-causative factors in cancer, diabetes, cardiovascular diseases, autoimmune diseases, neurodegenerative disorders, aging and several degenerative diseases has led to a medical revolution that is promising for a new paradigm of healthcare. In the recent years, antioxidants have gained a lot of importance because of their potential as prophylactic and therapeutic agents in many diseases. Antioxidants can cancel out the cell-damaging effects of free radicals and will help quench all these inevitably free radicals in the body thus, improving the health by lowering the risk of various

diseases, above all degenerative. This statement is supported by studies performed in a variety of areas, including physiology, pharmacology, nutrition and even food processing.

I have analyzed the effects of a natural antioxidant on two swine target organs: the small intestine (examined at the end of the growing-finishing period) and the liver (analyzed during the weaning period), and I have demonstrated that the studied natural antioxidant substance could improve intestinal barrier function and hepatocellular antioxidant status.

In all cases, the morpho-functional approach to the analysis of selected structural aspects of the swine digestive system has been shown valid in judging the value of experimental dietary treatments. Specially, histometrical techniques, utilizing objective quantitative data, appear a noticeable mean in verifying the efficacy of dietary supplements in the food animal species, together with other, more conventional criteria.



# CHAPTER 7

## Summary



## 7. Summary

The aim of my PhD thesis was to give a contribution in the field of improving pig welfare in different crucial phases of the rearing period. This on the basis that: i) keeping farm animals healthy is highly recommended for obtaining animal products that are safe for human consumers, and ii) pathologies and difficulties in food animal species growth result in severe economic losses in farming; really, a correct nutritional management may play a key role in animal production, above all when performed utilizing a multidisciplinary approach. We already know that the appropriate dietary composition and amount of nutrients are determinant for an optimal growth of animals. After the banning (2006) from EU countries of the use of chemotherapeutics as growing substances, the interest of scientific researchers for alternative nutritional strategies appears more and more increasing. Various nutritional approaches with different dietary integrations have been tested in the recent years for finding alternatives to the limited use of chemotherapeutics in animal rearing, as well as for enhancing the intrinsic quality of a food product of animal origin. In the last year most literature focus on the so called nutraceuticals. Here I present three assays aimed at evaluating the effects of different dietary supplementations in pigs in two different phases of the intensive farm industries: **weaning**, which is most often accompanied by acute stress, and **growth-finishing period**, which is characterized by a long term stress. In all these studies my approach was micro-anatomical, consistent with morpho-functional analyses of the pig alimentary tract. In addition with the description of important structural details, sometimes differently quantified (histometry) in treated animals in comparison with control ones, the used micro-anatomical approach let me to hypothesize in some instances mechanisms of action of the studied dietary interventions. Moreover I have worked to correlate the results obtained with the used micro-anatomical methods with those aimed at evaluating growth performance, thus respecting a multidisciplinary approach, which is useful when approaching the swine that is important both as a food animal species, and a largely used animal model for approaching biomedical themes important for human medicine.

In the first assay I studied the way to manage oxidative stress in the **growth-finishing phase**. The aim of the study was to investigate whether the use of verbascoside (VB), a polyphenolic plant compound, could modulate pig feeding oxidative and/or nitrosative stress in the gut. Eighteen male piglets were assigned to two groups, which were fed with either a control diet (CON) or a diet supplemented with 5 mg/kg of verbascoside (VB) for 166 days. At slaughter, duodenum and jejunum specimens were collected. Immunohistochemistry and Western Blot analyses were performed on the samples to evaluate free radical adducts, including acrolein (ACR), 8-

hydroxydeoxyguanosine (8-OHdg) and nitrotyrosine (NT). A KRL test was also used to assess the total blood antioxidant activity. Immunohistochemistry and Western blot showed that dietary treatment decreased the levels of nitrotyrosine in enteroendocrine cell populations ( $P < 0.05$ ). Characterization of the enteroendocrine cell typology was then performed, and serotonin immunoreactive cells were revealed to be directly involved in decreasing nitrosative stress status. This preliminary study demonstrates the important role of dietary VB in decreasing stress biomarkers in swine gut, thus highlighting a possible intervention aimed at building a large prospective for antioxidant dietary supplementation in food animal species.

In the second and third assays I studied how to manage the **weaning period** stressful condition focusing my attention on the intestine in the first work and on the liver in the second one. The effects of dietary supplementation of different copper sulphate forms ( $\text{CuSO}_4$ ) in post weaning piglets on growth performance, small intestinal morphology and ecosystem, as well as faecal copper content were studied. At weaning 90 piglets were allotted to three dietary treatments for 18 d: control diet (with no copper sulphate) and two diets supplemented with 150 mg/kg of copper sulphate in protected and unprotected form. Unprotected copper sulphate showed an increase in feed:gain ratio. Moreover, duodenum villi length and crypts depth revealed to be higher in the treated animals. Furthermore, the two copper diets showed a decrease in Streptococci, and an higher fecal copper content. The obtained results revealed a modulation in the intestinal environment caused by addition of unprotected copper sulphate, as well as positive structural changes in the duodenum.

In the third and last work the aim of the study was to investigate whether the use of verbascoside in weaning pig feeding could modulate oxidative stress in the liver. Twenty four weaned female piglets were assigned to three experimental groups: the first group were fed a diet with 9% of sunflower oil (T1), the second one were fed with the same diet of the T1 but with an integration of antioxidant mix (T2), in particular with a 5 mg/kg of verbascoside (VB), and the third group was fed a control diet (CTR), where the oil was substituted by starch. The trial went on twenty nine days, and at the end the animals were slaughtered and the liver specimens were collected. Immunohistochemistry and Western Blot analyses were performed on the samples to evaluate free radical adducts, including Heat Shock Protein (Hsp) 70, Hsp 90, lysozyme and desmin. These analyses showed an increase of the level of Hsp 70 in the liver treated with sunflower oil. A KRL test was also used to assess the total blood and plasma antioxidant activity, and showed a significant decrease in T1 and T2 groups than Control. We evaluated also the impact of the use of antioxidant integrations with analyses on some plasma and liver enzymes related to oxidative stress: superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX).



In the plasma compartment all enzymes were lower in the treated animals in comparison with control group. In the liver only SOD had significant results: T1 group presented a higher level of this enzyme related to other groups.

In conclusion, these three works support in my opinion the hypothesis that selected dietary integrations are possibly able to improve pig welfare in intensive farm, directing their actions, among other targets, upon the gut, and it is well known that a healthy gut may be responsible for a healthy individual. This in turn may be of a fundamental importance in the environment of the rearing farm that is so frequently rich of stressors, which may be with efficacy counteracted by nutraceuticals.



## **CHAPTER 8**

### **Additional Published Works**



During my PhD experience I have started on a collaboration with Dott. Rainer Schneider, a Veterinary Practitioner, who is the Scientific Responsible of Parco delle Cornelle's zoo. From this collaboration a scientific paper was born concerning morpho-functional aspects of the liver in wild-captive Ruminants:

**Carollo V**, Di Giancamillo A., Vitari F., Schneider R. and Domeneghini C. 2012. Immunohistochemical Aspects of Ito and Kupffer Cells in the Liver of Domesticated and Wild Ruminants. Open Journal of Veterinary Medicine, vol.2, n°3, 129-136.

In these years I have also collaborated with my research group, publishing a scientific paper that was concerning the pig:

Vitari F., Di Giancamillo A., Deponti D., **Carollo V**. and Domeneghini C. 2012. Distribution of ghrelin-producing cells in the gastrointestinal tract of pigs at different ages. Veterinary Research Communications, vol.36, n°1, 71-80.

Here enclosed there are the two additional published papers.

## 8.1 Immunohistochemical aspects of Ito and Kupffer cells in the liver of domesticated and wild ruminants

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## **Abstract**

The mammalian liver is a morphologically and functionally complex organ, made up of not only of the largely predominant parenchymal cells (hepatocytes) but also non-parenchymal cells. Although there are less non-parenchymal cells than hepatocytes, they nevertheless play an important role in regulating many hepatocyte functions, as well as in the immunology of the liver. We investigated the structural aspects of the liver and the morpho-functional characteristics of Ito and Kupffer cells in two domesticated ruminant species (cattle and goat) in comparison with four wild ruminant species living in captivity in a zoo in northern Italy. The liver specimens were studied using histological, histochemical and immunohistochemical methods. The liver parenchyma was structurally normal. Immunohistochemistry was performed for desmin, glial fibrillary acidic protein (GFAP), vimentin,  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), collagen I, lysozyme, CD68 and tumor necrosis factor  $\alpha$  (TNF $\alpha$ ). In all the studied ruminants, Ito cells reacted with desmin and vimentin antibodies, Kupffer cells were evidenced only with lysozyme-immunopositivity, and both displayed a characteristic distribution in the hepatic lobular/acinar structure. The results obtained, not only contribute to the knowledge of ruminant wild species, but also help to define a normal structure reference for the diagnosis and treatment of liver diseases.

## **Keywords**

liver non parenchymal cells, captive wild ruminants, histology, histochemistry, liver immunobiology

## Introduction

The mammalian liver is a morphologically and functionally complex organ, made up not only of the largely predominant parenchymal cells (hepatocytes), but also non parenchymal cells (NPCs), including Ito, Kupffer and sinusoidal endothelial cells (SECs), as well as other cell types that reside in the sinusoidal compartment [1,2,3]. Although present in a small percentage in the total liver volume (around 6%), non-parenchymal cells play an important role in the regulation of many hepatocyte functions [4,1] as well as in the immunobiology of the liver [5], in both normal and pathological conditions [6].

Hepatic stellate cells (HSCs) also called Ito cells, fat-storing cells, lipocytes [7], are vitamin A-storing cells located in the space of Disse between hepatocytes and sinusoidal endothelial cells, which is why they are also called perisinusoidal cells. These cells constitute approximately 5% of the total number of liver cells [2], their cytoplasm is especially rich in lipid droplets (long-chain fatty acid esters of retinal, retinyl palmitate), and show long, branched cytoplasmic processes that embrace the endothelial cells [8,1]. In addition they are able to modulate the turnover of parenchymal cells and regulate liver regeneration. Owing to their smooth muscle alpha-actin when contracting, these NPCs may reduce the lumen of sinusoid capillaries, in such a way modulating the liver sinusoidal blood flow. When the liver is damaged, the hepatic stellate cells change their shape and transform (via a process named “activation”) into the myofibroblast-like cells, which are the major cell type responsible for the onset of liver inflammatory fibrosis and eventually cirrhosis [9]. Myofibroblast-like cells are highly proliferating and secrete a large quantity of extracellular matrix proteins (collagens type I and III, proteoglycan, adhesive glycoproteins), as well as extracellular matrix degrading metalloproteinases, cytokines and chemokines, but lose their function with regard to the vitamin A metabolism [10]. They promote hepatic fibrogenesis, possibly together with portal fibroblasts and parenchymal cells, and parenchymal cells in parallel begin to be transformed into mesenchymal cells (epithelial to mesenchymal transition) [11].

Kupffer cells are the liver resident macrophages. They are intra-sinusoidal and display huge endocytic and non specific phagocytic activities, since they are a part of the reticulo-endothelial system. The liver contains one of the largest resident populations of macrophages [12], which are key components of the innate immune system [13] and derive from circulating monocytes [2]. Kupffer cells represent approximately 30% of NPC fractions and approximately 15% of all liver cells [2]. The distribution of Kupffer cells within the hepatic lobules/acini is variable and perhaps species-specific: in the rat, the periportal area contains 43% of the cells, the midzonal region approximately 28% and the remaining 29% of Kupffer cells are located in the centre of the hepatic



lobules/acini [14]. They remove senescent and damaged erythrocytes from circulation, which may lead to an excess of cellular iron deposits in some storage diseases, as the effect of either seasonal variations or metabolic dysregulation phenomena [15,16].

Kupffer cells phagocyte the great majority of bacterial products coming from the gut, and consequently are responsible for the onset of the acute phase response and produce a large variety of inflammatory mediators (IL-1, TNF-alpha, TGF-beta), which in turn may induce liver injury. As a response to inflammatory inputs, Kupffer cells (and in some species, also Ito cells) release prostaglandins from arachidonic acid via cyclooxygenases (COX)-1, -2. Prostaglandins affect the hepatic glucose and lipid metabolisms [17], and elicit oxidative stress molecules that are read by hepatocytes as apoptogenic stimuli. They have a limited local proliferating ability and, together with SECs, express scavenger, mannose, and membrane receptors for the Fc region of IgG and for the complement [2,6].

In summary, both Ito and Kupffer cells share a fundamental role in the occurrence of some pathological liver conditions, however, paradoxically, the histochemical and immunohistochemical aspects of both these cell types are better known in terms of their relationship to pathological rather than normal conditions.

Thus, also bearing in mind the complex heterogeneity (sometimes species-specific) of these non parenchymal cells, our aim was to investigate the structural aspects of the liver, and to detail the morpho-functional characteristics of Ito and Kupffer cells in two domesticated ruminant species (cattle, goat) in comparison with wild ruminant species living in captivity at a zoo in northern Italy. In addition the aim was to identify the fundamentals of the normal liver structure in mammals that to date have not been fully investigated, in order to improve the present structural framework, to which one can refer for describing possible hepatic diseases. This might then lead to a better quality of care and management of zoo animals, where captivity is fundamental for safeguarding endangered species, but which can also involve stressful environmental conditions.

## **Materials and Methods**

### *Animals and tissue processing*

Approximately 1 cm<sup>3</sup> of liver samples (similar lobes) from different ruminant species (two adult individuals for each species) were collected, promptly after death: Holstein Freisian cattle (*Bos taurus*) and Saanen goat (*Capra hircus*) livers were obtained at slaughter; giraffe (*Giraffa camelopardalis*), reindeer (*Rangifer tarandus*), scimitar oryx (*Oryx dammah*) and Mrs Gray's lechwe (*Kobus megaceros*) livers were obtained during necropsies, which were performed on the animals in

2010-2011 years at “Le Cornelle” park in the north of Italy. These captive wild ruminants had died for reasons unrelated to gastrointestinal diseases. The gross anatomy of the livers was in all cases judged to be normal. The hepatic liver samples were fixed by immersion in 10% neutral-buffered formalin, routinely embedded in paraffin, and then sectioned 4  $\mu\text{m}$  thick. The paraffin sections, after dewaxing and rehydration, were treated with histological, histochemical and immunohistochemical stains, as described below.

#### *Histological and histochemical analyses*

Dewaxed sections were stained with Haematoxylin and Eosin (HE) sequential stain, Masson’s trichromic stain, and Gordon and Sweet’s modified procedure for reticulum [18], the latter for revealing the liver architecture.

#### *Immunohistochemical analyses*

After dewaxing and endogenous peroxidase blocking with  $\text{H}_2\text{O}_2$ , 10% for 10 minutes, slides were pre-treated with either a microwave treatment (twice for 5 minutes at 450W in a citrate buffer pH 6, with a 20 minute interval between the two treatments; Table 1) or proteinase K (0.2% proteinase K in PBS pH 7.4 at room temperature for 5 minutes; Table 1) to induce antigen retrieval. Sections were then incubated overnight at room temperature in a humid chamber with the primary antibodies (see Table 1).

**Table 1.** Primary antisera used, aimed at identifying Ito and Kupffer cells

	<b>Code</b>	<b>Source</b>	<b>Dilution</b>	<b>Incubation</b>	<b>Antigen retrieval</b>
<b>CD68</b> clone KP1	H7122	Dakocytomation	1:50	Overnight at RT	Heat
<b>Collagen I</b>	7066	Chondrex	1:400	Overnight at RT	Heat
<b>Desmin</b> clone D33	H7094	Dakocytomation	1:50	Overnight at RT	Heat
<b>GFAP</b>	20334	Dakocytomation	1:500	Overnight at RT	Proteinase K
<b>Lysozyme</b> EC3.2.1.17	A0099	Dakocytomation	1:400	Overnight at RT	Proteinase K
<b><math>\alpha</math>SMA</b>	H7114	Dakocytomation	1:50	Overnight at RT	Heat

<b>TNF<math>\alpha</math></b>	Ab6671	Abcam	1:100	Overnight at RT	Heat
<b>Vimentin</b>	674M	Biogenex	1:10	Overnight at RT	Heat

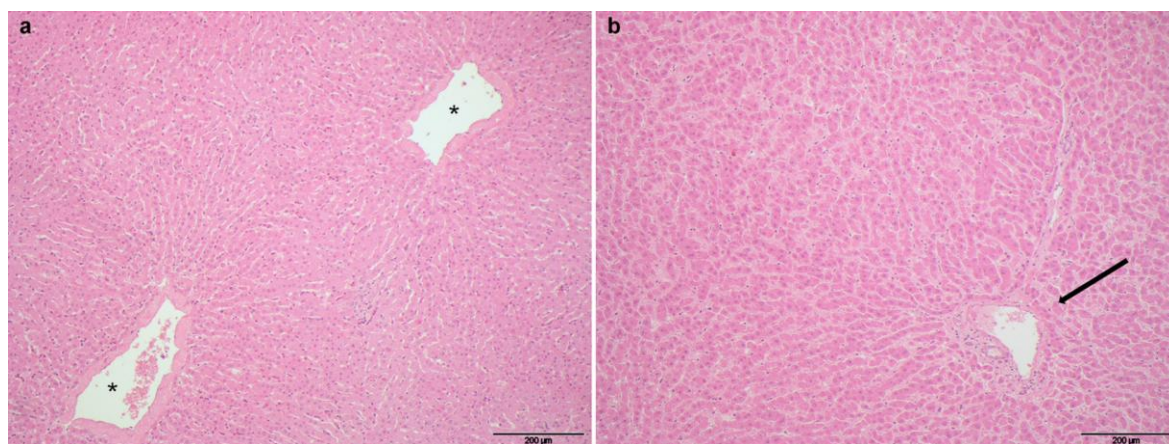
Sections were subsequently incubated with EnVision™ Detection Systems, Rabbit or Mouse (Dakocytomation, Italy) and the reaction products were visualized with a freshly prepared solution of 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma, Italy), 10 mg in 15ml of a 0.5M Tris buffer at pH 7.6, containing 1.5ml of 0.03% H<sub>2</sub>O<sub>2</sub>. To ascertain structural details, sections were slightly counterstained with Mayer's haematoxylin.

Porcine liver sections were used as a positive control. For the negative controls, other sections were processed simultaneously with the procedure described above, except that the primary antibodies were substituted with i) PBS, ii) preimmune sera. Both these procedures gave negative results.

## Results

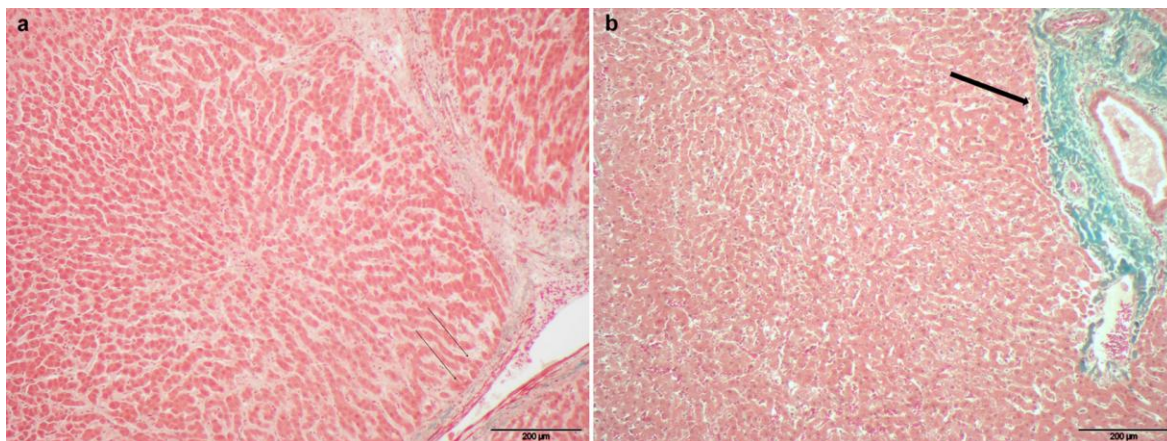
### *Histological and histochemical analyses*

The histological and histochemical observations confirmed the observations following gross anatomy examination: the liver parenchyma was structurally normal, the central vein and the portal spaces were always evident, and the connective component that accompanies the lobular structure was rather scarce (Figs. 1a,b).



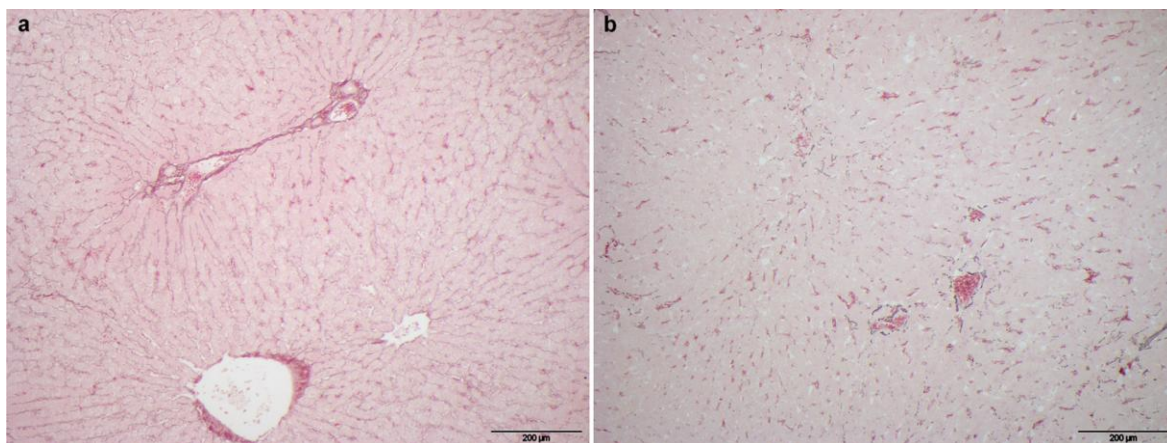
**Fig. 1** Ruminant livers. HE sequential stain. a) Giraffe: the liver structure is normal. Two central veins (asterisks) are visible. Scale bar 200 $\mu$ m. b) Reindeer: the hepatic parenchyma is normal. A portal area is present (arrow). Scale bar 200 $\mu$ m.

Masson's trichromic stain showed that in the cattle, goat and reindeer livers, the connective tissue was present small quantities in the capsule, portal areas, and delineating the lobular *septae* (Fig. 2a). In the giraffe, scimitar oryx and Mrs Gray's lechwe livers, the portal areas were more clearly characterized by the presence of connective tissue (Fig. 2b).



**Fig. 2** Ruminant livers. Masson's trichromic stain. a) Reindeer: the connective tissue component is very scarce (thin arrows). Scale bar 200µm. b) Scimitar oryx: the connective tissue is relatively abundant in a portal area (arrow). Scale bar 200µm.

The histochemical stain aimed at highlighting the reticular fibres that support the hepatic parenchyma also showed the normal architecture of the lobular structure (Figs. 3a,b).



**Fig. 3** Ruminant livers. Gordon and Sweets' modified method for reticulum. a) Cattle, b) Scimitar oryx: the normal architecture of the liver is demonstrated by the reticular fibers running. Scale bar 200µm.

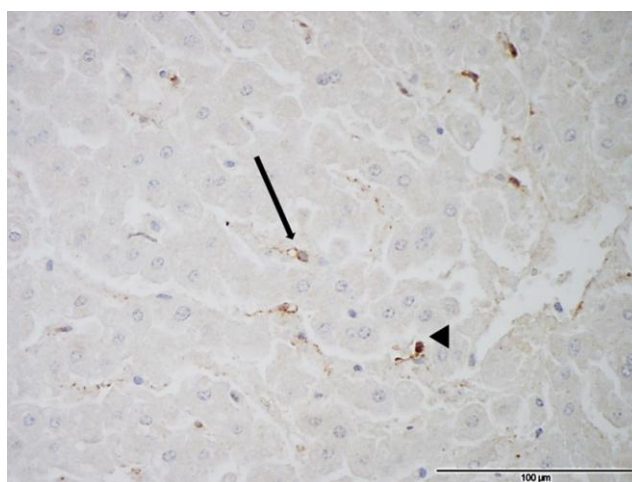
*Immunohistochemical analyses*

Immunohistochemistry was used to demonstrate the presence of Ito and Kupffer cells (see Table 2).

**Table 2.** Ito and Kupffer cells immunoreactivities in the six ruminant species

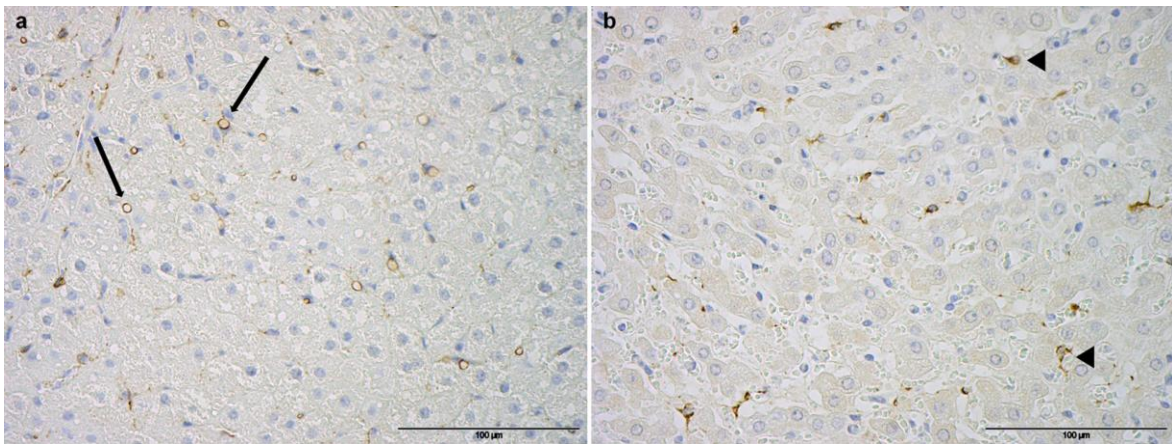
		Cattle	Goat	Giraffe	Reindeer	Scimitar oryx	Mrs Gray's lechwe
<b>Ito cells</b>	$\alpha$ SMA	-	-	-	-	-	-
	Collagen I	-	-	-	-	-	-
	GFAP	+	-	-	+	-	-
	Desmin	+	+	+	+	+	+
	Vimentin	+	+	+	+	+	+
<b>Kupffer cells</b>	Lysozyme	+	+	+	+	+	+/-
	TNF $\alpha$	-	-	-	+	-	-
	CD68	-	-	-	-	-	-

None cell type was revealed by applying anti- $\alpha$ SMA and anti-Collagen I antibodies (data not shown), whereas anti-GFAP immunohistochemistry showed small, roundish to irregular perisinusoidal cells in the cattle and reindeer livers (Fig. 4).

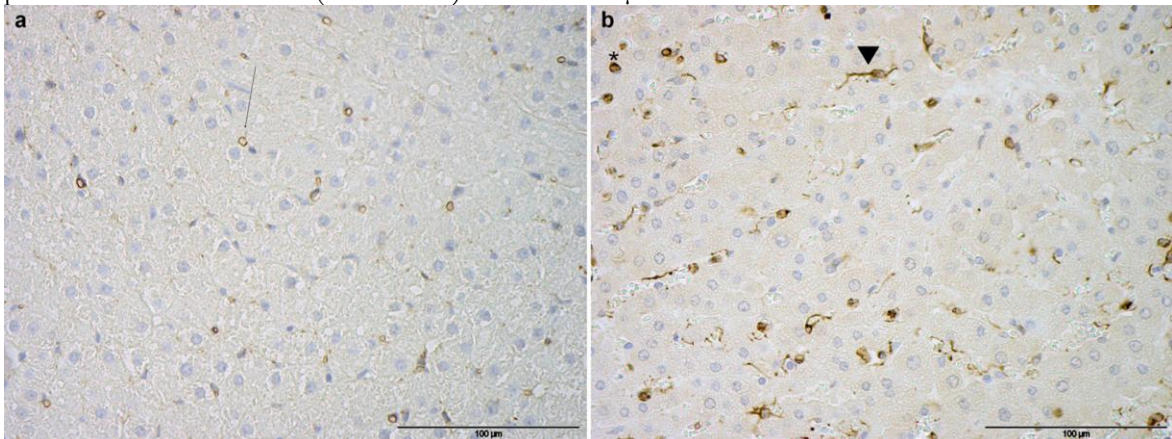


**Fig. 4** Ruminant liver. GFAP-immunohistochemistry. Reindeer: small, roundish (arrow) to irregular (arrowhead) immunopositive cells are visible in perisinusoidal localizations. Scale bar 100 $\mu$ m.

In addition, a number of similar, variously sized, perisinusoidal cells were found to be immunopositive to both anti-desmin (Fig. 5) and anti-vimentin (Fig. 6) antibodies in all the animals. In the bovine, goat and oryx livers these desmin (Fig. 5a)- and vimentin (Fig. 6a)- immunopositive cells were prevalently roundish, and contained cytoplasmic vacuoles that appeared to be devoid of contents after the applied routine procedure for paraffin embedding, and were thus interpreted as rich in lipid content. Giraffe, reindeer and Mrs Gray's lechwe presented another immunohistochemical feature: desmin (Fig. 5b)- and vimentin (Fig. 6b)- immunopositive cells predominantly exhibited a stellate shape with extensive long cytoplasmic processes running along or encircling the sinusoids.

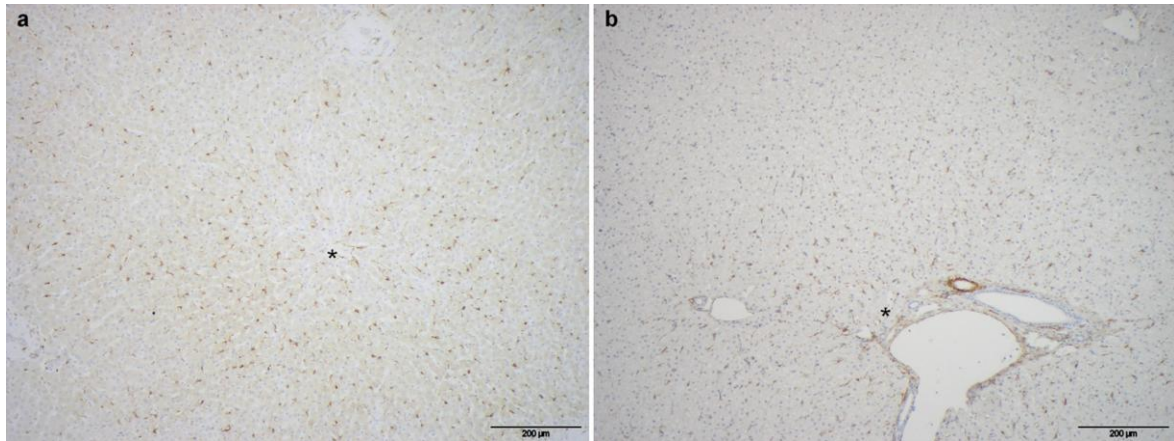


**Fig. 5** Ruminant livers. Desmin-immunohistochemistry. a) Goat: several small roundish (arrows) immunopositive cells are visible in perisinusoidal areas. The cytoplasm clearly shows a lipid content. Scale bar 100µm. b) Giraffe: Numerous irregularly shaped immunopositive cells are present in perisinusoidal localizations (arrowheads). Scale bar 100µm.



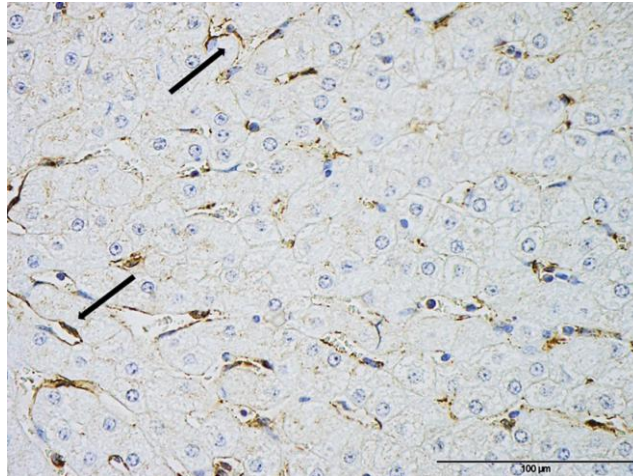
**Fig. 6** Ruminant livers. Vimentin-immunohistochemistry. a) Goat: small roundish (thin arrow) immunopositive cells are visible in perisinusoidal areas. The cytoplasm clearly shows a lipid content. Scale bar 100µm. b) Giraffe: numerous irregularly shaped immunopositive cells are present in perisinusoidal localizations (arrowhead). A smaller number of roundish immunopositive cells are also present (asterisk). Scale bar 100µm

In the latter animals, roundish desmin- and vimentin-immunopositive cells were also present, however in fewer numbers than the immunopositive stellate cells. The distribution of this type of perisinusoidal cells was different in the animals studied: in the cattle, giraffe (Fig. 7a) and scimitar oryx, desmin- and vimentin-immunopositive cells were more numerous in the pericentral than the periportal areas of the hepatic lobules. In contrast in the goat (Fig. 7b), reindeer and Mrs Gray's lechwe, these cells were more numerous in the periportal areas.



**Fig. 7** Ruminant livers. Desmin-immunohistochemistry. a) Giraffe: immunopositivity is mainly visible in a pericentral area (asterisk). Scale bar 200µm. b) Goat: the immunopositivity is mainly visible in a periportal area (asterisk). Scale bar 200µm.

One other non-parenchymal cell type was immunohistochemically found in all the studied ruminants, with a characteristic localization (in the sinusoid wall), and shape (irregular or spindled): this cell type was lysozyme-immunopositive (Fig. 8), although the intensity of the immunoreactions was not the same for all the animals studied, and was particularly scarce in Mrs Gray's lechwe. These non-parenchymal cells were more numerous in the periportal (Fig. 8) than the pericentral areas, and this distribution was uniformly observed in the liver of all the animals studied.



**Fig. 8** Ruminant liver. Lysozyme-immunohistochemistry. Cattle: numerous immunopositive, irregularly shaped cells are visible in the sinusoid walls (arrows). Scale bar 100 $\mu$ m

This cell type was also TNF $\alpha$ -immunopositive, but limited to reindeer liver, and was not immunopositive to the CD68 antibody (data not shown).

## Discussion

In this study we examined the liver of six different ruminants belonging to the *Artiodactyla* order. Judging from the histology and histochemistry, the architecture of the liver of these ruminants was normal. Its structure corresponded to what is known for ruminant species, especially concerning the generally limited quantity of connective tissue in the normal hepatic lobular/acinar structure. Considering the mutual relationship of the non-parenchymal cells in developing hepatic diseases, special attention was paid to the immunohistochemical features of the Ito and Kupffer cells that were revealed by panels of immunohistochemical markers.

In all the ruminant species analyzed in this study, the variously sized and shaped Ito cells, with their characteristic content of cytoplasmic fat droplets, showed a positive reactivity to anti-desmin and anti-vimentin antibodies, as also observed by Neubauer et al. [19] and Uetsuka et al. [20]. In the case of the cattle and reindeer, Ito cells were also immunopositive for GFAP, in accordance with Neubauer et al. [19], who identified this marker in the rat liver and *in vitro* experiments. In humans hepatic stellate cells are also detectable using GFAP-immunopositivity, however the intensity of the reaction increases when fibrosis develops [21].

Cattle, goat and scimitar oryx livers showed rounded lipid vacuoles in the cytoplasm of Ito cells, which were more abundant in the pericentral area.



Giraffe, reindeer and Mrs Gray's lechwe liver Ito cells exhibited a stellate shape with extensive long cytoplasmic processes running along or encircling sinusoids, and were more evident in the periportal area.

No positive immunostaining for  $\alpha$ SMA was observed in the Ito cells, which is in line with Uetsuka's study on bovine liver [20]. The absence of immunopositivity for both  $\alpha$ SMA and Collagen I is likely due to the absence of Ito cell activation, that is, Ito cells were not transforming into myofibroblast-like cells. Accordingly, the liver in all the ruminants of this study appeared to be fully normal and did not display fibrosis. Liver fibrosis has been described in ruminant species, above all in cattle [22,23,24], in which, as in other mammals, this disease represents the liver's response, via the activation of stellate cells, to inflammatory, toxic, infectious or metabolic stimuli [25,26].

Kupffer cells are fundamental in sustaining the immunobiology of the liver both in normal and pathological conditions. They are also able to modulate systemic immune tolerance, via their capacity to suppress T cell activation [27,6]. Together with Ito cells, the liver resident macrophages contribute to an assessment of liver fibrosis [28]. Ruminant Kupffer cells showed a clear lysozyme-immunoreactivity; only in Mrs Gray's lechwe's did they present a scarce immunopositivity to lysozyme. In the reindeer, Kupffer cells were also immunopositive for TNF $\alpha$ . Kushibishi [29] demonstrated in cattle that TNF $\alpha$  from activated Kupffer cells regulates inflammatory responses in mastitis, and affects metabolic disorders, such as acidosis. None of the ruminants livers showed an immunoreactivity to CD68, in contrast with other studies on different mammals [30,28]. This apparent immunohistochemical heterogeneity was expected, because it is well known that macrophages are differently detected in their various tissue localizations and species [31,32]. Ruminant Kupffer cells were located in the sinusoid walls, showed an irregular shape, and were more numerous in the periportal than the pericentral areas, in accordance with studies in other mammals [33]. When the liver is injured, Kupffer cells activate and contribute to the immune cell responses [34,35,6]. Kupffer cells have been shown to contain PrP(Sc) (a marker of prion disease) in experimentally infected sheep [35], and, when activated, may release a lot of inflammatory mediators in cattle [37]. Ruminant Kupffer cells are evidently damaged in intoxication phenomena, which sometimes give rise to lysosomal storage diseases [38,39], and poisoning [40,41].

To the best of our knowledge, this is the first description of the morphofunctional aspects and distribution of non-parenchymal cell types in wild ruminants. Since Sleyster and Knook's study on the rat [42], it has been well known that functional gradients exist in the lobular distributions of liver non-parenchymal cells, in particular of Kupffer cells, and these functional distributing differences may potentially help in elucidating pathogenic mechanisms [2].

In conclusion, we have shown the immunohistochemical features and morphological distribution of Kupffer and Ito cells in the liver of six different ruminant species. Four of these species were originally wild ruminants kept in a zoo in northern Italy. The examined ruminants belonged to three different feeding habits (sometimes overlapping), according to the classification by Hofmann and Stewart [43], updated by Hackmann and Spain [44]: reindeer, scimitar oryx and Mrs Gray's lechwe are grass- and roughage-eaters (grazers), the giraffe is a concentrate herbage and foliage selector, and cattle and goat are intermediate feeders (browsers). This classification, which above all concerns the morphology and morphometry of the gastrointestinal tract, does not seem to affect the liver parenchyma, whose normal structure is fundamentally similar in the six examined species. The non-parenchymal cells were well evidenced by immunohistochemistry, although with some differences possibly linked to either genetics or feeding habits/plans, which need further research. Ito cells were revealed with both desmin- and vimentin-immunohistochemistry, Kupffer cells were immunopositive to lysozyme, and both displayed a characteristic distribution in the hepatic lobular/acinar structure. We believe that our data contribute to the knowledge of wild species, and also help to define a normal liver structure reference for the diagnosis and treatment of liver diseases.

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## **Bibliography / References**

- [1] Z. Kmiec, “Cooperation of liver cells in health and disease,” *Advances in Anatomy, Embryology and Cell Biology* vol. 161, No. 1, 2001, pp. 1-151.
- [2] D. E. Malarkey, K. Johnson, L. Ryan, G. Boorman and R. R. Maronpot, “New insights into functional aspects of liver morphology,” *Toxicologic Pathology* vol. 33, 2005, pp. 27-34.
- [3] K. Uetsuka, S. Nishikawa, A. Yasoshima, H. Nakayama and K. Doi, “Histopathological characteristics of Ito cells and Kupffer cells in the feline liver,” *Journal of Veterinary Medical Science*, vol. 68, No. 3, 2006, pp. 235-242.
- [4] A. M. Neyrinck, L. D. De Wispelaere, V. P. Vanhulle, H. S. Taper and N. M. Delzenne, “Are Kupffer cells involved in the metabolic adaptation of the liver to

dietary carbohydrates given after fasting?," *Biochimica et Biophysica Acta*, vol. 1475, No. 3, 2000, pp. 238-44.

[5] G. A. Parker and C. A. Picut , "Liver immunobiology," *Toxicologic Pathology*, vol. 33, 2005, pp. 52-62.

[6] G. A. Parker and C. A. Picut , "Immune functions in nonlymphoid organs: the liver," *Toxicology Pathology*, vol. 39, 2011, pp. 1-11.

[7] H. Senoo, N. Kojima and M. Sato, "Vitamin A-storing cells (stellate cells)," *Vitamins and Hormones*, vol. 75, 2007, pp.131-159.

[8] M. L. Hautekeete and A. Geerts, "The hepatic stellate (Ito) cell: its role in human liver disease," *Virchows Archiv*, vol. 430, 1997, pp.195-207.

[9] K. Iwaisako, D. A. Brenner and T. Kisseleva, "What's new in liver fibrosis? The origin of myofibroblasts in liver fibrosis," *Journal of Gastroenterology and Hepatology*, vol. 27, 2012, pp.65-68.

[10] H. Senoo, K. Yoshikawa, M. Morii, M. Miura, K. Imai and Y. Meezaki, "Hepatic stellate cell (vitamin A-storing cell) and its relative—past, present and future," *Cell Biology International*, vol. 34, 2010 , pp. 1247-1272.

[11] F. Tacke and R. Weiskirchen, "Update on hepatic stellate cells: pathogenic role in liver fibrosis and novel isolation techniques," *Expert Review of Gastroenterology and Hepatology*, vol. 6, 2012, pp. 67-80.

[12] M. Naito, G. Hasegawa, Y. Ebe and T. Yamamoto, "Differentiation and function of Kupffer cells," *Medical Electron Microscopy*, vol. 37, 2004, pp. 16-28.

[13] I. Kiki, B. Z. Altunkaynak, M. E. Altunkaynak, O. Vuraler, D. Unal and S. Kaplan, "Effect of high fat diet on the volume of liver and quantitative feature of Kupffer cells in the female rat: a stereological and ultrastructural study," *Obesity Surgery*, vol. 17, 2007, pp. 1381-88.

[14] L. Bouwens, M. Baekeland, R. De Zanger, E. Wisse, "Quantitation, tissue distribution and proliferation kinetics of Kupffer cells in normal rat liver," *Hepatology*, vol. 6, No 4, 1986, pp.718-22.

- [15] B. Borch-Johnsen and K. Thorstensen, "Iron distribution in the liver and duodenum during seasonal iron overload in Svalbard reindeer," *Journal of Comparative Pathology*, vol. 141, 2009, pp. 27-40.
- [16] P. Olias, A. T. Weiss, A. D. Gruber and R. Klopffleisch, "Iron storage disease in red deer (*Cervus elaphus elaphus*) is not associated with mutations in the HFE gene," *Journal of Comparative Pathology*, vol. 145, 2011, pp. 207-213.
- [17] A. M. Neyrinck, S. Margagliotti, C. Gomez and N. M. Delzenne, "Kupffer cell-derived prostaglandin E2 is involved in regulation of lipid synthesis in rat liver tissue," *Cell Biochemistry and Function*, vol. 22, 2004, pp. 327-332.
- [18] H. Gordon and H. H. Sweet, "A simple method for the silver impregnation of reticulin," *American Journal of Pathology*, vol. 12, 1936, p. 545.
- [19] K. Neubauer, T. Knittel, S. Aurisch, P. Fellmer and G. Ramadori, "Glial fibrillary acidic protein – a cell type specific marker for Ito cells in vivo and in vitro," *Journal of Hepatology*, vol. 24, 1996, pp. 719-30.
- [20] K. Uetsuka, S. Nishikawa, K. Isobe and H. Nakayama, "Histopathological characteristics of Kupffer cells and Ito cells in the porcine and bovine liver," *Journal of Veterinary Medical Science*, vol. 69, No. 7, 2007, pp. 767-70.
- [21] S. Morini, S. Carotti, G. Carpino, A. Franchitto, S. G. Corradini, M. Merli and E. Gaudio, "GFAP expression in the liver as an early marker of stellate cells activation," *Italian Journal of Anatomy and Embryology*, vol. 110, 2005, pp. 193-207.
- [22] A. C. Bourque, I. C. Fuentealba, R. Bildfell, P. I. Daoust and P. Hanna, "Congenital hepatic fibrosis in calves," *The Canadian Veterinary Journal*, vol. 42, 2001, pp. 145-146.
- [23] M. B. Torres and K. I. Coelho, "Foamy macrophages in the liver of cattle fed *Brachiaria brizantha* hay," *Veterinary and Human Toxicology*, vol. 45, 2003, pp. 163-164.
- [24] S. Haywood, T. Müller, A. M. Mackenzie, W. Müller, M. S. Tanner, P. Heinz-Erian, C. L. Williams and M. J. Loughran, "Copper-induced hepatotoxicosis with hepatic stellate cells activation and severe fibrosis in North Ronaldsay lambs: a model for non-Wilsonian hepatic copper toxicosis of infants," *Journal of Comparative Pathology*, vol. 130, 2004, pp. 266-277.

- [25] K. Neubauer, B. Saile and G. Ramadori, "Liver fibrosis and altered matrix synthesis," *Canadian Journal of Gastroenterology*, vol. 15, 2001, pp. 187-193.
- [26] N. R. Tomanovic, I. V. Boricic, D. C. Brasanac, Z. M. Stojic, D. S. Delic and B. J. Brmbolic, "Activated liver stellate cells in chronic viral C hepatitis: histopathological and immunohistochemical study," *Journal of Gastrointestinal and Liver Disease*, vol. 18, No. 2, 2009, pp. 163-67.
- [27] C. Ju, "The role of haptic macrophages in regulation of idiosyncratic drug reactions," *Toxicology Pathology*, vol. 37, 2009, pp. 12-17.
- [28] C. Liu, Q. Tao, M. Sun, J. Z. Wu, W. Yang, P. Jian, J. Peng, Y. Hu, C. Liu and P. Liu, "Kupffer cells are associated with apoptosis, inflammation and fibrotic effects in hepatic fibrosis in rats," *Laboratory Investigation; a journal of technical methods and pathology*, vol. 90, 2010, pp. 1805-1816.
- [29] S. Kushibiki, "Tumor necrosis factor- $\alpha$ -induced inflammatory response in cattle," *Animal Science Journal*, vol. 82, 2011, pp. 504-511.
- [30] J. H. Lefkowitz, J. H. Haythe and N. Regent, "Kupffer cell aggregation and perivenular distribution in steatohepatitis," *Modern Pathology*, vol. 15, No 7, 2002, pp. 699-704.
- [31] J. Yamate, H. Yoshida, Y. Tsukamoto, M. Ide, M. Kuwamura, F. Ohashi, T. Miyamoto, T. Kotani, S. Sakuma and M. Takeya, "Distribution of cells immunopositive for AM-3K, a novel monoclonal antibody recognizing human macrophages, in normal and diseased tissues of dogs, cats, horse, cattle, pigs, and rabbits," *Veterinary Pathology*, vol. 37, 2000, pp.168-176.
- [32] P. Zelnickova, J. Matiasovic, B. Pavlova, H. Kudlackova, F. Kovaru and M. Faldyna, "Quantitative nitric oxide production by rat, bovine and porcine macrophages," *Nitric Oxide: biology and chemistry*, vol. 19, 2008, pp. 36-41.
- [33] P. F. Moore, "Characterization of cytoplasmic lysozyme immunoreactivity as a histiocytic marker in normal canine tissue," *Veterinary Pathology*, vol. 23, 1986, pp. 763-69.
- [34] G. L. Su, "Lipopolysaccharides in liver injury: molecular mechanisms of Kupffer cell activation," *American Journal of Physiology. Gastrointestinal and Liver Physiology*, vol. 283, 2002, pp.G256-G265.

- [35] L. C. Kabaroff, A. Rodriguez, M. Quinton, H. Boermans and N. A. Karrow, "Assessment of the ovine acute phase response and hepatic gene expression in response to *Escherichia coli* endotoxin," *Veterinary Immunology and Immunopathology*, vol. 15, 2006, pp. 113-124.
- [36] S. J. Everest, A. M. Ramsay, M. J. Chaplin, S. Everitt, M. J. Stack, M. H. Neale, M. Jeffrey, S. J. Moore, S. J. Bellworthy and L. A. Terry, "Detection and localization of PrP (Sc) in the liver of the sheep infected with scrapie and bovine spongiform encephalopathy," *PLoS One*, vol. 12, 2011, p. e19737.
- [37] M. Yoshioka, T. Ito, S. Miyazaki and Y. Nakajima, "The release of tumor necrosis factor-alpha, interleukin-1, interleukin-6 and prostaglandin E2 in bovine Kupffer cells stimulated with bacterial lipopolysaccharide," *Veterinary Immunology and Immunopathology*, vol. 66, 1998, pp. 301-307.
- [38] D. Driemeier, E. M. Colodei, E. J. Gimeno and S. S. Barros, "Lysosomal storage disease caused by *Sida carpinifolia* poisoning in goats," *Veterinary Pathology*, vol. 37, 2000, pp. 153-159.
- [39] A. G. Armien, C. H. Tokamia, P. V. Peixoto, J. D. Barbosa and K. Frese, "Clinical and morphological changes in ewes and fetuses poisoned by *Ipomea carnea* subspecies *fistulosa*," *Journal of Veterinary Diagnostic Investigation*, vol. 23, 2011, pp. 221-232.
- [40] M. Yamada, M. Nakagawa, M. Haritani, M. Kobayashi, H. Furuoka and T. Matsui, "Histopathological study of experimental acute poisoning of cattle by autumn crocus (*Colchicum autumnale* L.)," *Journal of Veterinary Medical Science*, vol. 60, 1998, pp. 949-952.
- [41] S. D. Stoev, N. Grozeva, R. Sieonov, I. Borisov, H. Hubenov, Y. Nikolov, M. Tsaneva and S. Lazarova, "Experimental cadmium poisoning in sheep," *Experimental and Toxicologic Pathology*, vol. 55, 2003, pp. 309-314.
- [42] E. C. Sleyster and D. L. Knook, "Relation between localization and function of rat liver Kupffer cells," *Laboratory Investigation; a journal of technical methods and pathology*, vol. 47, 1982, pp. 484-490.
- [43] R. R. Hofmann and D. R. M. Stewart, "Grazer or browser: a classification based on the stomach structure and feeding habits of east African ruminants," *Mammalia*, vol. 36, 1972, pp. 226-240.

[44] T. J. Hackmann and J. N. Spain, "Invited review: ruminant ecology and evolution: perspectives useful to ruminant livestock research and production," *Journal of Dairy Science*, vol. 93, 2010, pp. 1320-1334.

## 8.2 Distribution of ghrelin-producing cells in the gastrointestinal tract of pigs at different ages.

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## **Abstract**

Ghrelin is involved in many biological processes, ranging from appetite regulation and the release of growth hormone to the regulation of gastrointestinal motility and secretion processes. Ghrelin expression is not homogeneously distributed throughout the gastrointestinal tract; expression is species specific and can also depend on the animal age. This study was performed to investigate ghrelin immunolocalization in the gastrointestinal tract of pigs at different ages: 1 day (birth), 28 days (weaning), 2 months, 4 months, and 7 months (pre-puberty). Tissue samples were collected along the entire gastrointestinal tract and were examined by immunohistochemical and double-immunofluorescence staining. Histometry was performed by counting the number of endocrine ghrelin immunopositive cells in the gastrointestinal mucosa. Ghrelin was found to be present along the swine alimentary canal from the stomach to the caecum. In all regions of the alimentary canal of the animals studied, ghrelin-immunoreactive (IR) cells co-localized with chromogranin-A and were therefore identified as endocrine cells. In the gastric fundus, ghrelin-immunoreactivity was partially detected in co-localization with H-K-adenosine triphosphatase and pepsinogen. Ghrelin-IR endocrine cells were abundant in the oxyntic mucosa but less present in the small intestine and rare in the large intestine. The cell density of the ghrelin-IR endocrine cells was lowest in the oxyntic mucosa of 1-day-old pigs. We can conclude that gastric ghrelin expression is not related merely to age but could also potentially be influenced by food intake.

**Key words:** oxyntic mucosa, immunohistochemistry, double immunofluorescence, histometry

## Introduction

Ghrelin is a 28-amino-acid peptide hormone acylated with an n-octanoyl group (C8:0) in the serine residue at position 3 and was originally isolated from rat stomach (Kojima et al., 1999; Date et al., 2000). The acylation is necessary for the biological activity of ghrelin. These activities depend on the binding of ghrelin to the growth hormone secretagogue receptor (GHS-R), which is expressed in the hypothalamus and pituitary gland (Kojima et al., 1999). Through this receptor, ghrelin acts as a growth-hormone-releasing peptide and food intake modulator (Kojima and Kangawa, 2005). It has been demonstrated that ghrelin stimulates growth hormone release from the pituitary gland in rats, humans and pigs (Kojima et al., 1999; Takaya et al., 2000; Salfen et al., 2004) and that ghrelin exerts a central orexigenic effect through the activation of neuropeptide Y/agouti-related peptide hypothalamic neurons (Horvath et al., 2001).

Recent studies have demonstrated that GHS-R is also expressed in other areas of the central nervous system (Ferrini et al., 2009) and in peripheral tissues, suggesting that ghrelin is a multifunctional molecule involved in many biological processes. Studies have demonstrated that in the peripheral tissues, small amounts of circulating ghrelin are derived from the small and large intestine, pancreas, kidney, immune system, placenta, testis and ovary (Casanueva and Dieguez, 2002). The major site of acylated bio-active ghrelin production, however, is the gastric mucosa, which is consequently the main peripheral source of circulating ghrelin (Ariyasu et al., 2001). Tanaka- Shintani and Watanabe (2005) reported that ghrelin-producing cells were distributed mainly in the fundic region of the human stomach, and their distribution was correlated with that of the parietal cells. Du et al. (2006; 2007) showed that ghrelin may significantly increase the H<sup>+</sup>-K<sup>+</sup> -ATPase activity in swine gastric mucosal cells. Ghrelin plays a modulating role in cardiac and gastrointestinal functions, affects carbohydrate and lipid metabolism, acts on reproductive apparatus, and regulates cell proliferation and differentiation (van der Lely et al., 2004; Leite-Moreira and Soares, 2007; Dong et al., 2009). Moreover, ghrelin promotes a positive energy balance by acting on adipose tissue, where it stimulates adipogenesis (Thompson et al., 2004) and decreases lipolysis (Muccioli et al., 2004) and adipocyte apoptosis (Kim et al., 2004). These effects may be mediated through the GHS-R independent pathways (Tschop et al., 2000; Thompson et al., 2004).

Considering the reported discrepancies and the inhomogeneous data found among the different studied mammalian species, the present study was performed to investigate by immunohistochemical methods the presence and distribution of ghrelin immunopositive cells in the gastrointestinal tract of pigs

at different ages and to possibly relate ghrelin-producing cells with other cell types.

## Materials and methods

### *Animals and tissue sampling*

A total of 30 cross-breed (Large white X Landrace) male pigs (live weight: table 2) , from the same husbandry, were used in this study. Before slaughtering, pigs fasted for the same duration (12 hours) were selected on the basis of age: 1 day (n=6), 28 days (n=6), 2 months (n=6), 4 months (n=6) and 7 months (n=6). All the animals were treated in accordance with the policies and the principles of laboratory animal care consistent with the European Union guidelines (86/609/EEC) that have been approved by the Italian Ministry of Health (Law 116/92).

Immediately after slaughtering, small specimens from the stomach (cardiac, oxyntic and pyloric regions), small intestine (duodenum, jejunum, and ileum), caecum, ascending colon (second in-going coil) and rectum were taken from each animal. The samples (total number = 270) were promptly fixed in 4% para-formaldehyde in 0.01 M phosphate-buffered saline (PBS), pH 7.4, for 24 h at 4°C, dehydrated through a graded series of ethanol, cleared with xylene and embedded in paraffin. Consecutive microtome sections (4 µm-thick) were obtained from each sample and collected onto slides treated with poly-L-lysine. One section of each sample was stained with hematoxylin and eosin (HE) and examined under a light microscope (Olympus BX51, Olympus, Italy) to assess the morphology and exclude pathological aspects. The other ones were treated as follows in “Immunohistochemistry and cell counts” as well as in “Double Immunofluorescence” paragraphs. :

In addition, a mucosal scraping (approximately 1 gr) was taken from all the sampled organs of the gastrointestinal tract. These samples were vacuum-packed and stored at -80°C until Western blot analysis could be performed (see below).

### *Immunohistochemistry and cell counts*

Immunohistochemical tests were conducted to identify the presence and localization of ghrelin-secreting cells and to count them (histometry).

The dewaxed sections were treated with 3% H<sub>2</sub>O<sub>2</sub> in distilled water for 20 min and then incubated with normal goat serum (NGS, DakoCytomation, Italy) diluted at 1:20 in a Tris-HCl buffered saline (TBS; 0.05 M, pH 7.4, 0.55 M NaCl) for 30 min to reduce non-specific background staining. Next, the sections were incubated with rabbit anti-ghrelin (porcine) antibody (1:2000;

Phoenix Pharmaceuticals Inc., USA) in TBS plus 1% bovine serum albumin (BSA) overnight at room temperature (RT). The antigen–antibody complexes were detected using a peroxidase-conjugated polymer, which carries secondary antibody molecules directed against rabbit immunoglobulins (EnVision™+, DakoCytomation). This polymer was applied for 60 min at RT. Appropriate washing with TBS was performed between each step, and all incubations were performed in a moist chamber. Peroxidase activity was detected using a pre-dosed diaminobenzidine liquid chromogen (DAB, DakoCytomation) as the substrate. Finally, the sections were weakly counterstained with Mayer's hematoxylin, dehydrated, and permanently mounted. To determine the density of the ghrelin-immunoreactive (IR) endocrine cells in the mucosa of the different organs of the gastrointestinal tract, their number was evaluated in five randomly selected visual fields of the mucosa (each field representing a tissue section area of 77323,25  $\mu\text{m}^2$  at 400X) for each section, and the density was expressed as number of cells/ $\text{mm}^2$  of mucosa. All the observations were made by a single investigator, unaware of the ages of the pigs from which samples were obtained, using an Olympus BX51 microscope (Olympus Italia Srl) equipped with a digital camera and DP software for computer-assisted image acquirement and management.

The control experiments were performed as follows: (1) removal of the primary antibody, and (2) substitution of the primary antibody with a 0.05 M Tris-BSA buffer. The controls were run on the sections immediately after treatment with the primary antibody.

#### *Double Immunofluorescence*

Double immunofluorescence tests were conducted to identify possible co-localizations.

Serial paraffin-embedded sections were de-waxed and incubated with the first-step primary antibody, 1:2000 anti-porcine-ghrelin antibody (see above), overnight at 4° C. The sections were then washed in TBS, and then, the secondary antibody, DyLight®488 Anti-rabbit IgG (9  $\mu\text{g}/\text{ml}$  of 10 mM HEPES, 0.15 M NaCl, pH 7.5, 0.08% sodium azide, Vector Laboratories Inc., UK) was applied for 1 h at RT. The sections were then washed in TBS and, if the second step primary antibody was from rabbit (see Table 1), incubated with rabbit IgG for 2 h (Vector Laboratories Inc.; Zhao et al., 2008).

**Table 1.** Primary antibody use in double immunofluorescence analyses (HDC: histidine decarboxylase, GIP: gastric inhibitory polypeptide).

<b>Antibody</b>	<b>Tissue</b>	<b>Dilution</b>	<b>Incubation</b>	<b>Code and Manufacturer</b>
Anti-ghrelin	Stomach Small intestine Large intestine	1: 2000	Overnight at RT	H03152 Phoenix Pharmaceuticals, USA
Anti-chromogranin A	Stomach Small intestine Large intestine	Ready to use	Overnight at RT	AM126-5M Biogenex, USA
Anti-HDC	Stomach	1: 500	Overnight at RT	sc-34458 Santa Cruz, USA
Anti-serotonin	Stomach Small intestine	1: 100	Overnight at RT	AB938 Chemicon, USA
Anti-somatostatin	Stomach Small intestine	1: 800	Overnight at RT	AB1976 Chemicon, USA
Anti-H <sup>+</sup> /K <sup>+</sup> -ATPase	Stomach	1: 100	Overnight at RT	Ab2866 abCam, UK
Anti-pepsinogen	Stomach	1: 100	Overnight at RT	P327205 USBiological, USA
Anti-GIP	Small Intestine	1: 500	Overnight at RT	T4340 Bachem, Switzerland

This step was performed according to the methods proposed by Zhao et al. (2008) to inhibit the binding of the second primary antibody to the goat anti-rabbit IgG used in the first sequence. For the second step of the double immunofluorescence procedure, the sections were treated with other antibodies, as detailed in Table 1. After a washing step with the TBS, the sections were treated with 9 µg/ml DyLight®594 anti-rabbit IgG or DyLight®594 anti-mouse IgG (Vector Laboratories Inc.) for 1 h at room temperature or with the appropriate secondary biotinylated antibody IgG (Vector Laboratories Inc.) 10 µg/ml in TBS for 1 h at RT. In this last case, after rinsing in TBS, the sections were treated with DyLight®594 streptavidin

(Vector Laboratories Inc.), 10 µg/ml in 10 mM HEPES, 0.15 M NaCl, pH 7.5, 0.08% sodium azide, for 1 h at RT. Finally, the sections were embedded in Vectashield Mounting Medium (Vector Laboratories Inc.) and observed using a Confocal Laser Scanning Microscope (FluoView FV300, Olympus, Italy). The immunoreactive structures were excited using Argon/Helio-Neon-Green lasers with the excitation and barrier filters set for the used fluorochromes. Images showing the superimposition of the fluorescence were obtained by sequentially acquiring an image slice from each laser excitation or channel. The specificity of the double immunofluorescence was verified by incubating sections with PBS instead of the second antibody as second step of procedure. The results of these controls were negative (i.e. staining was abolished).

#### *Protein extraction and Western blot*

The samples were homogenized in a 2 ml solution containing ice-cold lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40, pH 7.4) supplemented with a protease inhibitor cocktail (EuroClone, Italy). The homogenates were centrifuged at 10,000 g for 5 min to discard cellular debris, and the supernatant was collected. The protein concentration of each sample was determined using a BCA protein assay (EuroClone). After addition of 0.05% bromophenol blue, 10% glycerol, and 2% β-mercaptoethanol, 50 µg of each sample was boiled and loaded onto 15% SDS-polyacrylamide gel, and Western blot tests were performed by separating the proteins with SDS-polyacrylamide gel electrophoresis using 15% Tris-Glycine polyacrylamide gel. The proteins were transferred onto nitrocellulose membranes (cod 126-0146, Bio-Rad Laboratories, Italy) that were incubated with 5% non-fat milk for 1 hour at room temperature to block the non-specific sites. The membranes were then incubated with primary polyclonal rabbit anti-ghrelin antibody (Phoenix Pharmaceuticals Inc.) and polyclonal anti-GAPDH antibody (sc-137179, Santa Cruz Biotechnologies, Inc., USA) for 2 hours at RT. Next, the membranes were washed and incubated for 1 hour at RT with a horseradish peroxidase-conjugated secondary antibody (1:5000, Bio-Rad Laboratories, Italy). The blots were developed using the ECL system (GE Healthcare, Italy): immunoreactivity was detected by chemiluminescence autoradiography according to the manufacturer's instructions.

#### *Statistical analysis*

A statistical analysis of the obtained quantitative data were performed using the general linear model of the SAS (v 8.1, Cary Inc., NC, USA). Histometrical analyses (cells counts) were analyzed by ANOVA using the PROC MIXED of the SAS package. The mixed model included the fixed effects of the age and the random effect of the pig. The individual pig values were considered to be

the experimental unit of all the response variables. The data were presented as least squared means  $\pm$  pooled SE. Differences between means were considered significant at  $P < 0.05$ .

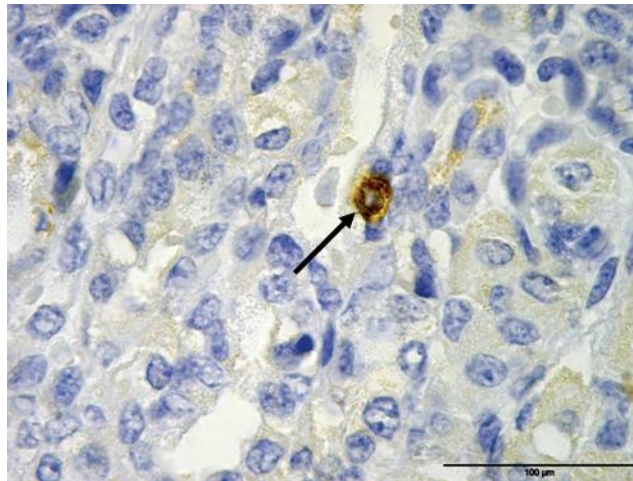
## Results

The structure of the different swine gastrointestinal organs was judged to be fully normal in the different studied ages.

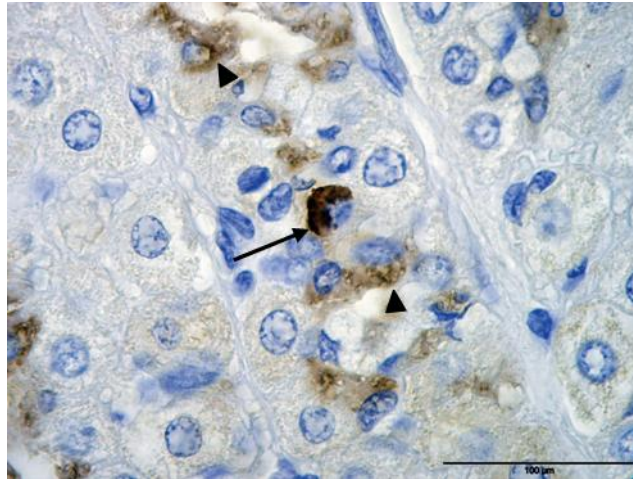
### *Immunohistochemistry and cell counts*

#### *Stomach*

In all the studied animals, ghrelin-immunoreactive (IR) endocrine cells were detected in all three glandular regions of the gastric mucosa. In the 1-day-old animals, the ghrelin-IR endocrine cells were localized in the lower half of the gastric glands in the oxyntic mucosa. In the older pigs (from 28 days to 7 months old), the gastric mucosa contained immunopositive endocrine cells, which were distributed from the neck to the base of the oxyntic, cardiac or pyloric glands. In all the glandular regions of the gastric mucosa, the cells were small and roundish and showed the typical morphology of closed-type endocrine cells, which were devoid of elongation contacting the lumen (Figs. 1, 2).



**Fig. 1.** Oxyntic mucosa of a 1-day-old animal. One ghrelin-IR endocrine cell is small and roundish and shows the typical morphology of the closed-type endocrine cells (arrow).

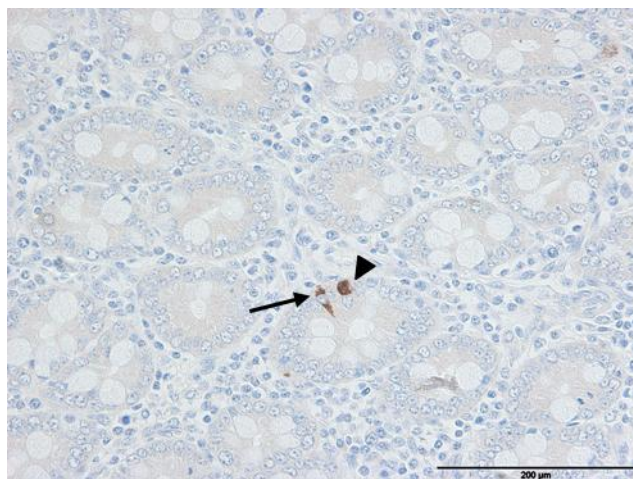


**Fig. 2.** Oxyntic mucosa of a 1-month-old animal, showing one closed-type ghrelin-IR endocrine cell (arrow). Ghrelin-immunopositivity is present also in some exocrine cells (arrow head).

In addition, the oxyntic mucosa of the animals aged from 28 days to 7 months showed a number of exocrine cells that displayed ghrelin-immunopositivity (Fig. 2).

#### *Intestine*

Ghrelin-IR endocrine cells were observed in the lower half of the intestinal glands all along the small intestine and caecum. Few endocrine cells were found in the villi of the duodenum, jejunum, and ileum. No ghrelin-IR endocrine cells were detected in the ascending colon and rectum mucosa. The intestinal ghrelin-IR endocrine cells exhibited both closed-type and lumen-contacting open-type cells (Fig. 3).



**Fig. 3.** Duodenal mucosa of a 2-month-old animal, showing intestinal ghrelin-IR endocrine cells of the closed-type (arrow head) and lumen-contacting open-type (arrow).



### Histometry

The immunoreactive cell count data are presented in Table 2 .

Table 2. Density of ghrelin-immunopositive endocrine cells (n/mm<sup>2</sup> of mucosa) in the pig stomach and intestine at different ages.

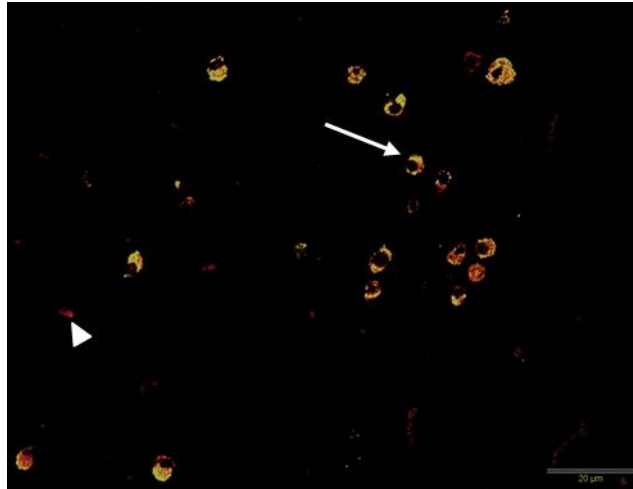
<b>Age: Measure:</b>	<b>1 day</b>	<b>28 days</b>	<b>2 months</b>	<b>4 months</b>	<b>7 months</b>	<b>Poo led SE</b>	<b>P value</b>
<u>Body Weight (Kg)</u>	1.16 <sup>c</sup>	8.07 <sup>d</sup>	39.78 <sup>c</sup>	74.83 <sup>b</sup>	123.56 <sup>a</sup>	1.81	<0.01
<u>Stomach</u>							
Cardias	-	51.89	45.38	50.31	43.74	5.26	0.653
Corpus	41.74 <sup>b</sup>	97.22 <sup>a</sup>	87.38 <sup>a</sup>	85.21 <sup>a</sup>	84.79 <sup>a</sup>	4.71	<0.01
Pylorus	-	55.19	57.14	46.10	53.25	4.64	0.386
<u>Small intestine</u>							
Duodenum							
Jejunum							
Ileum							
	20.52	28.84	27.88	26.11	23.94	5.94	0.864
	1.54	3.31	4.56	4.27	4.89	1.83	0.704
	0.87	1.73	0.92	0.76	0.81	0.99	0.948
<u>Large intestine</u>							
Caecum							
Ascending colon	<1	<1	<1	<1	<1	-	0.99
Rectum	0	0	0	0	0	-	-
	0	0	0	0	0	-	-

<sup>1</sup>Values are means  $\pm$  Pooled SE; n = 120 for Cardias and Pylorus, n = 150 for remaining tracts.

histometrical analysis revealed that the largest number of ghrelin-IR endocrine cells was present in the oxyntic mucosa. The next-to-largest numbers were observed in the cardias, the pylorus, and the duodenum in all the studied animals (from 1-day-old to 7-months-old). Small numbers of ghrelin-IR endocrine cells were observed in the jejunum and ileum, and they were scarcest in the caecum. None were detected in the ascending colon and rectum. Moreover, the cell count showed that the density of the ghrelin-IR endocrine cells was lowest in the oxyntic mucosa of the 1-day-old animals than in the other age groups (P<0.01).

### Double immunofluorescence

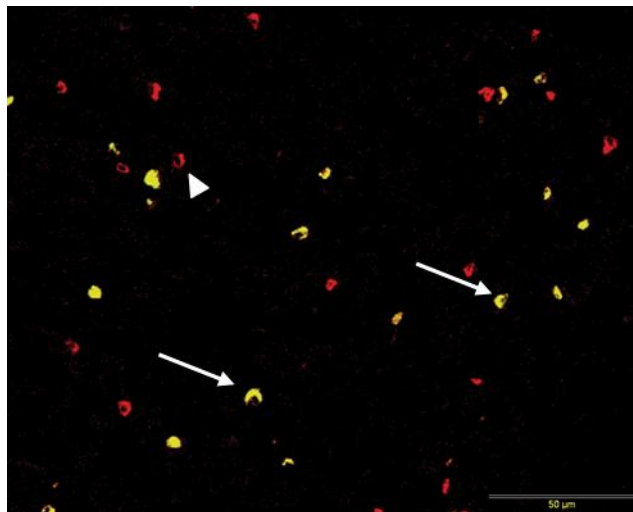
Double immunofluorescence showed that all the ghrelin-IR endocrine cells were also immuno-positive for chromogranin-A (but not *vice versa*) (Fig. 4).



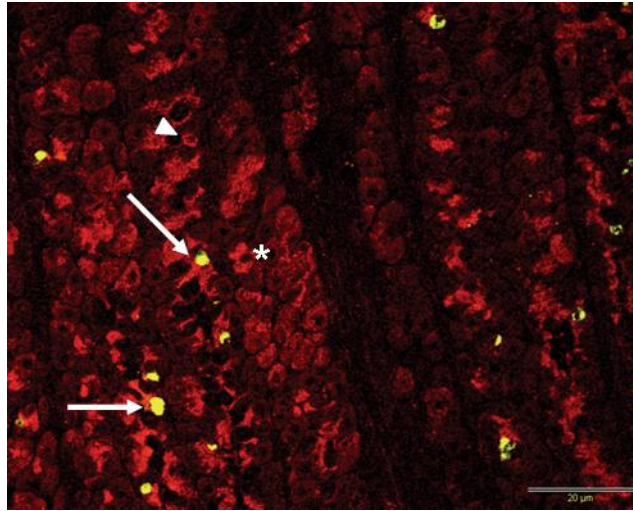
**Fig. 4.** Pyloric mucosa of a 28-day-old animal, showing double immunofluorescence for ghrelin (green) and chromogranin-A (arrow head, red). Here, ghrelin cells show the typical morphology of the closed-type cells (arrow) only and all ghrelin-IR cells are positive also for chromogranin-A (arrow, yellow) confirming their endocrine nature.

*Stomach*

Double labeling for ghrelin and somatostatin (Fig. 5), as well as for ghrelin and histidine decarboxylase (HDC) showed that ghrelin co-localized totally with somatostatin and partially with HDC (Fig. 6), the marker of histamine-secreting endocrine cells.



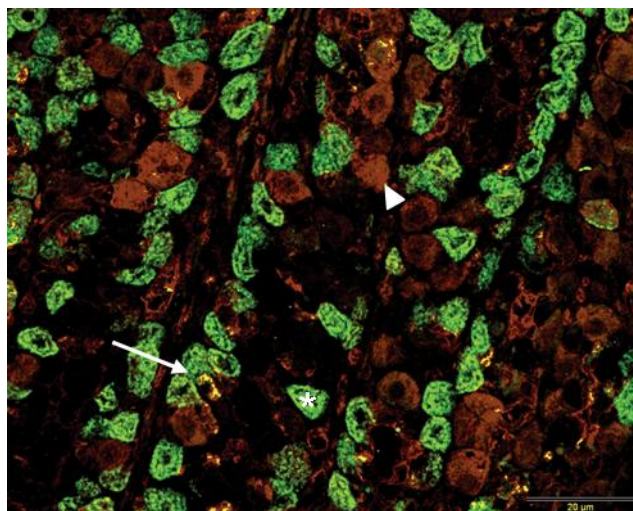
**Fig. 5.** Pyloric mucosa of a 4-month-old animal showing double immunofluorescence for ghrelin (green) and somatostatin (red). Ghrelin-immunoreactivity totally co-localizes with somatostatin-immunoreactivity (arrow, yellow) but some somatostatin-IR endocrine cells are not positive for ghrelin (arrow head).



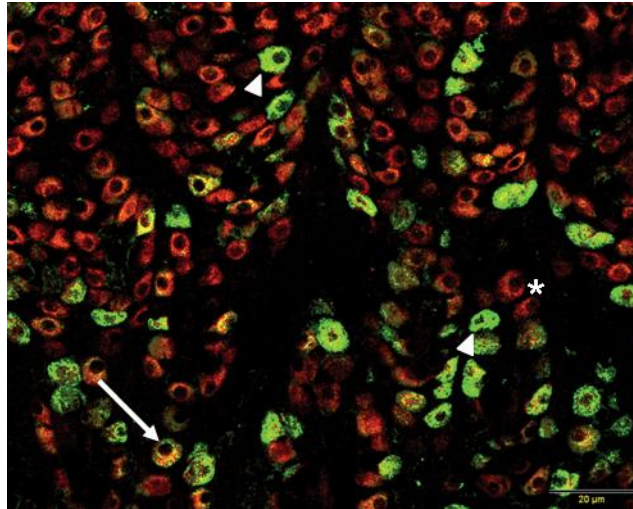
**Fig. 6.** Oxyntic mucosa of a 7-month-old animal, showing double immunofluorescence for ghrelin (red) and HDC (green). In oxyntic mucosa is evident that ghrelin immunoreactivity is present in both gastric endocrine (arrow and arrow head) and exocrine cells (asterisk). Ghrelin-immunoreactivity partially co-localizes with HDC-immunoreactivity (arrow, yellow).

Conversely, ghrelin never co-localized with serotonin, showing that the ghrelin-secreting cells constitute an endocrine cell type separate from the type secreting serotonin (EC cell).

Double immunofluorescence tests for ghrelin antibody versus both pepsinogen (principal cell marker) (Fig. 7) and H-K-adenosin triphosphatase ( $H^+/K^+$ -ATPase, parietal cell marker) (Fig. 8) antibody were performed.



**Fig. 7.** Oxyntic mucosa of a 4-month-old animal, showing double immunofluorescence for ghrelin (green) and  $H^+/K^+$ -ATPase (red) that identify parietal cells (arrow head). Ghrelin-immunoreactivity is evidenced in gastric exocrine cells (asterisk) and partially co-localizes with  $H^+/K^+$ -ATPase-immunoreactivity (arrow, yellow).

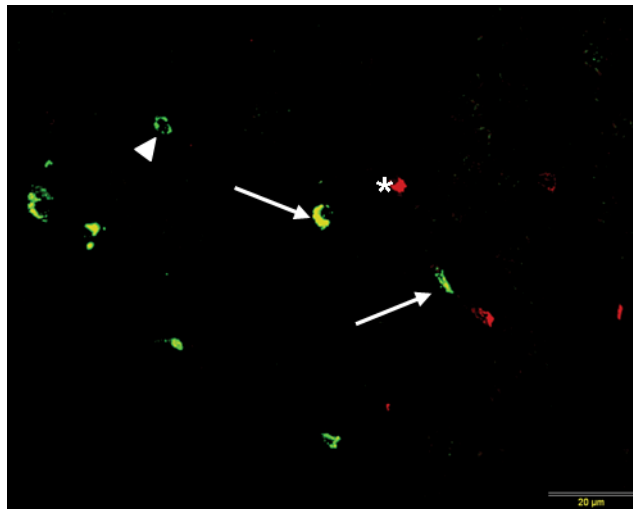


**Fig. 8.** Oxyntic mucosa of a 4-month-old animal, showing double immunofluorescence for ghrelin (green) and pepsinogen (red) that identify principal cells (arrow head). Ghrelin-immunoreactivity is evidenced in gastric exocrine cells (asterisk) and partially co-localizes with pepsinogen-immunoreactivity (arrow, yellow).

These reactions showed that the ghrelin immunoreactivity partially co-localized with  $H^+/K^+$ -ATPase-immunoreactivity and, to a lesser extent, with pepsinogen-immunoreactivity. This was true for all the studied ages, except for 1-day-old animals.

#### *Intestine*

In the mucosa of the small intestine, the ghrelin-IR endocrine cells completely co-localized with somatostatin and partially co-localized with gastric inhibitory polypeptide (GIP) in both closed- and open-type endocrine cells (Fig. 9).

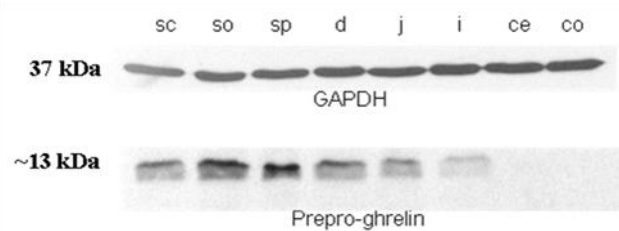


**Fig. 9.** Duodenal mucosa of a 4-month-old animal, showing double immunofluorescence for ghrelin (arrow head, green) and GIP (asterisk, red). Some ghrelin-IR endocrine cells, both closed and open-type, are positive to GIP also (arrow, yellow).

The ghrelin-IR endocrine cells did not co-localize with the serotonin antibody. No co-localization was observed in the intestinal mucosa of the large intestine.

#### Western blot analysis

Western blot analysis revealed a specific band at 13 kDa, which identified a ghrelin preproform expression (Fig. 10).



**Fig. 10.** Western blot analysis reveals a specific band at 13kDa in the different gut organs. (Sc= stomach, cardiac mucosa; So= stomach, oxyntic mucosa; Sp= stomach, pyloric mucosa; D= duodenum mucosa; J= jejunum mucosa; I= ileum mucosa; Ce= caecum mucosa; Co= colon mucosa)

According Dong et al., (2009) porcine ghrelin is the 25-52 peptide segment of the preproghrelin.

### **Discussion**

In all the studied mammalian species, ghrelin-secreting cells in peripheral (other than central nervous system) localizations are largely predominant in the stomach (Date et al., 2000; Tomasetto et al., 2000; Dornonville de la Cour et al., 2001; Hayashida et al., 2001; Rindi et al., 2002).

In the present study, we performed a thorough mapping and quantification of cell distribution in the porcine gastrointestinal tract at different ages. We confirmed that the greatest number of ghrelin-secreting endocrine cells was present in the oxyntic mucosa, where they were distributed from the neck to the base of the glands. In the cardiac and pyloric glands, the distribution of ghrelin-secreting endocrine cells was similar in all pigs at different ages, as was also observed by Hayashida et al. (2001). In addition, ghrelin-secreting endocrine cells were observed in the small intestine mucosa, especially in the duodenum and, to a lesser extent, in the large intestine (caecum) as well, indicating that the intestine (principally the duodenum) is a considerable additional source of ghrelin from birth. The density of ghrelin immunopositive endocrine cells along the different tracts of the alimentary canal in different

mammalian species, as well as at different ages, was found to be variable (Kotunia and Zabielski, 2006). Zhao and Sakai (2008) reported that gastric ghrelin cell density in male rats increased in an age-dependent manner after birth, and the increase was in concert with an increase in the ghrelin expression level. In addition, Fak et al. (2007) suggested a positive relation between the gastric ghrelin expression, body weight and solid food intake in rats at weaning, and Reynolds et al. (2010) showed the influence of a feeding pattern on ghrelin plasma concentrations in pigs.

When we compared the different age groups, ghrelin-immunostained cells were shown to be present in the lowest number in the oxyntic mucosa of 1-day-old pigs. Different studies have shown that the density of ghrelin endocrine cells in the stomach of rats and humans is low until the weaning period when the ghrelin cell population is greatly expanded (Bjorqvist et al. 2002; Hayashida et al., 2002; Wierup et al., 2002; Wierup et al., 2004). Similarly, we have observed a significant increase in ghrelin cell density in the oxyntic and intestinal mucosa of 28-day old animals, and this is the approximate age for weaning in reared swine. Fak et al. (2007) have demonstrated that the increase in gastric ghrelin expression seen in rats at weaning was associated with the onset of solid food intake and not only to age.

Fak et al. (2007) have also demonstrated a relationship between the ghrelin cell density in the oxyntic mucosa and body weight development. The amount of ghrelin cells in the glandular part of the stomach increased in an age-dependent manner from neonatal to adult stages in male rats (Zhao and Sakai, 2008). Here we have shown that this does not occur in swine after weaning. In our study, the mucosal ghrelin cell density did not change significantly after weaning in any tracts or in relation to age. This suggests that ghrelin expression may not be tightly and positively linked to age and body weight in swine. In humans, a number of studies have demonstrated a negative correlation between plasma ghrelin levels and some anthropometric indices, such as birth weight, body length or ponderal index, revealing that circulating ghrelin concentrations progressively increase during the first 2 years of life and then decrease until puberty (Kitamura et al., 2003; Soriano-Guillen et al., 2004). The trend of ghrelin cell distribution in the pig gastrointestinal tract during the period analyzed in this experiment (between the first day of life and age 7 months) looks similar to the trend of ghrelin plasma levels observed in humans.

We have observed that only the closed type of ghrelin-secreting endocrine cells are found in the stomach, and both closed- and open-types are found in the intestine. It is well known that open-type endocrine cells of the gastrointestinal tract require regulation by luminal signals, whereas the activity of closed-type endocrine cells are supposedly modulated by hormones,

neuronal stimulations or mechanical distention (Solcia et al., 2000). It is possible that ghrelin-secreting cells may be modulated by different stimulators and may play different physiological roles in various regions of the swine gastrointestinal tract.

In this study, double immunostaining tests were performed to examine the possible presence of co-localizations. As observed in the gastrointestinal tracts of humans and rats (Date et al. 2000, Dornonville de la Cour et al. 2001), ghrelin-secreting cells are positive for chromogranin-A, confirming their endocrine nature. Ghrelin and ghrelin-mRNA have been well documented in gastric endocrine cells, and previous studies using *in situ* hybridization have shown the endocrine nature of ghrelin-producing cells in the gastrointestinal tracts of rats, humans and pigs (Date et al. 2000; Wierup et al., 2007). In humans and rodents, the ghrelin-secreting endocrine cells are distinct from other better-known endocrine cell types of the mammalian gastrointestinal tract, namely, the histamine-producing enterochromaffin-like (ECL) cells, the somatostatin-producing D cells, and the serotonin-producing enterochromaffin (EC) cells (Date et al., 2000; Rindi et al., 2002). Ghrelin immuno-positive cells were mainly observed in the mammalian oxyntic mucosa, where were detected between the neck and the base of the oxyntic glands (Rindi et al., 2004). Ghrelin is stored in the secretory granules of gastric endocrine cell types, which have been identified as P/D1-type cells in humans, A-like types in rats, and X-types in dogs (Rindi et al., 2002). In addition, in rat and human gastric mucosa, ghrelin immunopositive cells have been shown to be a rather abundant separate cell type, independent from the endocrine somatostatin (D)-, serotonin (enterochromaffin, EC)- and histamine (enterochromaffin-like, ECL)-producing cells (Date et al., 2000; Rindi et al., 2002).

In the present study, we have shown that ghrelin-secreting endocrine cells are also involved in the synthesis of either somatostatin (stomach, intestine) or GIP (intestine) and that a partial co-localization exists with the histamine-secreting gastric ECL endocrine cells. The localization of different gastrointestinal peptides in the same endocrine cell type (possibly in different secretory granules) indicates that endocrine cells may differ in the way they modulate their response to either chemical or mechanical factors.

Our data do not fully agree with that found by Wierup et al. (2007). They presented data indicating that ghrelin was not co-localized with glucagon, somatostatin, CCK, GIP, secretin, serotonin or neurotensin in the gastrointestinal tract of 2-month-old pigs, showing that ghrelin peptide and mRNA expression were confined to a distinct cell type. A possible reason for these discrepancies may be related to different technical conditions, as well as gender-related and fasting-versus-feeding conditions.

The bulk of the ghrelin cells in the acid-producing mucosa of the stomach suggest that ghrelin may play a role in the control of gastric secretion (Sakata et al., 2002). In fact, ghrelin has been reported to participate in the central regulation of gastric acid secretion, although this effect is still under debate. Some authors (Masuda et al., 2000; Date et al., 2001) supported the notion that ghrelin stimulates basal gastric acid secretion in rats, while Sibilia et al. (2002) have in the same species demonstrated that ghrelin plays an inhibitory role in the central regulation of gastric acid secretion. Surprisingly, we have observed the presence of ghrelin-immunopositivity in both the parietal and, to a lesser extent, the principal cells of the gastric oxyntic mucosa, suggesting a process of ghrelin internalization in these exocrine cell types. We can consequently hypothesize that ghrelin could act locally to regulate gastric secretion without involving the central nervous system. Du et al. (2007) have recently demonstrated that cultured gastric mucosal cells from weaning piglets increased H<sup>+</sup>-K<sup>+</sup>-ATPase activity immediately after exposure to ghrelin. In addition, ghrelin presence was immunohistochemically identified in the parietal cells of the oxyntic mucosa in Mongolian gerbils by Suzuki et al. (2004). Other authors (Tanaka-Shintani and Watanabe, 2005) have shown that the distribution of ghrelin-immunoreactivity in the human stomach was positively correlated with one of the parietal cells.

In conclusion, the present study suggests that the presence and distribution of gastric ghrelin-secreting endocrine cells may be dependent on age and weaning (with the introduction of solid feed) in the pig. In the swine gastrointestinal tract, ghrelin is co-stored in the same cell type as other gastrointestinal peptides, suggesting that ghrelin acts in concert with them, even if acting on different targets. Given that ghrelin plays an important role in the regulation of food intake and adiposity, which are key aspects for pig production as a food animal species, further studies are necessary to elucidate the influence of the duration of breastfeeding upon ghrelin expression and also the regulatory mechanisms of ghrelin in different gastrointestinal functions in this species.

## References

- Ariyasu H, Takaya K, Tagami T, Ogawa Y, Hosoda K, Akamizu T, Suda M, Koh T, Natsui K, Toyooka S, Shirakami G, Usui T, Shimatsu A, Doi K, Hosoda H, Kojima M, Kangawa K, Nakao K . 2001. Stomach is a major source of circulating ghrelin, and feeding state determines plasma ghrelin-like immunoreactivity levels in humans. *J Clin Endocrinol Metab* 86: 4753-4758.
- Björkqvist M, Dornonville de la Cour C, Zhao CM, Gagnemo-Persson R, Håkanson R, Norlén P. 2002. Role of gastrin in the development of gastric



mucosa, ECL cells and A-like cells in newborn and young rats. *Regul Pept* 108: 73-82.

Casanueva FF, Dieguez C. 2002. Ghrelin: the link connecting growth with metabolism and energy homeostasis. *Rev Endocr Metab Disord* 3: 325-338.

Date Y, Kojima M, Hosoda H, Sawaguchi A, Mondal MS, Suganuma T, Matsukura S, Kangawa K, Nakazato M. 2000. Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. *Endocrinology*. 141: 4255-4261.

Date Y, Nakazato M, Murakami N, Kojima M, Kangawa K, Matsukura S. 2001. Ghrelin acts in the central nervous system to stimulate gastric acid secretion. *Biochem Biophys Res Commun* 280: 904-907.

Dong X-Y, Xu J, Tang S-Q, Li H-Y, Jiang Q-Y, Zou X-T. 2009. Ghrelin and its biological effects on pigs. *Peptides* 30: 1203-1211.

Dornonville de la Cour C, Björkqvist M, Sandvik AK, Bakke I, Zhao CM, Chen D, Håkanson R. 2001. A-like cells in the rat stomach contain ghrelin and do not operate under gastrin control. *Regul Pept* 99: 141-150.

Du GM, Liu MJ, Shi ZM, Zhang L, Wei XH, Zhao RQ. 2006. In vitro effect of ghrelin  $H^+ -K^+ -ATP$  ase and pepsin activity and mRNA expression of gastrin, somatostatin, receptors for GH and IGF-1 in cultured gastric mucosal cells of weanling piglets. *Anim Sci* 82: 823-828.

Du GM, Shi ZM, Wei XH, Liu MJ, Zhang L, Zhao RQ. 2007. Expression of gastric ghrelin and  $H^+ -K^+ -ATP$ ase mRNA in weanling piglets and effect of ghrelin on  $H^+ -K^+ -ATP$ ase expression and activity in gastric mucosal cells in vitro. *Res Vet Sci* 82: 99-104.

Fåk F, Friis-Hansen L, Weström B, Wierup N. 2007. Gastric ghrelin cell development is hampered and plasma ghrelin is reduced by delayed weaning in rats. *J Endocrinol*. 192: 345-352.

Ferrini F, Salio C, Lossi L, Merighi A. 2009. Ghrelin in central neurons. *Curr Neuropharmacol* 7: 37-49.

Hayashida T, Murakami K, Mogi K, Nishihara M, Nakazato M, Mondal MS, Horii Y, Kojima M, Kangawa K, Murakami N. 2001. Ghrelin in domestic animals: distribution in stomach and its possible role. *Domest Anim Endocrinol* 21: 17-24.

Hayashida T, Nakahara K, Mondal MS, Date Y, Nakazato M, Kojima M, Kangawa K, Murakami N. 2002. Ghrelin in neonatal rats: distribution in stomach and its possible role. *J Endocrinol* 173: 239-245.

Horvath TL, Diano S, Sotonyi P, Heiman M, Tschöp M. 2001. Minireview: ghrelin and the regulation of energy balance--a hypothalamic perspective. *Endocrinology* 142: 4163-4169.

- Kim MS, Yoon CY, Jang PG, Park YJ, Shin CS, Park HS, Ryu JW, Pak YK, Park JY, Lee KU, Kim SY, Lee HK, Kim YB, Park KS. 2004. The mitogenic and antiapoptotic actions of ghrelin in 3T3-L1 adipocytes. Mol Endocrinol 18: 2291-2301.
- Kitamura S, Yokota I, Hosoda H, Kotani Y, Matsuda J, Naito E, Ito M, Kangawa K, Kuroda Y. 2003. Ghrelin concentration in cord and neonatal blood: relation to fetal growth and energy balance. J Clin Endocrinol Metab 88: 5473-5477.
- Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. 1999. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 402: 656-660.
- Kojima M, Kangawa K. 2005. Ghrelin: structure and function. Physiol Rev 85: 495-522.
- Kotunia A, Zabielski R. 2006. Ghrelin in the postnatal development of the gastrointestinal tract. J Physiol Pharmacol 57: 97-111.
- Leite-Moreira AF, Soares JB. 2007. Physiological, pathological and potential therapeutic roles of ghrelin. Drug Discov Today 12: 276-288.
- Masuda Y, Tanaka T, Inomata N, Ohnuma N, Tanaka S, Itoh Z, Hosoda H, Kojima M, Kangawa K. 2000. Ghrelin stimulates gastric acid secretion and motility in rats. Biochem Biophys Res Commun 276, 905-908.
- Muccioli G, Pons N, Ghè C, Catapano F, Granata R, Ghigo E. 2004. Ghrelin and des-acyl ghrelin both inhibit isoproterenol-induced lipolysis in rat adipocytes via a non-type 1a growth hormone secretagogue receptor. Eur J Pharmacol 498: 27-35.
- Reynolds CB, Elias AN, Whisnant CS. 2010 Effects of feeding pattern on ghrelin and insulin secretion pigs. Domest Anim Endocrinol 39: 90-96.
- Rindi G, Necchi V, Savio A, Torsello A, Zoli M, Locatelli V, Raimondo F, Cocchi D, Solcia E. 2002. Characterisation of gastric ghrelin cells in man and other mammals: studies in adult and fetal tissues. Histochem Cell Biol 117: 511-519.
- Rindi G, Torsello A, Locatelli V, Solcia E. 2004. Ghrelin expression and actions: a novel peptide for an old cell type of the diffuse endocrine system. Exp Biol Med Maywood 229: 1007-1016.
- Sakata I, Tanaka T, Matsubara M, Yamazaki M, Tani S, Hayashi Y, Kangawa K, Sakai T. 2002. Postnatal changes in ghrelin mRNA expression and in ghrelin-producing cells in the rat stomach. J Endocrinol 174: 463-471.
- Salfen BE, Carroll JA, Keisler D,H, Strauch TA. 2004. Effects of exogenous ghrelin on feed intake, weight gain, behaviour, and endocrine responses in weanling pigs. J Anim Sci 82: 1957-1966.
- Sibilia V, Pagani F, Guidobono F, Locatelli V, Torsello A, Deghenghi R, Netti C. 2002. Evidence for a central inhibitory role of growth hormone

secretagogues and ghrelin on gastric acid secretion in conscious rats. *Neuroendocrinology* 75: 92-97.

Solcia E, Rindi G, Buffa R, Fiocca R, Capella C. 2000. Gastric endocrine cells: types, function and growth. *Regul Pept* 93: 31-35.

Soriano-Guillén L, Barrios V, Chowen JA, Sánchez I, Vila S, Quero J, Argente J. 2004. Ghrelin levels from fetal life through early adulthood: relationship with endocrine and metabolic and anthropometric measures. *J Pediatr* 144: 30-35.

Suzuki H, Masaoka T, Hosoda H, Ota T, Minegishi Y, Nomura S, Kangawa K, Ishii H. 2004. Helicobacter pylori infection modifies gastric and plasma ghrelin dynamics in Mongolian gerbils. *Gut* 53: 187-194.

Takaya K, Ariyasu H, Kanamoto N, Iwakura H, Yoshimoto A, Harada M, Mori K, Komatsu Y, Usui T, Shimatsu A, Ogawa Y, Hosoda K, Akamizu T, Kojima M, Kangawa K, Nakao K. 2000. Ghrelin strongly stimulates growth hormone release in humans. *J Clin Endocrinol Metab* 85: 4908-4911.

Tanaka-Shintani M, Watanabe M. 2005. Distribution of ghrelin-immunoreactive cells in human gastric mucosa: comparison with that of parietal cells. *J Gastroenterol* 40: 345-349.

Thompson NM, Gill DA, Davies R, Loveridge N, Houston PA, Robinson IC, Wells T. 2004. Ghrelin and des-octanoyl ghrelin promote adipogenesis directly in vivo by a mechanism independent of the type 1a growth hormone secretagogue receptor *Endocrinology* 145: 234-242.

Tomasetto C, Karam SM, Ribieras S, Masson R, Lefèbvre O, Staub A, Alexander G, Chenard MP, Rio MC. 2000. Identification and characterization of a novel gastric peptide hormone: the motilin-related peptide *Gastroenterology* 119: 395-405.

Tschöp M, Smiley DL, Heiman ML. 2000. Ghrelin induces adiposity in rodents. *Nature* 407: 908-913.

van der Lely AJ, Tschöp M, Heiman ML, Ghigo E. 2004. Biological, physiological, pathophysiological, and pharmacological aspects of ghrelin *Endocr Rev* 25: 426-457.

Wierup N, Svensson H, Mulder H, Sundler F. 2002. The ghrelin cell: a novel developmentally regulated islet cell in the human pancreas. *Regul Pept.* 107: 63-69.

Wierup N, Yang S, McEvelly RJ, Mulder H, Sundler F. 2004. Ghrelin is expressed in a novel endocrine cell type in developing rat islets and inhibits insulin secretion from INS-1 (832/13) cells. *J Histochem Cytochem* 52: 301-310.

Wierup N, Björkqvist M, Weström B, Pierzynowski S, Sundler F, Sjölund K. 2007. Ghrelin and motilin are cosecreted from a prominent endocrine cell population in the small intestine. *J Clin Endocrinol Metab* 92: 3573-3581.

Zhao CM, Furnes MW, Stenström B, Kulseng B, Chen D. 2008. Characterization of obestatin- and ghrelin-producing cells in the gastrointestinal tract and pancreas of rats: an immunohistochemical and electron-microscopic study. *Cell Tissue Res* 331: 575-587.

Zhao Z,, Sakai T. 2008. Characteristic features of ghrelin cells in the gastrointestinal tract and the regulation of stomach ghrelin expression and production. World J Gastroenterol 14: 6306-63

## **CHAPTER 9**

# **Acknowledgements**

## 9. Acknowledgements

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