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Effects of melon pulp rich in superoxide dismutase on piglets and poultry health

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CHAPTER 1

Foreword

Piglets weaning

Weaning pigs from the sow is one of the most stressful events in the pig's life that can contribute to intestinal and immune system dysfunctions. Weaning stress result in reduced pig health, growth, and feed intake, particularly during the first week after weaning. Modern technological improvements such as housing, nutrition, health, and management have been used to reduce some of the adverse effects of weaning stress, but a better understanding of the biological effect of stress is needed to improve approaches to overcome weaning stress in piglets.

Stressors during weaning

When it is weaned from the sow, the pig experiences significant physiological, environmental, and social challenges that can influence the pig to consequent diseases and other production and economic losses. Weaning is one of the most stressful periods in piglets which causes in gastro-intestinal, immunological, and behavioural changes. During this period, pigs are subjected to a number of stressors, for examples, an sudden separation from the sow, transportation and handling stress, a different food source, social hierarchy stress, co-mingling with pigs from other litters, a different physical environment (room, building, farm, water supply, etc.), increase number of exposure to pathogens, and dietary or environmental antigens. The piglet must familiarize to all above those stressors rapidly in order to be productive and efficient. When the different stressors of weaning are too higher for the pig to overcome, it can subsequently lead to poor growth performance and increased mortality.

Feed intake and weaning

The gastro-intestinal system has various functions, for example, digestion and absorption of nutrients and electrolytes, maintenance of bodily fluid balance, secretion of different digestive enzymes, mucin, immunoglobulins, and multiple other components, and to act as a barrier for the host to prevention of harmful pathogens and antigens.

When the piglet is weaned, the piglet must adapt abruptly from highly digestible and palatable liquid milk from its mother. That liquid milk is equally spaced throughout the day to a solid dry diet, which is more or less digestible and palatable. As a result, feed intake is usually decreased primarily after weaning and the piglet becomes mal-nourished with decreased transient growth performance rate. As reviewed by Le Dividich and Seve (2000), the amount and duration of reduced feed intake is inconstant. It is projected that by the end of the first week post-weaning,

metabolizable energy (ME) intake about 60-70% of pre-weaning milk intake and that it takes is approximately 2 weeks post-weaning to achieve full recovery to the pre-weaning ME intake level. There are the interrelationship between low feed intake with different diet compositions (lactose/protein ratios) and small intestinal barrier function (Spreeuwenberg et al., 2001). They estimated during the first 4 d post-weaning, that diet composition was not as important a factor to maintain intestinal barrier function; but that continued low feed intake was more important to influence the pig to intestinal barrier dysfunction. McCracken et al., (1999) determined that low feed intake during the post-weaning period may contribute to cause of intestinal inflammation and harmfully affect intestinal villous height and crypt depth.

Growth performance is reduced owning to low feed intake. In general, pigs lose about 100–250 g body weight (BW) the first day after weaning, regardless of weaning age and recover this loss in BW by about 4 d post-weaning (Le Dividich and Seve, 2000). Tokach et al., (1992) reported that body weight gain in the first week after weaning influences the total days to market (at approximately 110 kg BW). When pigs were gaining greater than 227 g/d during the first week after weaning, days to market was reduced about 6–10 d compared to pigs gaining 0 g/d to 150 g/d the first week.

Therefore, it is important to get pigs eating and growing as soon as possible after weaning. It is difficult to prevent some of the decline in BW as the pigs move from sow's milk to the starter diet. However, understanding the challenges and impact of low feed intake associated with weaning and the consequent influence on growth performance can help the nutritionist better design diets by utilizing various feed ingredients and additives proven to increase feed intake and help the producer to use other management techniques that help to reduce the weaning stress.

Structure and functional changes of intestine and weaning

Along with experiencing low feed intake, weaned pigs experience intestinal physiological changes in structure and function, mainly enzyme activities and absorption or secretion. As reviewed by Pluske et al., (1997), these physiological changes cause the absorptive capacity of the small intestine which can likely impact on feed efficiency. Pluske et al., (1997) and Boudry et al., (2004) reported that weaning period affects both acute and long-lasting small intestinal structural and functional changes, specifically villous atrophy and crypt elongation after weaning. (Hampson, 1986) confirmed that villous height can quickly reduction by about 25 to 35% of pre-weaning height within the first 24 h in pigs weaned at 21 d of age. The reduction in villous height constant until about 5 d after weaning, when the villi were approximately only half of the initial height. But, unweaned groups exhibited only small changes in villous height. Crypt elongation was also assessed with slower changes occurring over the first 11 d post-weaning. Moreover, numerous markers of

intestine associated with weaning that may allow research to be conducted to decrease the physiological changes associated with weaning (Montagne et al., 2007). Pigs brush border digestive enzyme activities also reduced after weaning (Pluske et al., 1997). Reductions in lactase and amino-peptidase activity occurred from d 2 to 15 post-weaning, whereas maltase activity was decreased for 2 d post-weaning, then increased d 8 to 15 post-weaning (Jean-Paul et al., 2004). Furthermore pancreatic secretions of pig had a transient reduce to d 15 after weaning, before trypsin and amylase activity began to increase. Alkaline phosphatase which act as a role in detoxification of pathogenic bacterial lipopolysaccharide endotoxin and impacts intestinal inflammation (Lallès, 2010), is also decreased in early weaned pigs (Lackeyram et al., 2010). Finally, these alterations can influence the ability of the small intestine's digestive, absorptive, secretory capacity and intestinal barrier function, which may contribute to post-weaning diarrhoea.

Dietary management of GIT disorders post-weaning

Protein sources

In weaned pigs, skim-milk powder and whey are excellent but luxurious protein sources for supporting a high growth performance parameter in weaned pigs. Also, spray dried plasma (SDP) combined at levels of 4-6% into starter diets is commercial product stimulating post weaning growth rate and feed intake (van Dijk et al., 2001, Torrallardona et al., 2003, Torrallardona et al., 2007). Small intestinal structural and physiological alterations and incidence and severity of post weaning diarrhoea are often reduced. Porcine SDP is more effective than bovine SDP or SDP of mixed origin as palatability and beneficial effects of SDP-supplemented diets of both SDP origin are mostly associated to high levels of immunoglobulin G (Pierce et al., 2005). It is possible that IgG help to prevents adhesion to potentially pathogenic *Escherichia coli* to intestinal epithelial cells (IECs). Remarkably, SDP showed low levels of basal immune activation as seen both locally (Jiang et al., 2000, Bosi et al., 2004) and systemically in supplemented piglets (Touchette et al., 2002). The growth of intestinal lactobacilli may or may not stimulate by SDP supplementation (Torrallardona et al., 2003, Torrallardona et al., 2007). Lastly, improved dietary protein utilisation in SDP-supplemented pigs may result from reduced protein catabolism by the enteric microflora (Jiang et al., 2000).

Bovine colostrum supplementation for protecting pig GIT may act as a another valuable alternative to in-feed antibiotics. It showed to stimulate growth rate and voluntary feed intake post weaning (Luron et al., 2004). However, pair-feeding experiments demonstrated that most of the valuable effects on the GIT may be due to an increased feed intake since there were only significant effects of colostrum

supplementation on gastric pH (decreased) and duodenal lactobacilli to coliform ratio (increased) (Huguet et al., 2006).

Most of the feed protein sources in for piglets originate from grains and bean seeds. But, few plant protein sources may have antagonistic effects on piglets GIT health after weaning. This has been reported for pea protein isolates which cause increased diarrhoea and mortality post weaning (Owusu-Asiedu et al., 2003a, Owusu-Asiedu et al., 2003b). Plant protein sources, in comparison to dairy ingredients, also reduced the magnitude of beneficial effects observed with SDP supplementation (van Dijk et al., 2001). Inadequately treated soybean products are causing antigen specific immune-mediated gut disturbances in weaned pigs (Dréau and Lallès, 1999). More recent surveys have shown that seeds from cowpea, lupine, field pea may stimulate antibodies to dietary antigens in blood plasma (Salgado et al., 2002a) but this did not seem to affect digestion, intestinal mucosa architecture and enzyme activities (Salgado et al., 2001, Salgado et al., 2002b). Soybean protein concentrates combined at high levels at the expense of soybean meals may decrease the palatability of starter diets and, therefore, growth performance (Lenehan et al., 2007).

Energy sources

The energy source mainly, glucose, lactose and starch mixed in starter diets stimulated principally similar results for growth, feed intake and GIT characteristics (Vente-Spreeuwenberg and Beynen, 2003). But, increase levels of lactose acceptable increasing mixture of protein in weaning diets and stimulated growth rate and intestinal integrity (Pierce et al., 2007). In this case, most lactose run-away digestion in the small intestine which was fermented in the large intestine. Such a diet stimulated bifidobacteria and lactobacilli and reduced E. *coli* counts in gut. Furthermore, it decreased protein fermentation and generation of unwanted nitrogenous compounds, but increasing butyrate production and improving small intestinal and integrity and architecture (Pierce et al., 2007).

Amino acids

Amino acids (AA) show three distinct but complementary roles in the body and GIT, most of the AA acting as building blocks, some (e.g. glutamine) being fuels for specific (e.g. epithelial) cells in the body and some AA supporting important specific functions (e.g. threonine and GIT mucin). In all cases, essential AA need to be provided in amounts sufficient for covering pig's requirements.

Glutamine showed many times to improve pig performance and intestinal integrity while reducing the incidence of diarrhoea. Moreover, glutamine affects proliferation and reduces apoptosis of both intestinal cells and mucosal immune cells

(Domeneghini et al., 2004). Densities of intestinal macrophages and intra-epithelial lymphocytes were also increased which suggesting a stimulation of both innate and acquired components of intestinal mucosal immunity. The important component of mucin is a threonine and its requirements are increased in disease states or following consumption of diets stimulating mucin secretion (Bannink et al., 2006). Interestingly, dietary deficiency in threonine affects reduced intestinal barrier function and reduced protein and reduced mucin synthesis in young pigs (Hamard et al., 2007).

Organic acids

The beneficial effects of several organic acids (such as formic, fumaric, citric acids, calcium diformate) after weaning have shown in many publications (Mroz et al., 2006). They are helped for decreasing luminal pH in proximal GIT and for their bactericidal properties. Among organic acids, sodium butyrate (SB) has numerous beneficial properties in the large intestine when provided orally. Also, SB found to improve feed to gain ratio post weaning, to increase gastric DM content which suggesting a delayed gastric emptying rate, however to reduce ileal and faecal digestibility of organic matter and starch (Castillo et al., 2006, Manzanilla et al., 2006). The diversity and the composition of the jejunal microbiota also induced changes by butyrate with reduced colonic fermentation in the colon. The authors suggested an improved intestinal barrier function associated, at least partly with the observed microbial changes. SB providing piglets during the suckling period rather than after weaning was more effective for improving growth performance, feed intake and diet digestibility post weaning, and for reducing the weight of the intestinal mucosa (Gall et al., 2007). These results suggest a growth promoting effect for SB, but its mechanisms of action are currently unknown.

Prebiotics and probiotics

Providing appropriate fermentable carbohydrates diets helps manipulating the GIT microbiota towards beneficial fermentation profiles and improved resistance of enteric pathogens to colonization (Bauer et al., 2006). As mentioned above, high levels of dietary lactose does act as a prebiotic and was ability to improve both pig performance parameters and intestinal integrity (Pierce et al., 2007). A high level of lactose helped increasing the protein content of the diet and improved pig performance. By contrast, increasing dietary lactose already containing inulin, another fermentable carbohydrate, did not ameliorate the results obtained by the inulin diet. Supplementing weaning diets with carbohydrates of varying solubility and fermentability (inulin, lactose, sugar beet pulp, wheat starch) was revealed to induce

the growth of lactobacilli (e.g. *Lactobacillus sobrius*) and to improve colonic microbial stability and diversity post weaning (Konstantinov et al., 2004, Konstantinov et al., 2006).

Lactobacilli isolated from weaned pigs showed to reduce gut *E. coli* counts and diarrhoea (Huang et al., 2004). *Enterococcus faecium* intensely decreased the incidence of diarrhoea in the first week post weaning (Taras et al., 2006). A combination of probiotics (mainly lactobacilli) was capable to decrease diarrhoea and faecal shedding of Salmonella and to progress clinical consequence in weaned pigs (Casey et al., 2007). The creep supplemented with probiotic *E. coli Nissle* 1917 before weaning eradicated diarrhoea in weaned piglets challenged with pathogenic *E. coli*, decreased jejunal secretory capacity and prevented the reduced in para-cellular permeability as detected after pathogen challenge (Schroeder et al., 2006). Diet supplementation with live yeast improved pig performance and intestinal integrity post weaning (Bontempo et al., 2006).

Plant extracts and natural substances

Plant extracts and natural substances (e.g. essential oils) helped for controlling post weaning diarrhoea and GIT disorders (Han et al., 2006). For instance, various type of mixtures of essential oils and/or plant extracts increased GIT lactobacilli (Manzanilla et al., 2004) and/or reduced coliforms (Namkung et al., 2004) without major effects on pig performance parameters (Oetting et al., 2006). Therefore, more work is required on the bioavailability of such substances.

Since the beginning of the European ban on in-feed antibiotic growth promoters, many studies have investigated various nutritional approaches for preventing or reducing GIT disorders and post weaning diarrhoea in young pigs. It appears that two types of substances, namely spray dried plasma and organic acids represent the most satisfactory alternative solutions found thus far for limiting problems post weaning in the absence of preventive use of antibiotics. Other substances (e.g. butyrate) may be promising but more work is required for understanding better the underlying mechanisms. Studies with prebiotics and probiotics provide increased evidence for their beneficial effects on the pig and GIT homeostasis. Conversely, many plant extracts and natural substances display clear anti-bacterial effects in vitro but they rarely support consistent effects *in vivo*. Therefore more research is needed in this area for understanding these inconsistencies.

Inflammation associated with weaning

Weaning induce a harmful effect on intestinal barrier function with beyond the compromised digestive and absorptive capacity (Spreeuwenberg et al., 2001, Boudry et al., 2004, Moeser et al., 2007a). The intestinal epithelial layer acts as the body's first line of defence for protecting the pig from several harmful microorganisms, toxins, or antigens that exist within the lumen of the small intestine. When the intestinal barrier is disrupted which cause increased permeability that permits toxins, bacteria, or feed-associated antigens to cross the epithelium resulting in inflammation, mal-absorption, diarrhoea, and reduced growth and production.

Nabuurs et al., (1994) reported that the effects of weaning and *Escherichia coli* infection on absorption capacity of the small intestine in pigs. Pigs were either weaned at 30 to 32 d of age or unweaned by remaining on the sow, while *Escherichia coli* infection consisted of infecting segments of the small intestine using perfusion procedures. In unweaned non-infected pigs, no differences were noted in net absorption of fluid, potassium or chloride. However, pigs that had been weaned but not infected with *Escherichia coli* had less fluid absorption on d 4, 7, and 14, while sodium and chloride absorption was less on d 4 and 7 in the intestinal segments compared to segments from unweaned non-infected pigs. In the *Escherichia coli* infected intestinal segments of weaned pigs, fluid absorption was less on d 11 and 14, while sodium and potassium on d 11, and chloride absorption on d 4 and 11 was less than that of infected intestinal segments from unweaned pigs. Furthermore, *Escherichia coli* infected weaned pigs had greater net reduced absorption compared to infected unweaned pigs. The authors concluded that after weaning the net absorption of fluid and electrolytes is temporarily decreased which may contribute to diarrhoea.

Studies conducted at North Carolina State University investigated the effects of stress-induced intestinal damage associated with a weaning stress model. Moeser et al., (2007a) evaluated intestinal dysfunction in 19 d old weaned pigs compared to unweaned pigs. Twenty–four h after weaning, the pig's intestinal barrier function was evaluated for secretory activity by transepithelial resistance (TER) and intestinal permeability by paracellular mannitol flux in the jejunum and colon. In both the jejunum and colon, weaned pigs had greater secretory activity and intestinal permeability than unweaned pigs. This research corresponds with (Boudry et al., 2004), who demonstrated a transient reduction in jejunal TER, but not in the colon. Moeser et al., (2007a) also evaluated stress hormones and intestinal barrier function over 7 d post-weaning. They reported increased serum corticotrophin releasing factor (CRF) and cortisol in weaned pigs indicating that weaning induces activation of the stress pathways, which may be mediating the intestinal dysfunction. Subsequent studies by Moeser et al., (2007b) demonstrated that weaning age can affect the intestinal stress response in the pig. Finally, Smith et al., (2010) utilizing the weaning stress model evaluated the effects of early weaning stress on intestinal barrier function and intestinal health. Pigs from 5 different weaning ages (15, 18, 21, 23, and 28 d of age) were utilized. At 35 d of age, all pigs were evaluated for secretory activity and intestinal permeability in the jejunum. The results indicate that as weaning age

was incrementally increased, improvements in intestinal barrier function were observed as indicated by improved TER and lower permeability as measured by mannitol and inulin flux. To evaluate if the intestinal dysfunction was sustained, 15 or 28 d old weaned pigs were evaluated after 9 wks of age. The results were similar to previous observations; earlier weaned pigs had a reduced TER and increased permeability. Thus, the research demonstrated that the stress resulting from weaning induces a breakdown in intestinal barrier function associated with increased permeability and mucosal inflammation and that weaning age can impact present and future mucosal barrier function (Moeser et al., 2007a, Moeser et al., 2007b, Smith et al., 2010).

Other immunological responses that occur during the weaning process are alterations in pro-inflammatory cytokines. Pro-cytokines have an impact on epithelial function and intestinal integrity because it relates to permeability and transport of nutrients in gut (McKay and Baird, 1999). Pie et al., (2004) assessed gene expression of IL-1β, IL-6, and TNF-α during the weaning process. They applied 28 d old pigs and measured gene expression over 8 d post-weaning. The research reported that weaning is associated with an up-regulation of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α). Pie et al., (2004) reported that increased TNF-α expression occurred in the proximal and mid intestine initially by d 1 followed by increases in the distal small intestine and proximal colon from d 2 to 8. Consequently, indicating that weaning is associated with an early up-regulation of pro-inflammatory cytokines gene expressions that may give to functional disorders in gut resulting in decreased subsequent performance and play a role in post-weaning diarrhoea.

Weaning also altered to regulation of metabolism when increased expression of pro-inflammatory cytokines occurs through inflammation. Both immune function and growth or metabolic processes regulate by pro-inflammatory cytokines (Johnson, 1997, Spurlock, 1997). Therefore, weaning stress influences both structural alterations and active immune responses. The intestine is a major site for amino acid oxidation, net synthesis, and alterations in amino acid metabolism occur after weaning (Montagne et al., 2007, Burrin and Stoll, 2003), which may influence protein synthesis and consequent tissue deposition. Some authors confirmed that when the immune system is activated growth, feed intake, feed efficiency, and lean tissue deposition is reduced. Thus, the reduction or mitigation of post-weaning stress and consequent effects in the structural changes of the intestine and activation of the inflammatory immune response is critical for improving swine performance from weaning to market (Williams et al., 1997a, Williams et al., 1997b, Williams et al., 1997c).

Biological alterations such as metabolism, immune system, and intestinal functions occur during and immediately after weaning which may have both short and long-term effects on consequent pig growth and health, regardless of age of pig at weaning. It is important that swine producers use appropriate health, nutrition, and management strategies to diminish the adverse effects of weaning stress and to improve swine productive measures all the way to market weight.

Footpad dermatitis or pododermatitis

Footpad dermatitis (FPD)/pododermatitis is a condition that causes necrotic lesions on the plantar surface of the footpads in growing broilers and turkeys. This condition not only causes downgrades and condemnations of saleable chicken paws, the portion of the leg below the spur, but is also an animal welfare concern in both the United States and in Europe. Revenue from chicken paws in 2008 alone was worth $280 million. Harvesting large, unblemished paws has become a priority to poultry companies all over the world. Research on this subject has been on-going since the 1940s and has looked into many different areas including nutrition, environment, and genetics. Early research looked at nutritional deficiencies such as riboflavin and biotin mainly in turkeys. This early research was most likely looking at a separate form of dermatitis than what is being investigated now. Recent findings have suggested that there is a myriad of interacting factors that lead to FPD. Litter moisture appears to be the most likely culprit in the onset of this condition. Research has also shown a possible genetic link in the susceptibility to development of FPD lesions. Current chicken paw prices have skyrocketed due to a large export market in Asia. To produce unblemished paws for both increased profit and comply with current animal welfare recommendations, further research is needed to understand how the condition develops and what strategies can be used to prevent it.

Histopathological findings

In turkey poults, hyperkeratosis and separation of the keratin layers was seen at 6 week of age. Hyperkeratosis refers to a rapid turnover of keratinocytes that are undergoing apoptosis to produce keratin, resulting in a thickened layer of underdeveloped keratin. This is thought to be in response to an external trauma. Lesions tended to be more superficial at this age, but by 16 week, there were more severe ulcerations. Lymphocyte, granulocyte, and lymph follicle populations increased in the dermis adjacent to the lesions. Mild lesions show heterophils in the stratum germinativum and also defects in keratin formation (Martland, 1984). Heterophils were also found in the dermis, subepidermis, and epidermis along with basophilic cells in the stratum corneum (Greene et al., 1985). Vacuoles containing heterophils have also been found in the epidermis and inside blood vessels of the footpad (Harms and Simpson, 1975, Martland, 1984, Martland, 1985, Greene et al., 1985). Greene et al. (1985) observed complete destruction of the keratin and epidermal layer in the centre of the lesion, with necrotic tissue exposed and a mass of heterophils.

In severe lesions, there was acute inflammation with a more dense cellular infiltration and a thickening of the stratum corneum, which were referred to as

horned pegs (Martland, 1984, Fielding et al., 1990). The epidermis was more eroded and the dermis was filled with fluid. There was congestion and dilation of blood vessels that were sometimes found to be necrotic (Fielding et al., 1990).

Environmental factors associated with pododermatitis

Litter material

Litter management is an important aspect in rearing broilers to market age. It serves several functions that include thermal insulation, moisture absorption, protective barrier from the ground, and it allows for natural scratching behaviour. Bedding material must not only be a good absorber of moisture but also have a reasonable drying time (Grimes et al., 2002, Bilgili et al., 2009). Although litter refers to the mixture of bedding material, faecal droppings, and moisture, the term is used interchangeably with bedding materials. Litter will refer to both fresh bedding material and that which has faecal material and moisture. Litter material and depth is an important area of research for the understanding and prevention of FPD. Litter materials vary by region with regard to cost and availability. The most commonly used litter material is pine shavings in the United States, but straw is frequently used in Europe. Rice and peanut hulls are 2 other materials used regularly as bedding materials where it is economically feasible (Grimes et al., 2002).

Various materials have been examined for use as broiler litter and are generally tested for moisture absorption, caking, and bird performance. Caking refers to the compression of litter layers into a single wet layer on the very top of the bedding material. This thick, dense layer usually holds most of the moisture and fecal material in the litter. Therefore, a common management practice is to remove this caked litter between flocks, providing drier floors and better air quality for the next flock. The best-performing material was pine shavings and was followed by the following: rice hulls, ground corncobs, stump chips, pine sawdust, bark and chips, pine bark, and clay (Grimes et al., 2002). Differences in particle size of these materials were proposed to be the most important factor. No differences in paw quality or performance were observed between hay, bark, and wood chip litter as long as the particle size was less than 1 inch (2.5 cm). Lower FPD scores have been observed in pine shavings when compared with straw in broilers (Su et al., 2000, Sirri et al., 2010, Meluzzi et al., 2008a) and in turkeys (Mayne et al., 2007b). One explanation of this observation is that straw tends to have higher moisture content initially when compared with other materials such as pine shavings, rice hulls, and peanut hulls (Andrews and McPherson, 1963, Grimes et al., 2002).

Recycled paper products have been found, with proper management practices, to be as effective as pine shavings (Grimes et al., 2002). More recently, Grimes et al. (2006) looked at litter materials made from cotton waste, gypsum, and newspaper as

a comparison to pine shavings. There was no significant difference in the occurrence of FPD lesions between the different materials used; however, there was more caking with the cotton waste products.

Particle size of some litter materials has been examined as a contributing factor in the development of FPD. Used particleboard, a by-product of secondary wood products, has been evaluated in turkeys as a possible litter material. Large litter particles were between 0.32 and 1.27 cm and the fine particles were similar to fine sawdust or powder. Turkeys raised on fine particleboard had significantly lower incidence of leg abnormalities than those raised on the coarse size. The highest incidence of FPD was found with the coarse particleboard treatment (Hester et al., 1997). However, increased poultry mortality was observed due to gizzard compaction from consumption of fine particles. Sand was found to be an acceptable litter alternative to pine shavings, consistently showing a lower incidence of footpad lesions compared with broilers raised on shavings (Bilgili et al., 1999b, Bilgili et al., 1999a). Particle size is significantly different between these 2 materials and may explain why sand performed better as a litter material for broilers in that study. A more recent study looked at pine shavings, pine bark, chipped pine, mortar sand, chopped wheat straw, ground hardwood pallets, ground door filler, and cotton-gin trash. It was found that mortar sand and the ground door filler had significantly lower incidence of FPD than did the other treatments. It was theorized that the ground door filler performed well because of its moisture-holding capacity and the mortar sand performed well because of its ability to release moisture (Bilgili et al., 2009).

Litter moisture

Several factors, which include but are not limited to stocking density, ventilation, and drinker design, can affect litter moisture. One thing that is common among most previous research is that litter moisture is a significant factor in the onset of FPD. Martland (1985) found that wet litter appeared to be the only factor resulting in ulceration of broiler feet. Similar to findings with broilers, turkeys raised on wet litter have higher rates of FPD than those raised on dry litter (Martland, 1984). Mayne (2005) suggested that continually standing in wet litter will cause the footpad to soften and become more prone to damage, predisposing the bird to developing FPD. Drying out the litter and moving birds from wet litter to dry litter was observed to reverse the severity of FPD (Greene et al., 1985, Martland, 1985). Footpad dermatitis lesions have been found to be more severe as litter moisture increases, especially when the litter contains high moisture with sticky fecal droppings (Abbott et al., 1969, Harms et al., 1977, Greene et al., 1985, McIlroy et al., 1987, Ekstrand et al., 1997, Wang et al., 1998, Sørensen et al., 2000, Dozier et al., 2005, Dozier et al., 2006, Meluzzi et al., 2008b, Meluzzi et al., 2008a, Allain et al., 2009). Although most of the literature suggests that litter moisture is a critical component in the development of contact dermatitis, other studies have found no significant correlation between litter

moisture and the incidence and severity of FPD (Eichner et al., 2007, Nagaraj et al., 2007b).

Drinker design and management

Drinker design can play an important role in the overall moisture of the litter and thus the occurrence of FPD. Ekstrand et al. (1997) found that flocks reared with small drinker cups had a higher prevalence of FPD than did those reared on nipple drinkers. Nipple drinkers, however, have been shown to result in more scratches than other drinkers (Allain et al., 2009). In turkeys, small water cups have been shown to have a lower occurrence of FPD than bell drinkers (Ekstrand and Algers, 1996). Nipple drinkers with drip cups were most efficient and resulted in better litter conditions than nipple drinkers alone and bell drinkers. Drinkers that are too low or have the water pressure set too high tend to result in wetter floors. Water lines that may have a biofilm or other particulates can result in leaky drinkers, which will result in increased litter moisture. Regular flushing and sanitizing of the water lines will reduce water leakage. This will keep litter drier and improve its quality, subsequently resulting in better paw and hock quality (Tucker and Walker, 1992, Mayne et al., 2007b).

Stocking Density

Stocking density in general is a significant factor in broiler performance (Bilgili and Hess, 1995, Sørensen et al., 2000, Feddes et al., 2002, Heckert et al., 2002, Tablante et al., 2003). A survey of broiler reduction in Ireland over a 2-year period reported that flocks stocked at a higher density (≤0.48 ft^2/bird) had 10% ore hock lesions and 20% more breast lesions when compared with flocks at a lower stocking density [≥0.49 ft^2/bird (0.15 m^2/bird)]. Although no FPD data were recorded in this study, it was stated that when litter quality suddenly deteriorated, the level of hock lesions doubled when compared with flocks in which litter quality did not suddenly deteriorate (Bruce et al., 1990). Some studies have reported that higher stocking densities are associated with a greater incidence of FPD than lower stocking densities (McIlroy et al., 1987, Ekstrand et al., 1997, Sørensen et al., 2000, Dozier et al., 2005, Dozier et al., 2006, Haslam et al., 2007, Meluzzi et al., 2008a)**,** whereas other studies have suggested that stocking density plays little or no role in the formation of footpad lesions (Martrenchar et al., 2002, Sirri et al., 2010, Meluzzi et al., 2008b). Buijs et al., (2009) found that FPD was only negatively affected when density reached 56 kg/m^2, whereas Dawkins et al., (2004) reported that some leg health issues are compromised at or above a stocking density of 42 kg/m^2. The sudden onset of poor litter conditions associated with higher stocking densities is considered to be the biggest

influence on the development of FPD. Litter conditions deteriorate rapidly and litter moisture increases as stocking density increases (Bessei, 2006). Feddes et al., (2002) found that as stocking density increased, water consumption increased per bird. As birds drink more water, their faeces may become more watery and thus contributes to overall litter moisture. However, although more birds in a house makes litter quality difficult to maintain, it has been concluded that stocking density has little effect as long as appropriate environmental conditions are maintained (Dawkins et al., 2004).

Seasonal Effect

The time of year flocks are raised has been suggested as a contributing factor associated with the incidence of FPD. Dermatitis has been found more frequently during winter months than in summer and footpad condition has a high correlation with RH inside and outside the broiler house. When outdoor RH levels increased in winter months, there was an increase in paw lesions (Ekstrand and Carpenter, 1997). A 28% increase in the incidence of hock lesions has been observed in winter when compared with summer flocks (Bruce et al., 1990). Similar results were reported in other studies in which the incidence of paw lesions was greater in cold weather (Greene et al., 1985, McIlroy et al., 1987, Martrenchar et al., 2002, Dawkins et al., 2004, Haslam et al., 2007, Meluzzi et al., 2008b). Although outside RH is important, it is related to temperature so it is difficult to ascertain whether the main effect is RH or increased RH due to low outside temperatures. These seasonal effects are most likely caused by an increase in broiler house RH, which is due to decreases in ventilation rates typically observed in cold weather as operations try to avoid reducing house temperature and save on heating costs. Similar seasonal trends have been observed, with higher incidences of hock and breast lesions occurring during the winter months when compared with summer months (Mayne, 2005). Not all research has found the incidence of FPD elevated in the winter months. Wang et al., (1998) observed no cases of FPD in White Leghorn chickens when outside temperatures were between 48 and 59°F (9 and 15°C), but more birds with FPD were found when the temperature was warmer, between 68 and 79°F (20 and 26°C). It was suggested by the authors that a certain temperature may be required for FPD to develop regardless of litter moisture.

Litter Depth

Most research agrees that litter quality and type are important predisposing factors in the onset of FPD. Less focus has been given to the actual depth of the litter being used. In one study, litter material was found not to influence the

prevalence of FPD in broilers; instead, litter depth appeared to have more of an effect. Flocks reared on a thin layer (<5 cm) of litter had a lower prevalence of FPD than those raised on deeper layers (>5 cm) (Ekstrand et al., 1997). A similar study in France reported that high-quality flocks were raised on thin layers of litter and adding large amounts of litter may be a risk factor for FPD, but whether that was caused by litter conditions degrading was not determined (Martrenchar et al., 2002). In contrast to these results, Meluzzi et al., (2008a) found that broilers raised on deeper litter had a lower occurrence of FPD than those raised on a thin layer. This suggests that litter depth may be an important factor in foot health. An increase in final litter depth was found to have an overall lower hock burn score; with every centimetre increase in final depth, there was a corresponding decrease in hock burn score of 0.015 points (Haslam et al., 2007). Tucker and Walker (1992) noticed lower hock burn scores when shavings were at a depth of 10 cm when compared with 2.5 and 5 cm. No data were recorded on FPD lesions.

The studies that involved litter depth and its relationship with incidence of FPD were conducted in Europe, where poultry houses have concrete floors, an aspect that differs from the packed dirt floors commonly found in the United States. Meluzzi et al., (2008a) gave a weight per volume measurement (kg/m^2) for the amount of bedding material used. The initial depth could normally be explained by this measurement, but in this case, initial litter moisture was not taken into account, making it difficult to compare with other studies. The authors suggested that the experimental design confounded the actual effect of the litter depth because stocking density and photoperiod varied among treatments.

Litter Amendments

Litter amendments are often used in poultry production to reduce litter pH to control ammonia and as an intervention method in houses with a recurring disease issue such as gangrenous dermatitis. The most common type of litter amendments are litter acidifiers. These compounds lower the pH of the litter, inhibiting bacterial growth, which produces ammonia as a by-product of their metabolism. Some common litter amendments include aluminum sulfate, sodium bisulfate, and ferric sulfate. Sodium bisulfate influence on the incidence and severity of FPD in broilers has been evaluated. Application rates of $NaHSO_4$ were 0.22 or 0.44 kg/m^2 at chick placement, whereas a third treatment had 0.22 kg/m^2 at both 0 and 21 d. However, no significant FPD differences were noted between the treatments. The researchers stated that there was a trend of decreasing incidence and severity of FPD with the use of $NaHSO_4$ (Nagaraj et al., 2007c).

Nutritional factors associated with pododermatitis

Nutrition is considered to be a major factor in the onset of FPD along with poor litter conditions. Early FPD research took place with turkey poults and focused on soybean meal inclusion in diets and also nutritional deficiencies such as biotin and riboflavin (Patrick et al., 1943, Patrick et al., 1944, McGinnis and Carver, 1947, Abbott et al., 1969, Jensen et al., 1970, Murillo and Jensen, 1976). This dermatitis may not be the same as FPD, which is believed to be more of a contact dermatitis rather than a dermatitis caused by a deficiency. Biotin serves many roles in avian species, one of which is skin integrity, as reviewed by Mayne (2005). Research has branched from earlier nutritional work that focused mainly on deficiencies and has looked at many different areas. Some areas include different protein sources and levels, different diet densities, mineral and vitamin supplementation, and also the use of enzymes.

Nutritional Deficiencies

Deficiencies of vitamins and amino acids such as biotin, riboflavin, methionine, and cystine in the diets of growing birds have been reported to affect the incidence of FPD. Diets deficient in biotin have produced FPD lesions in turkeys (Patrick et al., 1943). When turkey poults were fed diets deficient in riboflavin and biotin, FPD was prevented by biotin supplementation but not with riboflavin supplementation (Patrick et al., 1944). Later, McGinnis and Carver (1947) found that riboflavin supplementation into turkey diets prevented dermatitis in poults. Jensen and Martinson (1969) observed severe dermatitis of the feet and around the head in poults that were fed a diet deficient in biotin. Additional supplementation of biotin was not found to alleviate FPD in several poults. Additional research has also shown that supplementation of biotin does not reduce the occurrence or severity of FPD lesions (Atuahene et al., 1984, Mayne et al., 2007a). An interaction between biotin supplementation and litter quality may exist. In a study by Harms and Simpson (1977), supplemental biotin resulted in significantly reduced footpad scores when given to poults grown on dry litter but was not observed when given to poults grown on wet litter. This finding either suggests that biotin alone is not responsible for the occurrence of these lesions or that it is not effective in conditions that are known to directly increase the incidence and severity of FPD.

Grain Sources

The addition of the feed ingredient soybean meal has been researched as a possible cause of FPD. There are some indications that sticky indigestible carbohydrates from plant sources (primarily soybean meal) may be caustic and contribute to FPD. These carbohydrates are referred to as non-starch polysaccharides (NSP) and are found in higher concentrations in wheat, barley and other grains when compared with soybean meal. As the diet NSP concentrations increase, gut viscosity increases, resulting in manure that adheres more readily to the footpads of the birds. Diets containing wheat that have increased levels of viscous NSP tend to have lower ME values and higher digesta viscosity than normal wheat diets. These diets can be improved with addition of NSP-degrading enzymes, showing significantly lower digesta viscosity than the wheat diet alone (Choct et al., 1995). The viscosity of gut contents can affect faecal dropping adhesion to the foot and over time may deteriorate the epidermis and keratin layers. When diets contain high levels of soybean meal, the incidence of dermatitis is very high with turkey poults, and it appears that the dermatitis is caused by manure sticking to the feet of the birds (Jensen et al., 1970).

Abbott et al., (1969) found that lesions were the result of wet, crusty litter and not dietary treatments differing in the amount of soybean meal fed to poults. These contradicting results suggest that dermatitis may be associated with independent and combined effects of soybean meal content in feed and litter moisture.

Vitamin, mineral, and amino acid supplementation

Nutrients such as biotin, riboflavin, pantothenic acid, and sulfur amino acids have been shown to affect the structural components of the skin. The addition of vitamins and trace minerals did not significantly reduce FPD and it was concluded that factors other than nutrition might be involved (Burger et al., 1984). Footpad dermatitis in young poults has been associated with methionine deficiency, but the supplementation of sulfate and cystine to the diet yielded no improvement in FPD (Chavez and Kratzer, 1974, Murillo and Jensen, 1976). Footpad condition never fully corrected with the addition of the methionine either, but contact of the bird's feet with the excreta was suggested to play a major role in FPD (Abbott et al., 1969, Jensen et al., 1970). Hess et al., (2001) supplemented broiler diets with a zinc amino acid complex and observed no significant difference in FPD scores in males but did detect a decrease in lesions when given to females.

Protein level and source

The incidence and severity of FPD is significantly affected by protein level and source (Nagaraj et al., 2007b). Birds reared on a low-protein diet and fed a diet based on vegetable and animal proteins showed the lowest incidence of FPD compared with other treatments. The most severe cases were associated with birds fed a high-protein diet consisting of only plant-based proteins (Nagaraj et al., 2007b). Eichner et al., (2007) observed similar results but found that the addition of corn gluten meal to an all-vegetable diet reduced the incidence of FPD when compared with a vegetable- and animal-based diet. Birds raised on an all-vegetable diet had a higher incidence of FPD than did birds raised on a mixed animal and plant diet. Studies on protein level and source have provided inconsistent results. For example, a second study by Nagaraj et al., (2007a) evaluating the effect that feed-grade enzymes may have on protein digestion and paw quality observed no differences between the high- and low-protein diets. However, it was noted that the litter moisture was greater in this study, possibly due to increased water consumption in response to high environmental temperatures experienced during that trial.

Diet density

In a study that examined the effects of diet density, 2 density levels were examined while keeping the feed formulation isocaloric and isonitrogenous. Diet density is related to the level of fat in the diet, with low-density diets having less fat than a high-density diet. Broilers raised on the low-density diet had significantly less incidence of paw lesions compared with the high-density diet due to reduced faecal viscosity from lower soybean meal content in the ration (Bilgili et al., 2006).

Enzymes

Nagaraj et al., (2007a) evaluated a feed-grade enzyme in diets with or without animal protein on the subsequent incidence of FPD. The incidence of lesions was lower with the addition of the enzyme to the all-vegetable diet, with no differences noted when enzyme was added to the vegetable and animal protein diet. The improvement in footpad condition was noted in the later stages of the flock and could be confounded with healing of the lesions. It is unclear at this time whether the rate of healing is affected by these dietary treatments or if it was a direct effect on faecal composition that would influence footpad condition. Additional research on feed enzymes to enhance feed utilization and reduce nitrogen in the litter is needed to better understand the effect of these feed additives on footpad condition.

Electrolyte imbalances

Harms and Simpson (1982) found that dietary salt content had a direct influence on the severity of footpad lesions and that dermatitis was more severe with higher levels of salt. Birds with diets containing high salt content had faecal droppings containing more moisture, resulting in poor litter conditions. They observed a reduction in both body weight and FPD with the supplementation of salt, suggesting that body size is a predisposing factor in the development of lesions.

Sex, body size and strain-cross

Sex and body size

The sex and size of broilers have been investigated as possible factors for the onset of FPD. It has been shown that male broilers tend to have higher incidence and severity of FPD than females (Harms and Simpson, 1975, Greene et al., 1985, McIlroy et al., 1987, Bilgili et al., 2006, Nagaraj et al., 2007b). The increased incidence of FPD in male broilers could be related to body size because males are typically heavier than females and thus more weight is placed on their footpads. This leads to increased surface area contact with the litter, possibly causing an increase in the incidence of burns and lesions. Body weight has been shown to be positively correlated with hock burns ($r = 0.353$) (Broom and Reefmann, 2005). Bruce et al., (1990) found that the prevalence of both hock and breast lesions was significantly higher in male broiler flocks than female broiler flocks.

Some research alternatively suggests that females have a higher incidence of footpad lesions than males (Harms et al., 1977, Kjaer et al., 2006). In contrast to their earlier findings (Nagaraj et al., 2007b) in which males had a higher incidence than females, Nagaraj et al., (2007c) observed a higher incidence of FPD in females than males (Nagaraj et al., 2007c). Other studies such as Martland (1985) and Nagaraj et al. (2007a) reported no relationship between body size and sex in the incidence of FPD. Because of the inconsistent results reported from research that has evaluated body size and sex on the incidence and severity of FPD, it is currently believed that these factors are not significant contributors in the occurrence of FPD. Ask (2010) stated that continued selection for increased BW without considering FPD in the breeding goal is likely to result in increased cases of FPD in broilers in the future.

Strain-cross

Bilgili et al., (2006) looked at the effect of strain-cross (SC) on the development of FPD along with diet densities. They found a significant SC × diet density interaction at 42 d of age, which suggested that the susceptibility to FPD may vary by SC. Similar data have been reported by Kestin et al., (1999), in which FPD scores varied between 4 different crosses, which suggested that FPD was not merely the product of poor management but that there may be a difference between various strains in susceptibility to developing FPD. Sanotra et al., (2003) found a lower prevalence of FPD in Swedish Cobb chicks when compared with Swedish or Danish Ross chicks. The authors mentioned, however, that differences in housing conditions may have confound their findings. Ross 308 broilers had higher rates of FPD and hock burns than did a slow-growing dual-purpose strain (Kjaer et al., 2006). It was stated that it should be possible to decrease the incidence of FPD through genetic selection. Similar conclusions were made by Allain et al., (2009) when looking at a fast-growing strain versus a slow-growing strain, with the fast-growing strain having higher rates of FPD but fewer breast blisters. Genetic variation between and within 10 commercial broiler lines was present for both FPD and hock burns (Ask, 2010). The authors stated that it may be possible to select against both FPD and hock burns without negatively affecting BW. Chavez and Kratzer (1972) found that Large White turkey poults had more severe FPD lesions than did Broad Breasted Bronze poults when reared in the same conditions on wire floors.

Cellulitis

Cellulitis in broiler chickens is only detectable at slaughter, once the carcass has been plucked and scalded. The inspector will first notice an area of yellow and thickened skin on the lower abdomen. Closer examination of the area will reveal a plaque of pus underneath the skin, and underlying muscles will often display small haemorrhages. The degree of inflammation and the size of the lesion vary tremendously from one carcass to another, with some showing chronic, localized and well-demarcated pea-size lesions, and others exhibiting an extensive seropurulent inflammation covering most of the abdomen and breast muscles. Although in some cases the carcass might be trimmed, it is most often discarded. Indeed, since the *E. coli* bacteria is isolated from these lesions, the risk of carcass contamination is considered a public health concern by the Canadian Food Inspection Agency; hence the condemnation of the whole carcass.

Epidemiological trends and economical losses related to cellulitis

Although the condition was first recognized in 1981 by Agriculture Canada's meat hygiene directives, the term cellulitis did not show up on condemnation records until 1986. Cellulitis ranked 10th among all condemnation categories in Canada that year, with only 160,405 chickens (0.048% of all slaughtered broilers) condemned for that reason. Ten years later, more than 2.6 million Canadian chickens affected with cellulitis (0.56% of total slaughter) did not pass inspection a 12-fold increase in frequency. Cellulitis is now the first cause of condemnation in broiler chickens in Canada, which makes it a source of major financial losses.

Aetiology of avian cellulitis

Cellulitis is a pathological condition resulting from a subdermal colonization of bacteria developing in areas of compromised skin (i.e., scratches or punctures) through skin-litter (bedding and faeces) contact. Norton (1997) described cellulitis as a bacterial disease affecting the skin and resulting in a diffuse, spreading, oedematous, suppurative inflammation of deep subcutaneous tissues with resulting abscess (plaque) formation. Although typically associated with the skin, cellulitis infection may extend into subcutaneous muscle tissue and is usually found on the abdomen, medial thigh, skin surrounding the cloaca, and back (Carr et al., 1996, Onderka et al., 1997, Fallavena et al., 2000). *Escherichia coli* has been implicated as the primary bacterial species causing cellulitis; however, other bacteria including *Enterobacter agglomerans*, *Pasteurella multocida*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, and *Streptococcus dysgalactiae*, have also been isolated from cellulitis lesions (Messier et al., 1993, Gomis et al., 1997). Although also once believed to be associated with poor hatchery hygiene, current research has supported the hypothesis that the occurrence of cellulitis in commercial poultry is caused primarily by dermal scratch-litter (faeces) contact during broiler grow out (Norton et al., 1999). Thus, cellulitis control strategies are being directed toward the broiler live production segment of the industry. Proliferation of immune cells at the bacterial entry point produces a plaque-like deposit which must be removed during bird processing, with the extent of downgrading dependent on severity. Fallavena et al., (2000), in a histological study of skin lesions on downgraded and condemned broiler carcasses, found cellulitis contributed to 45.3% of identified skin lesions. Onderka et al., (1997) estimated that 30% of cellulitis lesions examined on broiler carcasses were considered trimmable (localised to a particular area) without carcass contamination. Excessively large or non-localised cellulitis lesions result in whole carcass condemnation, with most cellulitis lesions measuring between 1 and 112 cm^2. Trimmable lesions were considered to be between 1 and 80 cm^2 (with most characterised between 33 and 48 cm^2). In the study by Onderka et al., (1997), flocks with a high incidence of cellulitis also had high levels of condemnations caused by other conditions, including

cyanosis, ascites, and emaciation. Cellulitis alone has not been shown to contribute to mortality in broilers; however, some weight decrease was noted as cellulitis *E. coli* exposure level increases (Johnson et al., 2001). Likewise, cellulitis causing *E. coli* has not been associated with human illness; however, some *E. coli* isolates from cellulitis lesions are serologically similar to those causing human septicaemia and meningitis (Kumor et al., 1998).

A particular problem for broiler producers in preventing and treating cellulitis is its lack of association with diagnostic signs. Because affected birds appear clinically normal, a definitive diagnosis can only be determined upon bird processing (Gomis et al., 1997). Treatment regimes, therefore, are often impractical and cost prohibitive. Although scratches have been implicated as the primary contributing factor to the development of cellulitis, other associations can influence its incidence. Many of these, such as housing densities, feed and water availability, bird stress level, and lighting programme, indirectly contribute to increased skin scratching. Elfadil et al., (1996) examined farm management parameters which could affect cellulitis incidence and found bird sex, feeding programme, growth promotant, litter type, time between subsequent flocks, and disease presence significantly influenced the occurrence of cellulitis. Although seemingly divergent, the management factors, which influenced cellulitis either, affected the amount of skin scratches (e.g. greater incidence of cellulitis in males), environmental hygiene (e.g. short times between flocks caused greater incidence of cellulitis), or bird immune status (e.g. presence of other diseases contributed to greater incidence of cellulitis). Birds fed zinc bacitracin (a growth promotant) had a higher incidence of cellulitis apparently due to its antimicrobial affect on Gram-positive bacteria allowing proliferation of Gram-negative organisms (e.g. *E. coli*) in the intestines and, likewise, the bird's environment (Elfadil et al., 1996).

Clearly, reductions in bird-to-bird scratching will produce a significant reduction in the incidence of cellulitis. However, the economics and logistics of commercial broiler production is not always conducive to lowering bird densities to an extent which would ultimately reduce bird-to-bird scratching. Thus, more realistic reduction strategies must be examined or a combination of strategies must be employed.

Trace mineral strategies to control cellulitis

To ensure adequacy in meeting bird requirements, trace mineral supplementation has been a longstanding practice in broiler diet formulation. However, considerable evidence indicates certain trace minerals, such as zinc, play significant physiological roles beyond simply meeting metabolic requirements for optimum bird growth and liveability. Zinc has been the primary trace mineral of focus in the control of cellulitis because of its role in wound healing, skin dynamics, and immune system potentiation.

Zinc exhibits a wide array of metabolic and physiological functions in animal systems. Of primary importance, zinc is associated with enzyme systems involved in protein metabolism, nucleic acid synthesis, and carbohydrate metabolism (Chesters, 1989). When involved in enzyme systems, zinc acts as a cross-link between enzyme proteins, thereby stabilizing enzyme structure. Zinc may also act as a cofactor for enzyme activation. Beyond its role in enzyme systems, zinc also functions in testosterone, insulin, and adrenal corticosteroid production, immunity, water and cation balance, vitamin A metabolism, and behaviour. Zinc must also be available to maintain optimum growth rate and skin quality, and augment wound healing (Van den Broek and Thoday, 1986, Pimentel et al., 1991, McDowell, 2003).

Zinc is a transition metal (atomic number 30, atomic weight = 65.38, group 2B) found as a soil constituent (ZnS), and in relatively high cytoplasmic concentrations (Williams, 1989). Dietary zinc is absorbed along the entire length of the small intestine and, to a lesser extent, in the proventriculus. Absorption of zinc into the mucosa is carrier-mediated and involves chelation of zinc ions with proteins before absorption (Lönnerdal, 1989). Plasma transport involves binding of zinc ions with albumin and α-2-macroglobulin which enhances tissue uptake and liver storage as metallothionein and superoxide dismutase (as a cofactor). However, zinc is not stored to a large extent in animal tissues. Excretion of dietary zinc is principally faecal with little urinary loss (McDowell, 2003, Jackson, 1989).

Zinc has been implicated in enhanced immune function (Good, 1989). Clearly, zinc is required in adequate amounts to sustain organ systems through its function in enzymes, DNA/RNA synthesis, energy metabolism, and various other roles. Several reports show retardation of lymphoid tissues, in particular the thymus, spleen and bursa in zinc-deficient animals (Fraker et al., 1977, Gross et al., 1979, Beach et al., 1980, Chandra and Au, 1980, Burns, 1983). Antibody production (IgA, IgG and IgM) in chickens, rats and steers was also enhanced through adequate zinc supplementation (Fraker et al., 1977, Pimentel et al., 1991, Beach et al., 1980, Burns, 1983, Stahl et al., 1989, Spears et al., 1991). Beyond enhanced humoral immunity of animals consuming adequate zinc, Stahl et al. (1989) showed an increase in passive immunity (measured as antibody titres to sheep erythrocytes) of progeny chicks from hens supplemented with 38 ppm dietary zinc. Further supplementation of hen diets with zinc, however, did not enhance humoral immune response or progeny performance.

Additionally, an adequate level of zinc seems to enhance lymphocyte (cell-mediated immune response) and phagocytic macrophage activities. In rats, the cytotoxic response of splenic, thymic, and blood lymphocytes was increased as zinc supplementation levels increased towards adequacy (Gross et al., 1979, Beach et al., 1980, Chandra and Au, 1980). Conversely, Pimentel (1991) showed no influence for 8 and 125 ppm zinc on primary and secondary cell-mediated immunity in chicks. Further, zinc-methionine supplementation (165 ppm) of turkey poults increased *in vitro* phagocytic macrophages from 59.6 to 63.8% when compared with a control diet (130 ppm zinc as $ZnSO_4$) (Kidd et al., 1994).

The effect of zinc on skin health and the importance of skin mechanics in physical protection from foreign invasion is well established. The involvement of zinc in the nutriture of the skin is complex. Zinc, as a component of a variety of metalloenzymes, is linked to collagen synthesis and crosslinking (glycine incorporation), cystine incorporation into epidermal protein, biosynthesis of keratin, epidermal energy metabolism (enzyme systems for carbohydrate metabolism), essential fatty acid (particularly arachidonic acid) metabolism, and dermal antioxidation as a component of zinc-superoxide dismutase for reduction of solar radiation damage (Codner and Thatcher, 1993). For its part in the interaction between skin health and protection from invasive organisms at times of skin compromise (e.g. punctures and scratches), adequate zinc is critical for proper wound healing (Sandstead and Shepard, 1968, Rahmat et al., 1974, Weismann, 1978). Because of its role in wound healing, zinc has received considerable attention as a strategy for the reduction of cellulitis. Simply put, complete and rapid healing of wounds associated with bird-to-bird scratching will reduce the number of invasive organisms implicated in cellulitis lesion formation. Maximisation of wound healing through zinc supplementation may prevent prolonged invasion of organisms (e.g. *E. coli*) at points of compromised skin integrity.

Research conducted at Auburn University (Alabama, USA) has sought to quantify the efficacy of zinc (alone and in combination with vitamin E) supplementation of broiler diets on the incidence of cellulitis. In two separate experiments, using a standardised cellulitis induction model developed by Norton et al., (1999) at Auburn University, the addition of 40 ppm zinc (as a zinc amino acid complex) to typical broiler starter and grower diets reduced the overall incidence of cellulitis lesion development. In the first experiment, the quantity of severe lesions was reduced 13%, which contributed to the overall reduction (across all lesion types) in cellulitis lesion occurrence of 10.8%. The zinc-mediated control of cellulitis in experiment 2 was less than the first and zinc-amino acid supplementation resulted in only a 1.7% reduction in overall cellulitis lesion incidence. In experiment 2, zinc supplementation showed the most significant reduction, among lesion types, in the occurrence of mild lesions (3.9%) (Downs et al., 2000, Downs et al., 2003). Analysed zinc levels of treated diets were 176% higher than control diets in experiment 1 (88 vs. 243 ppm), whereas treated diets in experiment 2 contained only 23% greater concentration of zinc (81 vs. 100 ppm). The lower reduction of cellulitis in experiment 2 was attributed to this variation in dietary zinc level. Although a consistent quantity of supplemental zinc was added to experimental diets, feeds were more variable in zinc concentration.

Although typical zinc supplementation of broiler diets results in apparent adequacies for bird maintenance and growth requirements, the physiological potential of zinc for affecting avian cellulitis is maximized at higher than recommended zinc levels. Although not determined in these studies, an implied bioavailability enhancement of zinc amino acid versus an inorganic zinc salt could have contributed to the cellulitis reduction attributed to zinc supplementation. Increased tissue/cellular concentration of zinc apparently maximizes the zinc

cofactor enzyme activation mechanisms responsible for zinc associated wound healing and immune response, thereby reducing the *E. coli* invasive pressure (i.e., rapid wound healing and cellular immune responses) in areas of skin compromise.

Vitamin strategies to control cellulitis

Like minerals, adequacy in vitamin supplementation of broilers is critical for optimum metabolism and growth. The physiological potential of vitamin supplementation, however, may not be realised at typical supplementation levels. Vitamin supplementation strategies have focused on meeting the growth needs of broilers without consideration of their effect on immunocompetency. The impact of vitamin supplementation strategies to ameliorate the incidence of cellulitis is clearly focused on their effects in cellular and humoral immune responses.

The impact of vitamin E deficiency on immune function has been well established (Moriguchi and Muraga, 2000). Vitamin E inadequacies result in impaired humoral and cellular immunity. In particular, T and B-cell mitogenesis, macrophage phagocytosis, and antibody titres were decreased during vitamin E deficiencies. The activities of vitamin E on immune response are primarily associated with its potent antioxidant effect. The presumed activity of vitamin E for increasing resistance to infection is through stimulation of the cell-mediated immune response. In particular, anti-oxidation activity in immune cell (e.g. phagocytic cell) membranes is enhanced, and immune cells are less susceptible to oxidative by-products (e.g. superoxides and hydroxyl radicals) produced by infective organisms during phagocytosis and blood clearance. Vitamin E reduces free radical production and fatty acid peroxidation, which would otherwise diminish cellular communication, membrane fluidity, secondary messenger elaboration, and gene expression (Benedich, 1990, Watkins, 1991, Packer and Suzuki, 1993, Klasing, 1997). Vitamin E has also been implicated in protective immunity to invasive bacteria (e.g. *E. coli*), protozoa (e.g. *Eimeria tenella*), and viruses (e.g. Newcastle disease) (Tengerdy and Nockels, 1975, Colnago et al., 1984, Boren and Bond, 1996).

Vitamin E has been the focus in the control of cellulitis because of its established role in avian immune response. Leshchinsky and Klasing (2001) indicated that the addition of vitamin E (up to 50 IU/kg) to broiler diets enhanced cellular and humoral immunity as reflected by an increase in antigen-antibody responses, a reduction in lymphocyte proliferation, and a lessening of heterophilia induction. Vitamin E supplementation levels beyond 50 IU/kg (i.e. 100 and 200 IU/kg) did not prove successful in enhanced immunity beyond the control. Expectations would lead to an obvious deduction that if vitamin E supplementation influences cellular and humoral immune response in broilers, it may be a practical control mechanism to reduce the invasive pressure of cellulitis bacteria or increase clearance of infective organisms. Macklin et al., (2000), in an evaluation of graded vitamin E levels fed to broilers and the impact on cellulitis lesion development using a scratch induction

model, found a varying impact of vitamin E level (from 9 to 141 ppm) on the development of cellulitis. A reduction in cellulitis was noted at 42 and 75 ppm vitamin E; however, cellulitis lesion development was enhanced when vitamin E was supplemented beyond the 75 ppm level. This is consistent with previous research pointing to a reduction of immune response at high vitamin E levels (Friedman et al., 1998).

Additional work at Auburn University showed that supplementing broiler starter and grower diets with 48 IU/kg vitamin E resulted in a 9.5 and 6.7% reduction (two experiments) in induced cellulitis lesions compared with a control diet supplemented with vitamin E at only 8 IU/kg (Downs et al., 2000, Downs et al., 2003). In these studies, the number of mild and moderate lesions was reduced the most through vitamin E supplementation. Mild lesions were reduced by 11.1 and 3.5%, respectively, in experiments 1 and 2; while moderate lesions were reduced by 3.5% in experiment 2, their numbers remained unchanged in experiment 1. Vitamin E supplementation beyond recommended levels apparently potentiates the immune system to control the entry of cellulitis bacteria at the wound site. More specifically, research with vitamin E and cellulitis suggests that macrophage and lymphocyte activity against invading bacterial cells is increased, with a concomitant increase in B-cell mediated antibody production. Therefore, invading bacteria are eliminated at the site of invasion with a reduction in cellulitis lesion number and severity.

Vitamin and mineral synergy

With benefits of vitamin or mineral supplementation alone on the incidence of cellulitis comes the obvious question of potential synergistic effects when particular vitamins and minerals are supplemented in combination. Supplementation of broiler diets with vitamin E and zinc has produced the most profound reductions in cellulitis occurrence. In two experiments, overall cellulitis lesion level was reduced by an average of 15.2% when broilers were supplemented with a combination of vitamin E (at 48 IU/kg) and zinc (as zinc amino acid at 40 ppm) (Downs et al., 2000, Downs et al., 2003). The greatest reductions in cellulitis (comparing vitamin E and zinc supplementation alone) resulted from the combined supplementation of vitamin E and zinc. The supplementation of vitamin E with zinc resulted in a 2.1 and 3.9%, respectively, greater overall cellulitis reduction when compared with vitamin E or zinc supplemented alone. Apparently, the potentiation of the immune response from vitamin E supplemented at higher than recommended levels and the enhanced wound healing attributed to zinc supplementation, contributed to the highest reduction in cellulitis incidence measured in these two experiments.

Oxidative stress

The term "oxidative stress" is defined as a physiobiological situation in which the generation of oxidizing species is higher than the capacity of the organism to detoxify them. The metabolism of oxygen produces such type of oxidizing species. Multicellular organisms have acquired a much higher efficiency in energy metabolism by using the reactive potential of oxygen. Damage cells and tissues occur via the oxygen-driven reactions that bear the potential of forming intermediate species. During evolution processes, a number of detoxifying mechanisms evolved aimed at reducing the damaging capacity of reactive oxygen species (ROS) or repairing ROS-related damage. The idea "oxidative stress" applies only if these protective or repair systems are overwhelmed. Oxidative stress may be occurred from increased ROS formation, or from the failure of the scavenging systems. As a consequence of this, and due to the fact that oxidants have multiple generation sites and the detoxifying systems involve several dozen of enzymes and compounds, oxidative stress is a complex phenomenon, difficult to assess and, therefore, hard to measure (Grune and Berger, 2007).

ROS and Oxidative cellular damage

The body cell is effected to a large variety of ROS and reactive nitrogen species (RNS) from both exogenous and endogenous sources (Figure 1) (Kohen and Gati, 2000). The threat of uncontrolled oxidation of biomolecules largely comes from the so-called reactive oxygen species (ROS). The term ROS is used to cover both the free radical and non-radical oxidants; a free radical composed of at least one unpaired electron in the shells around the atomic nucleus and are able of autonomous existence. The radical group includes species for example 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 peroxynitrite ($ONOO^{-}$), hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) (Table 1).

From a chemist's point of view, the formation and bioreactivity of ROS is rather intriguing. Molecular oxygen in air is contain triplet dioxygen; although it is a diradical, and needs a total of four electrons for reduction to water, it reacts slowly with many micromolecules (e.g. DNA, proteins and membranes). If reduced, however, O_2 is turned into highly aggressive ROS, which are more reduced yet, at the same time, more oxidizing than triplet oxygen. One-electron reduction causes in the superoxide anion, two-electron reduction in hydrogen peroxide and three electron reduction in the highly destructive hydroxyl radical.

ROS are more frequently converted into each other, such as, superoxide into peroxide (by dismutase enzymes) and peroxide into hydroxyl radicals (a metal catalysed Fenton reaction). Also, reactions of ROS with themselves or other

molecules produce secondary reactive species, such as peroxynitrite, which result from the condensation product of superoxide and nitric oxide radicals and a range of reactive sulfur species formed from ROS and cysteine residues. Moreover, ROS attack on metalloproteins frequently results in the release of their metal ions, such as Zn^{2+} and redox-active $Fe^{2+/3+}$ and $Cu^{+/2+}$. The latter two are capable to participate in Fenton-type reactions (a), that is, Fe^{2+} and Cu^{+} reduce H_2O_2 to form $HO\cdot$ (Winyard et al., 2005).

$$H_2O_2 + Fe^{2+}\ (Cu^{+}) \longrightarrow HO\cdot + HO^{-} + Fe^{3+}\ (Cu^{2+})\ (a)$$

Most of the transition metals composed of unpaired electrons and can, therefore, with the exception of zinc, be considered radicals by definition. Those transitional metal ions can be participated in the chemistry of radicals, which convert relatively stable oxidants into powerful radicals. Among the various transition metals, copper and especially iron are present in relatively high concentrations, which are major players in the Fenton reaction and the metal-mediated Haber-Weiss reaction. The metal ions participating in this reaction are those bound to the surface of proteins, DNA, and other macromolecules or chelates. These particular ions can still undergo the reduction-oxidation process, interacting with oxygen derivatives (Kohen and Nyska, 2002).

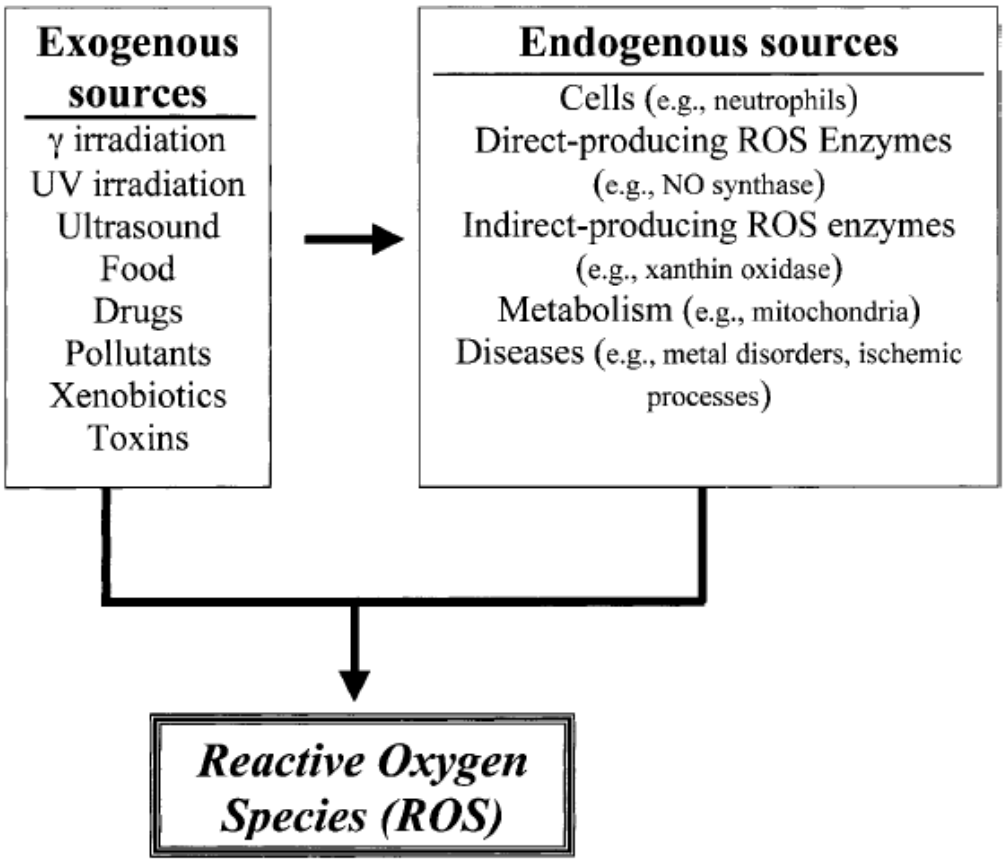

Figure 1. Exogenous and endogenous sources of reactive oxygen species (ROS) (Kohen and Nyska, 2002).

Under aerobic conditions, all cells in living body are continuously effected to increase numbers of oxidants which are derived from different endogenous and exogenous sources (Halliwell and Gutteridge, 1999). The endogenous sources of oxidants are several type which include the respiratory chain in the mitochondria that converts molecular oxygen to water. There are a few percent of oxygen molecules continuously leaks from the electron transport chain of mitochondria as a ROS intermediates and this process alone results in a substantial basal level of oxidants in vivo.

Moreover, immune reactions may be caused significantly to the generation of oxidants, specifically during infections time or as a result of autoimmune responses. For instance, activated blood neutrophils undergoing the respiratory burst to release ROS, which intended to target foreign pathogens, on the other hand, the lack of specificity in these reactions also results in tissue damage to the host organism. Furthermore, xanthine oxidase and nitric oxide synthase release $O_2^{\cdot-}$ and $NO^{\cdot}$, those two radicals which can energetically combine to form the deleterious species $ONOO^-$ (Halliwell, 2009). Moreover, foreign microorganisms stimulate secondary oxidant formation which release in the host through the immune system, as mentioned above, in addition to their occasionally directly oxidising capabilities (Lykkesfeldt and Svendsen, 2007).

Name	Symbol
Oxygen radicals	
Oxygen (bi-radical)	$O_2^{\cdot\cdot}$
Superoxide ion	$O_2^{-\cdot}$
Hydroxyl	$OH^{\cdot}$
Peroxyl	$ROO^{\cdot}$
Alkoxyl	$RO^{\cdot}$
Nitric Oxide	$NO^{\cdot}$
Non radical oxidants	
Hydrogen peroxide	H_2O_2
Organic peroxide	ROOH
Hypochlorous acid	HOCL
Ozone	O_3
Aldehydes	HCOR
Singlet oxygen	$^1O_2$
Peroxynitrite	ONOOH

Table 1. Radical and non-radical oxidants (Kohen and Nyska, 2002)

Oxidative stress, as previously reported, is commonly defined as an imbalance between oxidants and reductants (antioxidants) at the cellular or individual level. Oxidative damage is one result of such an imbalance and includes oxidative modification of cellular macromolecules, cell death by apoptosis or necrosis and structural tissue damage. The presence of free radicals and non-radical reactive molecules at high concentrations in host tissue is dangerous due to 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 in host physiology. Interestingly, various ROS-mediated reactions protect the cell from oxidative stress, which also serve to stabilize redox homeostasis. In more developed organisms NO and ROS act as signal transducing molecules which effect 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).

Because most radicals are short-lived species in host, they react quickly with other biomolecules. Few numbers of the oxygen-derived radicals are highly reactive with a short biological half-life. Moreover, the life span of other radicals is short which depends on the environmental medium. Also, non-radical metabolites such as HClO possess a relatively short half-life varying from parts of seconds to hours. The half-life of ROS depends on the physiological environment such as pH and the presence of other species. The high reactivity of radicals with their short life span cause the potential toxic effect which is difficulties in preventing oxidative damage.

To overcome the interaction between radicals and biological targets in host, the antioxidant should be present at the location where the radicals are being produced in order to compete with the radical for the biological substrate (Kohen and Nyska, 2002). The cellular macromolecules, mainly DNA, proteins and lipids, are natural targets of oxidative damage.

ROS can interact with stable, well-protected DNA molecule which cause various types of damage: modification of DNA bases, single- and double-strand DNA breaks, loss of purines (apurinic sites), damage to the deoxyribose sugar, DNA-protein cross-linkage, and damage to the DNA repair system. Not all ROS can cause damage; most is attributable to hydroxyl radicals (Kohen and Nyska, 2002). It is predictable that ROS are responsible for about 10000 DNA base modifications per cell per day. Oxidation or methylation of bases effect to the most serious phenotypic consequences. Mitochondrial DNA appears to be particularly vulnerable, in part due to its proximity to the site of most uncontrolled ROS generation, and because of the low level of repair that occurs. Moreover, telomeres, the caps at the chromosome end that are critical for genome stability, are vulnerable to attack from ROS, and the accelerated reduction in telomere length that results from oxidative stress can hasten cell senescence (Monaghan et al., 2009).

Interaction between proteins with ROS can cause direct and indirect damage, such as, peroxidation, damage to specific amino acid residues, changes in their tertiary structure, degradation, and fragmentation. The magnitude of the damage will depend in part on the location of the proteins relative to the site of ROS generation, and their composition and structure (Droge, 2002). Some amino acids, for example, tryptophan, tyrosine, histidine and cysteine, are much more susceptible to more oxidation than others, and ROS can also alter the secondary and tertiary structure of proteins (Droge, 2002). The $OH^{\cdot}$, $RO^{\cdot}$, and RNS also predominantly cause protein damage. The consequences of protein damage as a response mechanism to stress are loss of enzymatic activity, altered cellular functions such as energy production, interference with the creation of membrane potentials and changes in the type and level of cellular proteins (Kohen and Nyska, 2002). Following protein oxidation, modified proteins are susceptible to many changes in their function. These include chemical fragmentation, inactivation, and increased proteolytic degradation of proteins.

Damage to lipids is also of great significance, as this can have major consequences for membrane structure and function in particular. Membrane composition, which is very important to the membrane function and possibly to metabolic rate, influences susceptibility to oxidative damage. Polyunsaturated fatty acids (PUFAs) are much less resistant to peroxidation than monounsaturated or saturated fatty acids, and so variation in the proportion of PUFA in membranes can influence the rate of oxidative damage. PUFA, which are easily oxidized and may stimulate chain reactions resulting in further oxidative damage, which can compromise the integrity of the cell. In this process, abstraction of a hydrogen atom by a ROS results in conjugated diene formation, which renders the lipid more susceptible to further oxidation process. Its subsequent reaction with molecular

oxygen results in formation of a lipid peroxyl radical capable of oxidising a neighbouring lipid and thus propagating the oxidative damage, involving a range of reactive intermediates that can then also cause protein and DNA damage (Lykkesfeldt and Svendsen, 2007).

Involvement of oxidative stress in porcine diseases

Oxidative stress is the cause or consequence of hundreds of diseases, in both acute and chronic conditions, from diabetes to Alzheimer's disease. A large amount of studies documented the role of oxidative stress in human diseases; few studies about oxidative stress in farm animals are available in literature. Farm animal diseases due to oxidative stress have primarily been researched in pigs, cattle and horses, with a focus on ascorbate levels and $NO^{\cdot}$ production. Studies in pigs and cattle have been somewhat sporadic and mainly with infectious diseases, such as pneumonia, enteritis, mastitis, endometritis and sepsis. Studies in horses, in particular racing horses and horses with airway obstruction has been more systematic, including recurrent airway obstruction (RAO), exercise-induced pulmonary haemorrhage (EIPH), racing induced oxidative stress, laminitis, arthritis and intestinal strangulation.

Pneumonia

Piglets and adult pigs used as models of the potential toxicity of combined treatment of human neonates with inhalation of high levels of O_2 and $NO^{\cdot}$ (Robbins et al., 1995) and for evaluation of a potential anti-inflammatory effect of inhaled $NO^{\cdot}$ in a porcine model of cardiopulmonary bypass-induced pulmonary inflammation (El Kebir et al., 2005).

Enteritis

Physiologically, enteric nervous system release of $NO^{\cdot}$ plays a role in propagation of intestinal contents, and vascular release of $NO^{\cdot}$ influences intestinal blood flow. The activity of a biomarker of granulocyte infiltration and intestinal inflammation, myeloperoxidase, paralleled the increase in nitrite levels. The nitric oxide synthase (NOS) inhibitor L-NAME reduced epithelial permeability. This suggests that intestinal nitrite production may be a useful biomarker of gut injury. In addition, $NO^{\cdot}$ may contribute to repair of the intestinal epithelial barrier after gut injury (Lykkesfeldt and Svendsen, 2007).

Sepsis

The sepsis syndrome in piglets is a relatively frequent event, characterized by altered vascular tone and organ perfusion and, in particular, pathologically elevated pulmonary pressure. Intestinal motility disturbances are also associated with sepsis. Porcine endotoxaemic shock seems to be associated with increased oxidative stress and damage. Basu and Eriksson (2000) found decreased antioxidant status, as measured by a-tocopherol, and increased lipid peroxidation, as measured by isoprostanes, in pigs with endotoxaemia. Eight isoprostanes were measured as a biomarker of lipid peroxidation and NOS inhibition attenuated the sepsis-induced increase in oxidative damage, thereby supporting a role for $NO^{\cdot}$ in the oxidative stress observed in sepsis (Matejovic et al., 2004).

Antioxidants with markers of oxidative status

Antioxidants are substances, which frequently counteract free radicals and inhibit the damage caused by oxidative stress. These can largely decrease the harmful damage due to oxidants by crumbling them before they react with physiobiologic targets, stopping chain reactions or inhibiting the activation of oxygen to highly reactive products (Azzi et al., 2004).

Antioxidants can be classified into two major groups, i.e., enzymatic and non-enzymatic. Some of these antioxidants are endogenously produced from the body, which include enzymes, low molecular weight molecules and enzyme cofactors. Non-enzymatic antioxidants are obtained from dietary sources. Dietary source of antioxidants can be classified into various classes (Liu, 2004), of which polyphenols is the largest class. Polyphenols composed of phenolic acids and flavonoids. Another classes of dietary antioxidants consist of vitamins, carotenoids, organosulfural compounds and minerals (Figure 2). However, the antioxidants that were used, tested and evaluated for the treatment and prevention of oxidative stress are presented in table 2.

Enzymatic antioxidants

Enzymatic antioxidants consist of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), etc. Antioxidant enzymes, SOD and CAT, are not consumed and both enzymes have high affinity and rate of reaction with ROS. Thus, it may be assumed that the enzymes afford more effective protection system against acute massive oxidative insults in the mammalian, example, hyperoxia or inflammation. In treating severe acute insults due to oxidative stress, antioxidant

enzymes are more potential agents (Christofidou-Solomidou and Muzykantov, 2006). SOD and CAT are the utmost strong antioxidants recognized in nature. There are three types of SODs namely cytosolic CuZn-SOD, mitochondrial Mn-SOD and extracellular SOD. CAT occurs abundantly in the liver, with the highest activity, followed by erythrocytes, then the lungs. SOD catalyses dismutation of superoxide into oxygen and hydrogen peroxide and it is widespread in nature in eukaryotic and prokaryotic organisms (McCord, 1986).

To protect cells, CAT catalysing hydrogen peroxide decomposition into molecular oxygen and water with no free radical production. Moreover, CAT produces on toxic compounds namely phenols, formic acid, formaldehyde and alcohols by peroxidative reaction. It is now well reported that the mitochondria are the main and major producers and also the main targets of ROS.

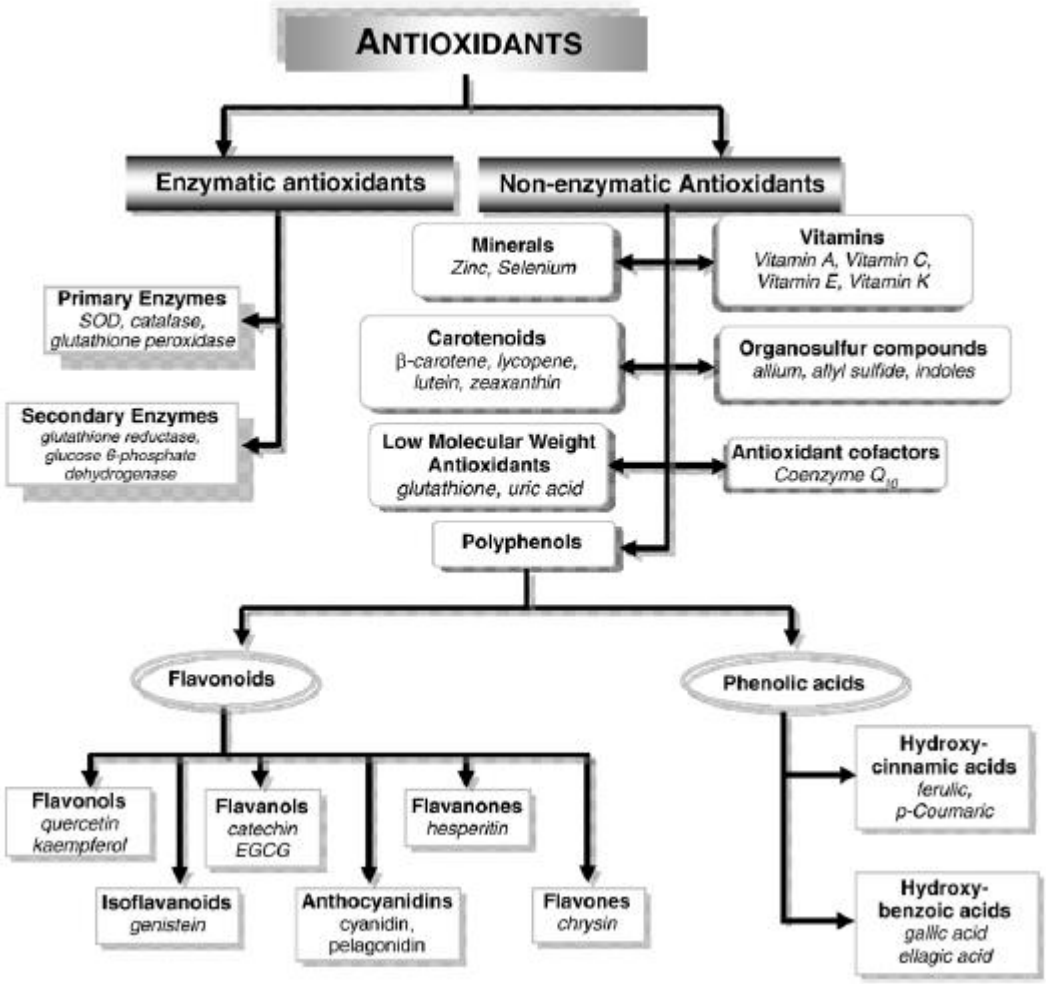

Figure 2. Classification of antioxidants. Some non-enzymatic antioxidants like uric acid, vitamin E, glutathione and CoQ10 are synthesized in the mammalian bodies and they can also be derived from dietary sources. Polyphenols are the major class of antioxidants which are derived from diet (Ratnam et al., 2006).

Many research data report that an excess production of ROS with free radicals in mitochondria result to increased expression of Mn-SOD. The stimulation of Mn-SOD gene expression due to oxidative stress may be one of the self-defence mechanisms to improve oxidative damage to mitochondria. Increase accumulation of ROS causes mitochondrial permeability transition and disorders the mitochondrial membrane potential, thereby causing cells to undergo apoptosis or necrosis.

Studies in intact animals and in humans shown that SOD and CAT give only modest advantage being potent antioxidants. The potentials of these agents are yet to be developed into effective, reliable and safe antioxidant therapies. The unfavorable results of animal and clinical studies can be recognized due to unfavourable pharmacokinetic profiles and inadequate delivery of SOD and CAT.

Antioxidant	Mechanism of action
SOD	Dismutation of superoxide to H_2O_2
CAT	Decomposes H_2O_2 to molecular oxygen and water
NAC	Scavenging of H_2O_2 and peroxide
	Deacetylation of precursor for GSH synthesis
GSH	Intracellular reducing agent
EGCG	Metal chelation
	Scavenging of superoxide, H_2O_2, OH and singlet oxygen
	Tocopherol regeneration
Lycopene	Trapping of singlet oxygen
Ellagic acid	Scavenging of H_2O_2
	Stimulation of glutathione-*S*-transferase
CoQ_{10}	Inhibition of lipid peroxidation
	Reduces mitochondrial oxidative stress
I3C	Inhibition of DNA-carcinogen adduct formation
	Suppression of free radical production
Genistein	H_2O_2 scavenging
Quercetin	H_2O_2 scavenging, one of the potent antioxidant among polyphenols
Vitamin C	Scavenging of superoxide anion by forming semidehydroascorbate radical which is subsequently reduced by GSH
Vitamin E	Direct scavenging of superoxide
	Upregulation of antioxidant enzymes
	Inhibition of lipid peroxidation

SOD=superoxide dismutase, CAT=catalase, NAC=*N*-acetyl cysteine, GSH=glutathione, EGCG=epigallocatechin-3-*O*-gallate, CoQ_{10}=coenzyme Q_{10}, I3C=indole-3-carbinol.

Table 2. Some selected antioxidants and their mechanisms of action (Ratnam et al., 2006).

For example, the enzyme CAT essentials to be transported to sites where the level of hydrogen peroxide increases in the vicinity of metastasizing tumour cells in order to succeed CAT-based inhibition/prevention of tumour metastasis (Nishikawa et al., 2005). SOD and CAT are poorly absorbed from and rapidly degraded during passage in GIT. Both of these enzymes have very short life spans in the blood stream after intravenous administration (Giri and Misra, 1984, Regnault et al., 1996, Baynes,

2005). Hepatic uptake and renal excretion are the major pathways for the elimination of antioxidant enzymes.

Non-enzymatic antioxidants

Endogenous non-enzymatic antioxidants are defined in two phases: lipophylic (vitamin E, carotenoids, etc.) and water soluble (vitamin C, glutathione, uric acid, etc.). Three antioxidant vitamins, A, C, and E, help defence in the body against oxidative damage. Vitamin C performances in the aqueous phase whereas vitamin E acts in the lipid phase which act as a chain breaking antioxidants. Vitamin C decreases $O_2^{\bullet -}$ and lipid peroxyl radical, but vitamin C is also a recognized as a synergistic agent for vitamin E (Baynes, 2005, Bielski and Cabelli, 1991). Vitamin E is the furthermost extensively distributed antioxidant in nature. Both Vitamin C and E work together to prevent lipid peroxidation reactions in plasma lipoproteins and membranes. Vitamin A is a potent free radical scavenger and is also lipophylic antioxidant (Baynes, 2005). Carotenoids, the precursor of vitamin A, can apply antioxidant effects, and also quench singlet O_2. There is considerable *in vitro* evidence for interaction of β-carotene with free radicals, for its properties as a chain-breaking antioxidant and in scavenging and quenching singlet oxygen (Miller et al., 1996).

However, a number of compounds synthesized endogenously function as non-enzymatic antioxidants. Whereas uric acid can directly scavenge $OH^{\bullet}$ and peroxy radicals, melatonin, the chief secretory product of the pineal gland, scavenges $O_2^{\bullet -}$, H_2O_2, $HO^{\bullet}$, peroxynitrite anion and lipid peroxides (Smith et al., 2005, Reiter et al., 1998, Reiter et al., 2003). Bilirubin is a sensitizer of singlet O_2 production. It behaves as an antioxidant especially the albumin bound fraction. Erythrocyte bilirubin acts as photosensitizer in the presence of phototherapy and causes oxidative damage (Dahiya et al., 2006). Although bilirubin is regarded as toxic when present at high concentrations, it has been postulated as a transitional antioxidant in the first few days of life before other antioxidant defence mechanisms mature (Turgut et al., 2004).

A number of different electron transport processes are present in the erythrocyte membrane. Redox cycling of drugs or other xenobiotics can generate ROS in red cells (Al-Omar et al., 2004). Some of these membrane transport systems have an antioxidant role. These processes appear sensitive to their immediate environment and cellular redox state as large variations in oxidoreductase (NADH and ascorbate oxidoreductases) activities have been reported in erythrocyte membranes. NADH, arachidonic acid (AA), flavonoids, ubiquinone, and a-tocopherolquinone act as electron donors to membrane redox systems (Kennett and Kuchel, 2003).

Mechanisms of defence against oxidative stress

Due to the considerable background exposure to oxidants resulting from a life depending on molecular oxygen, aerobic mammals have improved to constantly fighting a combat against oxidative stress. Advanced levels of cellular defence approaches have grown and gradually prolonged the potential lifespan for the individual species. The cellular defence mechanisms can be consisted into at least three levels according to their function of quenching oxidants, repairing/ removing oxidative damage or encapsulating non-repairable damage (Figure 3).

As a first level of body defence system against oxidants, the cell is prepared with a so-called antioxidant network. Antioxidants are able of donating electrons ion to oxidants, therefore quenching their reactivity under controlled conditions and making them harmless to cellular biomacromolecules. The antioxidants thereby become radicals themselves, but these are far more stable and are not capable of inducing cellular damage. The oxidised antioxidants are consequently recycled to their active reduced state through a number of efficient cellular processes powered by energy from NADPH. This recycling is the key to the power of the antioxidant network, which would otherwise deteriorate rapidly (Lykkesfeldt et al., 2003). The antioxidant network can be consisted into two primary groups, namely, the low molecular weight (non-enzymatic) and high molecular weight (enzymatic) antioxidants. Vitamins C and E and glutathione (GSH) are the low molecular weight antioxidants. Whereas, high molecular weight antioxidants are few and often possess specialized functions, e.g. superoxide dismutase catalyses the dismutation of two molecules of $O_2^{\cdot-}$ into one molecule of dioxygen and one of H_2O_2, but catalase and GSH peroxidase are directly targeted at removing H_2O_2 leaking from the electron transport chain. Dehydroascorbic acid reductase and GSH reductase help the recycling of the antioxidants vitamin C and GSH, respectively.

A second and highly important level of defence is the ability to detect and repair or remove oxidised and damaged molecules. Included in this part of the defence is a series of DNA repairing enzymes capable of detecting oxidised bases or misincorporations, cutting them out and inserting the correct undamaged base in the DNA. The extent of this activity is enormous; it has been estimated that the DNA of each living cell is subjected to 10,000 to 100,000 oxidative modifications per day, more than 99.99% of which are enzymatically repaired. Other means of second level defence include catabolism of non-functional or modified proteins and lipids.

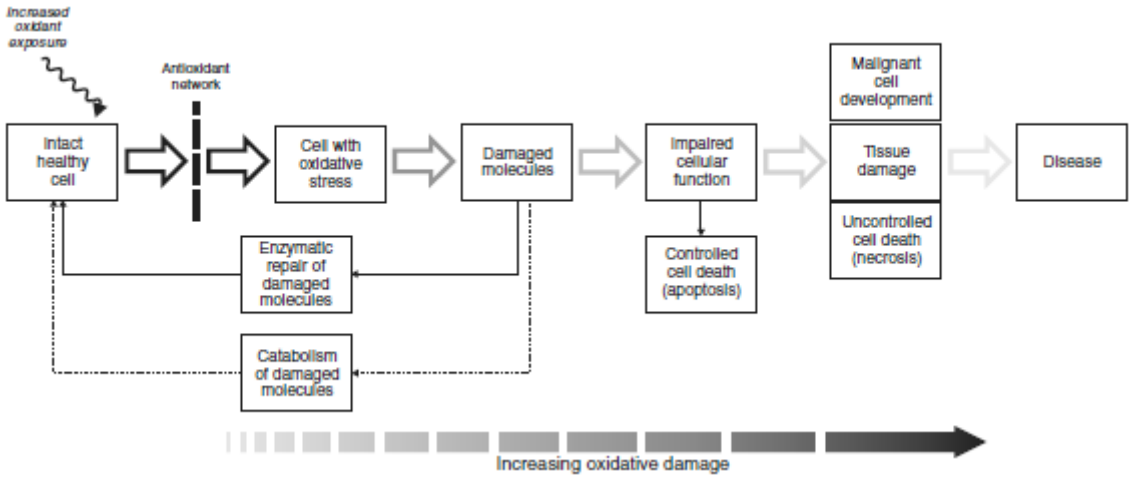

Figure 3. Schematic outline of cellular defences against oxidative stress-mediated cellular damage. Increased oxidative stress is initially counteracted by the antioxidant network. Damaged molecules are either repaired or catabolised. Controlled cell suicide can be initiated if further oxidative damage leads to impaired cellular function. When these signalling cascades are damaged or the oxidative damage exceeds the capacity of the defence mechanisms, uncontrolled cell death, tissue damage and malignant cell development can progress into disease (Lykkesfeldt and Svendsen, 2007).

Finally, if the degree of the oxidative damage exceeds the capacity of repair and removal, the organism is prepared with one final weapon, controlled cell suicide or apoptosis (Payne et al., 1995). The ability to induce programmed cell death is of major importance in a variety of bodily functions, including control of tissue growth, and is apparently under control by several signalling pathways. However, one of these appears to be that apoptosis is induced by increased oxidative stress and thus constitutes a final resort to encapsulate and isolate the damaged cells (Payne et al., 1995).

Additives to improve antioxidant status

The dietary intake of antioxidants is thought to play a major role in the antioxidant network. The indirect antioxidant effect (up-regulation of the antioxidant defence or repair systems) may be evoked by xenobiotics or components in vegetables that are not scavengers or even considered harmful; e.g., isothiocyanates are oxidants that stimulate cellular antioxidant proteins and detoxification enzymes. Antioxidants such as vitamin C, vitamin E, carotenoids, and flavonoids have been identified in many natural food products. Natural products also contain mixtures of other antioxidants and bioactive substances with unknown antioxidant properties (Moller and Loft, 2006). Plants contain high concentrations of numerous redox-active antioxidants, such as polyphenols, carotenoids, tocopherols, glutathione, ascorbic acid and enzymes with antioxidant activity, which fight against hazardous

oxidative damage of plant cell components. In animal cells, antioxidant production is much more limited. Therefore, plant-sourced food antioxidants like vitamin C, vitamin E, carotenes, phenolic acids, phytates and phytoestrogenes have been recognized as having the potential to reduce oxidative damage in animals. The intake of food rich in α- tocopherols, β-carotene and ascorbic acid has been associated with reduced oxidative-stress related diseases. Phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl, thus inhibiting the oxidative mechanism that lead to degenerative diseases (Pisoschi et al., 2009).

Many studies have reported that dietary supplements such as antioxidants, vitamins, and minerals prevent or at least attenuate organic impairment originated by excess oxidative stress (Girard et al., 2005). Inhibition of oxidative damage by supplementation of antioxidants becomes an attractive therapeutic strategy to reduce the risk of these diseases (Dıaz et al., 2004). There are, in addition, several nutritionally essential minerals incorporated into protective antioxidant enzymes. Zinc, copper, and manganese are required for activity of the two types of superoxide dismutases. Selenium, an essential component of glutathione peroxidase, is important in the decomposition of hydrogen peroxide and lipid peroxides. The level of dietary intake of all the antioxidant micronutrients directly affects the circulating level of these nutrients and the activity of the antioxidant metalloenzymes. Thus, low intakes of one or more of these antioxidant nutrients could reduce the body's defences against free radical damage and increase susceptibility health problems associated with free radical damage (Machlin and Bendich, 1987).

The use of antibiotics as growth promoters in animal feeds is facing reduced social acceptance due to the appearance of residues and resistant strains of bacteria. The use of antibiotics has been prohibited in the European Union since January 2006 (Regulation 1831/2003/EC). Natural, safe and inexpensive feed additives that do not endanger the environment with residues in wastes should be developed. There has been a revival of phytogenic feed additives (PFA) as a successful alternative to the prophylactic use of antibiotics. The action of PFA is a result of natural substances that contain low-molecular-weight reactive oxygen species-scavenging substances, where polyphenols are included among these. PFA influenced positively daily feed intake, daily weight gain, and feed utilization in growing pigs and improved growth performance in pigs (Wang et al., 2008). Most of these active secondary plant metabolites belong to the classes of isoprene derivatives, flavonoides and glucosinolates, and a large number of these compounds has been suggested to act as antibiotics or as antioxidants *in vivo* as well as in food (Wenk, 2003). Some antioxidants are used to protect the nutrients in the feed during storage. Others have their main activity in the digestive tract where they may also help that the substances sensible for oxidation can be absorbed. In the intermediate metabolism antioxidants are responsible for many functions like reduced aging or the protection of intact membranes. In farm animals antioxidants can have a direct influence on the product quality (Wenk, 2003). Amrik and Bilkei (2004) and Khajarern and Khajarern (2002) found an improvement in productive performance, feed conversion and feed intake

in sow fed with oregano;(Kyriakis et al., 1998) found that medication with oil of Origanum was effective in controlling post-weaning diarrhoea syndrome in piglets and also reduced the mortality rate. Use of antioxidant in animal feeding reduces lipid oxidation in meat, responsible of colour, flavour, nutritive value and in general improves meat pork (Mason et al., 2005, Corino et al., 1999, Corino et al., 2007), chicken (Botsoglou et al., 2002) and rabbit (Botsoglou et al., 2004) quality.

Functional Role of the Nrf2 System: From Comparative Animal Studies

Since the first finding of Nrf2 as a master regulator of indirect antioxidant genes, various comparative animal studies using *nrf2-/-* mice have been performed to investigate the role of Nrf2 in the mammalian defence system. It is now widely accepted that cells and animals with a *nrf2* null genotype are much more sensitive to environmental or oxidative stress conditions, leading to accelerated macromolecular damage, mutations, and apoptosis. The initial study by Chan and Kan (1999), demonstrated that *nrf2-/-* mice are highly susceptible to butylated hydroxytoluene (BHT)-induced lung damage and lethality in comparison to wild-type mice. A following independent study showed that Nrf2 is a critical factor for determining susceptibility to hyperoxic concentration of oxygen-induced lung injury in mouse (Cho et al., 2002a, Cho et al., 2002b). As a proof of its role in the CNS, astrocytes from *nrf2-/-* mice showed higher rates of cell death in response to hydrogen peroxide treatment (Lee et al., 2003). Murine embryonic fibroblasts (MEFs) isolated from *nrf2-/-* mice showed higher levels of cell death in response to treatment with the redox-cycling ROS generator menadione and GSH-depleting anticancer agent cisplatin (Cho et al., 2008, Kwak et al., 2004). Incubation with diquat dibromide, another redox cycling bipyridylium herbicide, MEFs displayed markedly decreased cell viability, increased lipid peroxidation and GSH oxidation in comparison to wild-type cells (Osburn et al., 2006).

Many electrophiles can cause oxidative stress leading to DNA mutations and carcinogenesis. As Nrf2 is a prime regulator for the expression of electrophile-detoxifying enzymes, the Nrf2 system has been recognized as a susceptibility determinant in response to chemical carcinogens. The first demonstration by Ramos-Gomez et al., (2001) showed that the incidence of gastric tumours was significantly increased in *nrf2-/-* mice following B[*a*]P treatment and B[*a*]P-DNA adduct levels were concomitantly increased in these mutant mice (Ramos-Gomez et al., 2001, Ramos-Gomez et al., 2003). Aoki et al., (2001) demonstrated that exposure of *nrf2-/-* mice to diesel exhaust particles, which are postulated as a probable causal factor in lung cancer, resulted in higher levels of oxidative DNA damage with concomitant increases in lung injury (Aoki et al., 2001). The incidence of urinary bladder carcinoma by BBN was significantly higher in *nrf2-/-* mice than in wild-type mice (Iida et al., 2004). When treated with arsenic, *nrf2-/-* mice showed more severe

pathological changes in the liver and bladder, and arsenic induced DNA hypomethylation was significantly elevated in the absence of *nrf2* (Jiang et al., 2009).

Recent reports support the role of Nrf2 in the inhibition of inflammatory injuries. Following treatment with lipopolysaccharide (LPS), peritoneal neutrophils from *nrf2*-/- mice exhibited increased NADPH oxidase-dependent ROS generation and levels of TNF-alpha, IL-6 and chemokines (Mip2 and Mcp-1) compared to wild-type neutrophils (Thimmulappa et al., 2006). *Nrf2*-/- macrophages were more susceptible to damage induced by reactive oxygen/nitrogen species, as well as acrolein and cadmium, macrophage toxins (Zhu et al., 2008). In addition to its role in inflammation, Nrf2 plays an inhibitory role in the fibrogenic process: bleomycin-induced pulmonary fibrosis and cyclosporine-mediated renal fibrosis were aggravated in *nrf2*-/- mice (Cho et al., 2004, Shin et al., 2010). All together these reports confirmed the critical role of direct and indirect antioxidant proteins, which are under the control of Nrf2, for cytoprotection against divergent arrays of oxidative damage.

Lipopolysaccharide

Lipopolysaccharide (LPS) also known as endotoxin which can enhance localized or systemic inflammation through the activation of receptors namely pattern recognition receptors. Moreover, LPS associated with inflammation can control intestinal epithelial function via altering epithelial barrier integrity, nutrient transport and utilization. Normally both Gram positive and negative bacteria can growth and multiply in the gastrointestinal tract, of which the Gram-negative bacteria provide as a source of LPS. Luminal LPS can enter circulation through three major routes: 1) nonspecific paracellular penetration via tight junctions in epithelial cell and 2) transcellular penetration by lipid raft membrane domains involving receptor mediated endocytosis, 3) through micellar assisted penetration when fat is consumed with diet. Dissociation of tight junction protein complexes help paracellular penetration of LPS resulting in reduced intestinal barrier integrity, which can be a result of enteric disease, inflammation, or environmental and metabolic stress. Transcellular penetration occur through specialized membrane regions which rich in glycolipids, sphingolipids, cholesterol as well as saturated fatty acids, is a result of raft recruitment of LPS-associated signalling proteins causing to signalling and endocytosis. Diet can be modifying for all permeability routes and sensitivity to LPS. Intestinal originated LPS and inflammation cause in suppressed appetite, stimulate of the immune system, and partitioning of energy and nutrients away from growth towards supporting the immune system requirements. Consequently, this leads to the suppression of growth, mainly, suppression of lean tissue accretion in livestock.

Various physical, social, and microbial factors are affected to the growth performance of animals and human health which influences animals to physiological or immunological stresses (Holck et al., 1998). Viruses, cell wall compound of live bacteria, and dead bacteria such as lipopolysaccharide (LPS) and peptidoglycans can

act as stressors that can attenuate the growth performance and alter metabolism (Schinckel et al., 1995, Smith, 1998). Significantly, the bacteria present in the intestinal lumen acts as a major source of LPS, on the other hand, the mucosal epithelium of the gastrointestinal tract serves as a major barrier to the LPS (Ravin et al., 1960, Schweinburg and Fine, 1960, Wiznitzer et al., 1960). The cell wall component (LPS) of gram-negative bacteria is a powerful immune stimulator in the circumstance of livestock production and human health. Different cells expressing the pattern recognition receptor, Toll-like receptor (TLR)4, and other proteins including LPS binding protein (LBP), cluster of differentiation 14 (CD14), and MD-2 that can help to recognized lipopolysaccharide in mammals. These proteins and receptors are also expressed by the intestinal epithelial cells and associated with the permeability of luminal LPS into circulation (Hornef et al., 2003, Neal et al., 2006). Moreover, LPS could be deactivated or detoxified by immune cells, such as macrophages, Kupffer cells or splenic cells, or by binding with plasma proteins after enter into general circulation (Buttenschoen et al., 2010, Rutenburg et al., 1967, Satoh et al., 2008). But, there is more permeability of LPS from the intestinal tract due to failure of systemic detection and deactivation which causing increased amount of circulating LPS can lead to systemic inflammation, results endotoxemia, multi-organ failure and even death (Rice et al., 2003, Zweifach and Janoff, 1965).

The chronic activation of the immune system by LPS to livestock production has been shown to antagonize the growth and performance of animals due to nutrients are being partitioned towards production of various immunological components such as cytokines, acute phase proteins, and other immune modulators rather than towards the anabolic processes that maintain milk and muscle synthesis (Johnson et al., 1977, Spurlock, 1997, Johnson, 1997). Moreover, LPS can cause different diseases such as laminitis in equines and also endotoxemia can cause of death in equine species finally (Sykes and Furr, 2005, Werners et al., 2005). Also lipopolysaccharide has been helped to stimulate the heterophils and up-regulate the pro-inflammatory cytokine with chemokine expression in poultry species (Kogut et al., 2005). In human population, presence of LPS in the circulation have been stimulated to contribute to the progress of chronic inflammatory processes that ultimately support the development of dysregulated metabolism due to many metabolic diseases such as type II diabetes and non-alcoholic fatty liver disease by the stimulation of TLR4 (Erridge, 2011).

Fascinatingly, dietary factors and stressors (heat stress, systemic disease and feed restriction or malnutrition) have been revealed to have effect on permeability of intestinal LPS (Cani and Delzenne, 2009, Hall et al., 2001). Dietary fat is the major factor that responsible to adapt the permeability of luminal LPS. Many authors indicated that increase percentage of dietary fat affected the concentration of circulating LPS (Amar et al., 2008, Erridge et al., 2007). Moreover, the ingested form of lipid may modulate the LPS permeability as well as emulsified lipids increasing LPS permeability (Laugerette et al., 2011). In case of ruminants, the permeability of LPS to the peripheral circulation has been revealed to enhance by feeding easily-digestible carbohydrates and grains (Khafipour et al., 2009, Zebeli et al., 2011).

Furthermore, dietary nutrients along with systemic increases in intestinal originated LPS could be caused to immunological and environmental stressors. Intestinal permeability and most probably, intestinal LPS permeability increases by hyperthermia (Lambert, 2004, Lambert, 2008). Plasma antibodies to LPS are inversely related to growth in malnourished young children and are associated with increased intestinal permeability and systemic immune system activation (Campbell et al., 2003). Further studies are needed to explore the relationship between LPS, growth, and metabolic changes.

Structure of lipopolysaccharide

Lipopolysaccharide consists of a glycolipid, which is present in the outer membrane of Gram-negative bacterial cell wall. Also it composed of a hydrophobic domain, interior lipid A, through which it is inserted into the outer leaflet of the outer membrane of the bacterial cell wall, a core polysaccharide (inner and outer core) and a distal polysaccharide (Elin and Wolff, 1976, Raetz and Whitfield, 2002). The most biologically active portion of the LPS molecule namely the hydrophobic interior lipid A domain which is synonymously known as endotoxin owing to its ability to stimulate the innate immune cells (Erridge et al., 2002).

In a wild type *Escherichia coli*, lipid A contains the following structural properties: 1) the backbone of the lipid A contains di-glucosamine, which is phosphorylated at positions 1 and 4'; 2) two 3-hydroxymyristate molecules are directly attached to each glucosamine, and 3) at positions 2' and 3', the hydroxyl groups of the fatty acids are substituted by laurate and myristate, and they form an acyloxyacyl bond with the primary fatty acid chains (Figure 1). Stimulators of the immune system have been effective by diphosphorylated hexaacyl lipid A due to optimally recognized by the mammalian immune system. Mono-phosphorylated or dephosphorylated LPS molecules exposed to substantially lose their potency and immune reactivity (Holst et al., 1996, Munford, 2005).

However, monophosphorylated lipid A acts as a potent adjuvant which is being formulated to use into human vaccines production. Another most important feature of lipid A is that it is mostly composed of all the fatty acyl chains which are made up of saturated fatty acids. LPS molecule could induce an attenuated immune response while the saturated fatty acids are replaced with unsaturated fatty acids (Kitchens et al., 1992, Munford and Hall, 1986).

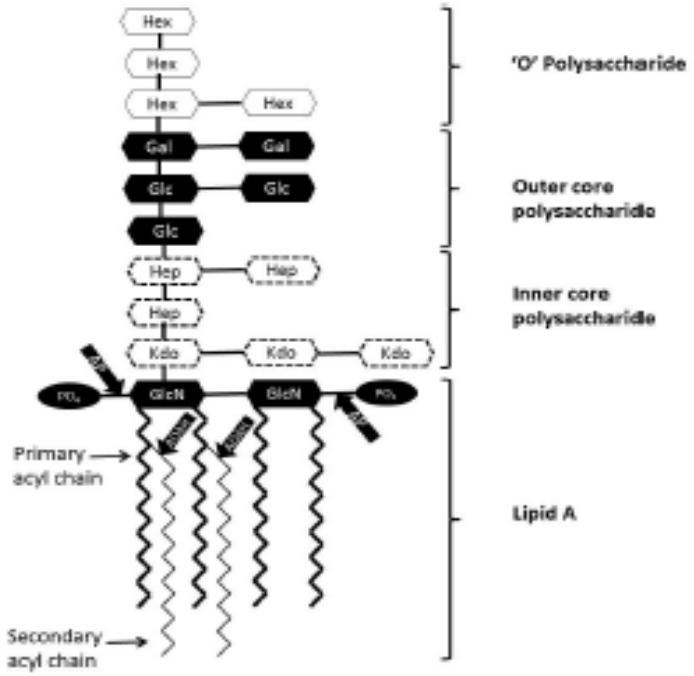

Figure 1. Simplified structure of lipopolysaccharide (LPS) from Gram negative bacteria such as *Escherichia coli*. Lipopolysaccharide contains a distal 'O' polysaccharide region, a core polysaccharide region divided into outer and inner core and an interior lipid A component through which LPS is inserted into the cell membrane. 'O' polysaccharide region is highly variable and contains approximately 10 to 25 repeated units and made up of common hexose (Hex) sugars. Outer core polysaccharide contains common hexose sugars such as glucose (Glc) and galactose (Gal) whereas inner core polysaccharide contains unusual sugar such as 3-deoxy-D-manno-octulosonic acid (Kdo). Lipid A structure is explained in the text. Arrows with acronyms AP (alkaline phosphatase) and acyloxyacyl hydrolase (AOAH) indicate the cleavage points where these enzymes cleave the phosphate and secondary fatty acyl chains respectively. GlcN - N-acetyl glucosamine; Hep – Heptose (Mani et al., 2012).

Lipopolysaccharide can come in systemic blood circulation from live bacteria, or as cell wall components of dead bacteria. Either way, if the amounts of LPS are too great causes ultimately antagonize anabolic growth (Kimball et al., 2003, Orellana et al., 2007) or lead to septic shock and death (Moore and Morris, 1992). Also lipopolysaccharide is released in another ways by bacterial death, growth and division, resulting it a ubiquitous contaminant (Petsch and Anspach, 2000, Yaron et al., 2000). Endotoxin units (EU) are the measurement unit of biological activity of LPS. For example, 100 pg of LPS is considered to have 1 EU of activity, or 10 EU is equivalent to 1 ng of LPS. Approximately 10^{-15} g of LPS generates from single Gram-negative bacteria and 10^5 equivalent numbers of bacteria can create 1 EU. It has been shown

that about 10^6 lipid A residues produce from single *E. coli* (Raetz et al., 1991). Moreover, the molecular size of the individual LPS molecules varies between 10 to 20 kDa in monomeric form and because of the amphiphilic nature; finally they can assemble themselves into large micellar structure achieving 1,000 kDa.

Innate immune response against lipopolysaccharide

The mammalian innate immune response is the first line of defence against any types of infectious diseases and foreign particles. Compared to adaptive immune system, innate immune system is immediate response and it usually activated within minutes to hours against a stimulus, on the other hand, the adaptive immune system is delay response which takes hours to days (Janeway and Medzhitov, 2002). There are five key elements for protection in innate immune response: 1) physical barriers (skin, epithelial layers, and mucus) to prevent the entry of pathogens; 2) antimicrobial enzymes like lysozyme and muramidase in body fluids to kill the invading foreign agents; 3) a recognition system such as germ line encoded pattern recognition receptors (PRRs) like TLRs and NOD-like receptors permit for immediate detection and response against foreign particles; 4) the complement system as a antimicrobial responses; and 5) the enrolment of other immune cells in the body for an enhanced response (Aderem and Ulevitch, 2000, Beutler, 2004, Heeg, 2007, Hoffmann et al., 1999). As like as adaptive response, the mammalian innate immune system can also be divided into cellular and humoral components. Cellular component is composed of hematopoietic and non-hematopoietic cells. Furthermore, hematopoietic cells like macrophages, dendritic cells, mast cells, neutrophils, eosinophils, natural killer cells and non-hematopoietic cells such as epithelial cells constitute the cellular component. But, humoral component consists of complement proteins, LPS binding proteins, C-reactive protein, collectins, and anti-microbial peptides like defensin's (Turvey and Broide, 2010).

The mammalian innate immune system was thought to act as a non-specific way; but this system was only partly true and that innate receptors identified a narrow range of particular organisms' components, e.g. TLR4 can distinguish LPS from many organisms that hypothesis proved by a sequence of discoveries in the late-1990s (Medzhitov et al., 1997). Macrophages, dendritic cells, B cells, and certain types of T cells contain specific receptors that could distinguish a particular pattern in the invading microbes which come to be known as PRRs (Medzhitov, 2001, Janeway and Medzhitov, 2002). Furthermore, myocytes and adipocytes also express as like as PRRs (Gabler and Spurlock, 2008). These 'patterns' present in the different microbial species contain these patterns which are necessary for their survival, called as pathogen associated molecular patterns (PAMP), later to be change named as a microbe associated molecular patterns (MAMP) to take account of all type of microbes including pathogens and non-pathogens (Akira et al., 2006, Ausubel, 2005). Jointly, the family of PRRs recognizes the attendance of a various type of molecules from the invading pathogens with commensals, and stimulating the secretion of

variety of immune mediators which regulates the immune response (Brikos and O'Neill, 2008). In recent time, PRRs have been shown to recognize cellular degradation products from the same organism along with pathogenic patterns and commensals, which are called as damage associated molecular patterns (Chen and Nunez, 2010, Rosin and Okusa, 2011). TLR4 was identified as a first Toll-like pattern recognition receptor, which identifies bacterial LPS, heat shock proteins and other proteins (Poltorak et al., 1998). At current situation, there are 11 human and 13 murine TLRs which identifies various pathogen components together with flagella, peptidoglycan, double-stranded RNA, and DNA (McGettrick and O'Neill, 2010, Moresco et al., 2011). PRRs recognized the inflammatory compounds, which lead to the activation of the master transcription factor nuclear factor-κB (NF-kB). Increased transcription and secretion of a class of pleiotropic molecules, known as cytokines occurs after NF-kB activation. Cytokines subsequently perform on other cells in the mammalian body to stimulate and enhance specific cellular immune response (Lenardo and Baltimore, 1989). Cytokines can make use of autocrine, paracrine, and even endocrine functions. There are many functions of cytokines in mammalian physiological systems which are include development of cellular and humoral immune response, induction of the inflammatory response, regulation of haematopoiesis, control of cellular proliferation and differentiation, and healing of wounds (Arai et al., 1990, Kindt, 2007). There are two types of cytokines, which can be divided into, pro- and anti-inflammatory and lead to the increase or decrease in the magnitude of the inflammatory response. Tumour necrosis factor-α (TNF-α), interleukin (IL)-6 and IL-8 are act as pro-inflammatory, but IL-10 and transforming growth factor-β are considered anti-inflammatory. Immune cells and many other cell types are secreted various classes of cytokines (Ashley et al., 2012). For instance, IL-1β secreted by monocytes, endothelial cells and epithelial cells, TNF-α secreted by macrophages, IL-12 secreted by macrophages and dendritic cells, interferon-β secreted by fibroblasts which are some of the examples of cytokines of innate immune cells. For the example of cytokines of adaptive system, interleukin-2, interferon-γ and IL-4 secreted by T cells, T_H1 cells and T_H2 cells with mast cells respectively. Overall, cytokines exert their function via acting on five classes of receptors: 1) immunoglobulin superfamily receptors, 2) class I and 3) class II cytokine receptor family, 4) TNF receptor family and 5) chemokine receptor family (Borish and Steinke, 2003, Dinarello, 2000, Miyajima et al., 1992). Chemokines are a sub-family of cytokines which composed of about 90-130 amino acids and mainly helps in the leukocyte recruitment (Allen et al., 2007).

Classically, innate immune response is characterized by inflammation which is associated with clinical signs such as redness, pain, heat, swelling, and loss of function. Mammalian inflammation can be acute or chronic which is depending on the duration it takes to remove the immune response. The inflammatory process is usually compartmentalized to the affected tissue (Kindt, 2007). Pro-inflammatory mediators secrete by the interaction of innate immune cells with the invading agent, when the tissue damage happen by the immune stimulant in case of acute inflammation period. This results cause the classical signs of inflammation and

bringing together of leukocytes, antimicrobial mediators such as interferons, defensins and cathelicidins, complement system, clotting, kinins and fibrinolytic proteins, lipid mediators like leukotrienes, platelet-activating factor, prostaglandins, peptides and amines like histamine, serotonin, neuropeptides and pro-inflammatory peptides and cytokines such as IL-1α, IL-1β, TNF-α and IL-6 which eventually act to obliterate the invading agent and microorganism. Neutrophils are the first immune cells to be recruited which followed by macrophages, eosinophils, and platelets, and followed by lymphocytes against invading agent or microorganism., the anti-inflammatory process mediated by cytokines like IL-10 and transforming growth factor-β recover to limit the damage to the surrounding tissues, if the invading agent is eliminated from the mammalian body (Ballou, 2012, Dolgachev and Lukacs, 2010, Kindt, 2007, Libby, 2002).

The systemic inflammatory response may occur, if the local inflammatory process is not contained within the affected tissue, otherwise known as acute-phase response. Tenure of the initial response, the cytokines secreted in the mammalian body, after that those cytokines enters systemic circulation, which act, on mainly bone marrow, hypothalamus and liver. Bone marrow secreted the increase number of leukocytes population during stimulation of bone marrow by cytokines which needed to fight against the infection. On the other hand, cytokines causes to activation of hypothalamus by directly via cytokine receptors or indirectly via vagus nerve, which stimulates the secretion of prostaglandins, results in a fever response. The prevention of the growth of the pathogen and increases the overall immune response that help by this febrile response (Johnson, 1997, Karin et al., 2006, Wright et al., 2000). Change in homeostasis of the body and a complex acute phase response which regulated by fever with temporary resetting of the body's thermostatic set point subsequently causing an increase in core body temperature (Hasday et al., 2000, Kluger et al., 1996). During oxidative phosphorylation, fever also causes in uncoupling of electrons which resulting in decreased ATP synthesis and decreased feed efficiency. Furthermore, fever decreases the appetite and, thus, reduces of iron supply indirectly from the diet, which is necessary by most pathogens. Moreover, an increase in the production of transferrin and lactoferrin occur by the acute phase response which additional restricts the availability of iron for the pathogen (Kozak et al., 2000). Furthermore, stimulates the secretion of corticosteroids from adrenal cortex happen by hypothalamus, which activates the pituitary gland causing in the release of adrenocorticotropic hormone (ACTH). The secretions of acute phase proteins (APP) take place primarily from the liver by the collective action of the cytokines and hypothalamus-pituitary axis. The major APP secreted from liver are C-reactive protein, serum amyloid A, fibrinogen, haptoglobin, mannose-binding protein, and complement components (Steel and Whitehead, 1994). Among these APP, particularly C-reactive protein and serum amyloid A concentration increases significantly after an acute phase response. Acute phase proteins regularly bind to the inflammatory agent, which in turn helps, facilitates its neutralization through the complement system (Balaji et al., 2000, Eckersall and Bell, 2010, Gabay and Kushner, 1999).

Lipopolysaccharide detoxification

After absorbed LPS in the intestinal wall, LPS is circulated by both lymph and blood; on the other hand, most of the LPS are reached to the liver through the portal system mainly portal vein where a major portion of the LPS detoxification process happens (Lemaire et al., 1999, Olofsson et al., 1986, Van Leeuwen et al., 1994). Endotoxemia take place subsequently, if the increase amount of LPS entering GIT which overwhelms the barrier and absorption functions of intestinal epithelial cells (IECs) and the detoxification capacity of liver (Olofsson et al., 1985). Either the intestinal mucosal surface or in systemic circulation of mammals have elaborate system to resistant and detoxify LPS. The down-regulation of proteins of LPS can help to tolerate LPS which participate in LPS signalling and the innate immune response (Fan and Cook, 2004). The detergent action of bile salts in bile play vital role to detoxifying LPS in the intestinal lumen. Furthermore, portal circulates LPS detect by hepatocytes and macrophages (Kupffer cells) in liver which then active and inactive forms of LPS may be transferred into neutralized to the bile and excreted into the lumen (Bertok, 2004, Maitra et al., 1981). Near about 7 % of the absorbed LPS by mammalian body are excreted by bile. There are four systems described by (Munford, 2005) by which LPS may be neutralized in mammalian body system. First, LPS binds with molecules that prevent it from engaging TLR4. Second, degrade lipid A occur by enzymes to decrease its activity. Third, LPS can be deactivated directly subsequently its uptake by the liver. Lastly, some target cell in mammals can adaptations that modify the response to LPS. Moreover, some authors indicated that LPS shows less pyrogenic and less inflammatory activities when incubation of LPS with plasma (Rall et al., 1957, Rudbach and Johnson, 1964, Ulevitch and Johnston, 1978). Furthermore, the inactivation and detoxification of LPS occur more specifically which able to bind with specific plasma proteins (Brade and Brade, 1985, Johnson et al., 1977, Rudbach and Johnson, 1966). During the acute phase response, serum amyloid A shown to increase which also able to binds with LPS monomers and eliminates this toxin via the liver (Coetzee et al., 1986, Emmanuel et al., 2008). In addition, proteins like collectins, along with bactericidal permeability increasing protein and neutrophil granules, are also plasma proteins that bind and neutralize LPS (Chaby, 2004, Munford, 2005).

Intestinal chylomicrons act as a transporting the absorbed fatty acids which promoted to the absorption of LPS (Ghoshal et al., 2009). But, the toxic effects of LPS mitigate by binding the LPS with chylomicrons and resulting its inactivation through contact and the action of bile (Harris et al., 1993, Read et al., 1993). Moreover, LPS binding protein (LBP) can bind to the chylomicrons, which enhance the binding of LPS to the chylomicrons, causes helps in reducing its bioactivity (Vreugdenhil et al., 2003). Low density lipoproteins (LDL) helps to the recognition of binding of LPS to the chylomicron and hepatocytes contain LDL associated

receptors, which encourage the endocytosis of LPS into the cell for its rapid clearance from body circulation (Harris et al., 2002). The chylomicrons is also contain apolipoprotein E which protective against LPS. Moreover, apolipoprotein E transfers the LPS in a straight line to hepatocytes, bypassing Kupffer cells and their pro-inflammatory cytokine production (Van Oosten et al., 2001). High density lipoprotein (HDL) is also bind with LPS for detoxification purpose, but the role of HDL in detoxifying LPS seems to be controversial (Ulevitch et al., 1979). It is hypothesized that HDL helps in sequestering and detoxifying LPS that has a more complicated way to clear from circulation (Birjmohun et al., 2007, Vreugdenhil et al., 2003). Further, LBP helps LPS to be transferred from HDL to LDL and phospholipids transfer proteins. Dyslipidaemia occur due to the transfer of LPS to LDL which the loss of HDL's capacity to bind cholesterol resulting to metabolic diseases (Levels et al., 2005).

Acyloxyacyl hydrolase (AOAH) acts as a major detoxification mechanism for LPS by its enzyme modification. This hydrolase enzyme is classified as a lipase and is widely distributed in macrophages, dendritic cells, neutrophils, Kupffer cells in liver and renal cortical tubule cells (Erwin and Munford, 1991). Interestingly, the renal cortical tubule cells can be produced AOAH where it is come into the urine for deacylate and neutralize LPS (Feulner et al., 2004). Acyloxyacyl hydrolase selectively removes the secondary fatty acyl chains from the lipid A moiety of a LPS structure which is capable of binding MD2/TLR4 but doesn't initiate the signal or only can be a partial agonist (Lu et al., 2005). It is reported that AOAH plays an important role in mediating macrophage tolerance to LPS due to increased AOAH mRNA levels in LPS-primed and -tolerant macrophages versus LPS-naïve macrophages (Mages et al., 2007). When compared with wild-type mice, AOAH lack in mice which are challenged with LPS, results enlarged livers and constant hepatic cytokine production which indicating that prolonged inflammatory reaction to LPS prevent by this enzyme (Shao et al., 2011). During localized inflammation in agricultural relevant species mainly cattle, AOAH activity is increased with its activity has been localized to neutrophils (McDermott and Fenwick, 1992). Further investigation in livestock will be needed to the regulation of AOAH by stressors and diet with its direct role in intestinal detoxification of LPS.

Intestinal alkaline phosphatase (AP) directly deactivates LPS, which support for further evidence that enzymes modification of LPS plays an important role in LPS neutralization and detoxification is supported by this recent reports (Bates et al., 2007, Goldberg et al., 2008). Mechanistically, LPS deactivated by AP through which dephosphorylating the diphosphoryl moiety of lipid A, rendering it inactive (Koyama et al., 2002, Munford et al., 2009, Poelstra et al., 1997). In zebra fish, alkaline phosphatase revealed to inactivate LPS (Bates et al., 2007) and its activity is increased during inflamed intestinal tissue (Sanchez de Medina et al., 2004). Furthermore, there is debate about how AP dephosphorylates LPS with its role in detoxification, which has limited evidence. Stress and dietary factors may be modulated about the expression and activity of intestinal AP (Lallès, 2010). The activity of intestinal AP regulate by dietary lipids. For instance, pigs fed a diet high in saturated fat (i.e., 15%

beef tallow) results greater activity of AP in comparison to pigs fed a diet high in unsaturated fat (i.e., 15% corn oil) (Dudley et al., 1994). Another example finding that dietary lipids regulated the activity of AP is that secretion of intestinal AP increased by n-3 fatty acid rich cod liver oil in feed (Kaur et al., 2007). Surprisingly, this may be explained by, AP activity induces by the increased expression of resolvin-E1, which is an anti-inflammatory n-3 fatty acid lipid mediator (Campbell et al., 2010). Furthermore, in case of obesity prone rodents, intestinal AP activity reduces through high dietary fat consumption (de La Serre et al., 2010). Interestingly, increase in plasma LPS and increased inflammation, which are associated with the decrease in ileal AP activity as assessed by myeloperoxidase activity (de La Serre et al., 2010).

Mechanistically, pro-inflammatory cytokines, such as IL-1β and TNF-α may be regulated the alteration of intestinal AP by dietary lipids, results inhibit the induction of AP (Malo et al., 2006). Reductions in feed intake occur by stress and disease in livestock which result decrease intestinal AP secretion (Goldberg et al., 2008, Lallès and David, 2011). It has been found that young age (i.e., 10 d) of weaning pigs decrease both the expression and activity of AP in the jejunum compared to 28 days of age of weaned pigs (Lackeyram et al., 2010). Decreased feed intake and increased intestinal pro-inflammatory cytokine expression occur in this same age period near weaning (Pie et al., 2004), perhaps both of which are responsible for decreased intestinal AP expression and activity that occurs with weaning in pigs. Finally, impact on intestinal and systemic inflammation and LPS concentrations found via alterations in mechanisms of detoxification and neutralization through dietary factors and stressors.

Pro-inflammatory cytokines

There are a variety of cells, mainly activated macrophages, release cytokines that are responsible for altering and modifying the host's metabolism through in response to antigenic challenge. Tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6) have profound behavioural, neuroendocrine and metabolic effects in mammalian body systems. There is data indication that cytokines and cytokine receptors are present in the neuroendocrine system and brain. Likewise, IL-1, IL-6 and TNF-α have been initiated to modulate intermediary metabolism of carbohydrate, fat and protein substrates, regulate hypothalamic-pituitary outflow system and operate in the brain causing to reduce food intake in laboratory animal species. Additionally, many research findings jointly to give significant interpretation that a most biological component of the growth inhibition experimentally found in immunologically assayed animals is controlled through pro-inflammatory cytokines.

Immunological stress and cytokines

Classic finding by Coates et al., (1963) reported that house with a germ-free environment of chicks grew faster than those housed in a conventional husbandry environment that also confirmed with earlier reports (Hill et al., 1953). Moreover, the sub-therapeutic levels of antibiotics in swine and poultry feeds has greater growth-promoting ability when animals are housed in dirty, poorly sanitized husbandry environments comparison with in clean, sanitary ones (Cromwell, 1991, Roura et al., 1992). In reality, antibiotics have slight or no effect on growth, when animals reared under clean or germ-free conditions (Hill et al., 1953, Roura et al., 1992). As antibiotics are supposed to help growth by the way of minimizing the multiplication and severity of host-pathogen interactions, pathogen-induced growth inhibition mechanism was recommended.

According to an old opinion, infectious pathogens, as an immunological challenge can directly disable or corrupt cytological function finally leading to the anorectic and metabolic effects. Even though this suggestion is still suitable to several ways, many authors have proposed that an immunological mechanism is at least partially responsible for this phenomenon because 1) many bacterial and viral organisms help to stimulate leukocytes to synthesize and secrete cytokines in body physiology, 2) animals growth loss due to anorectic cause either clinical or subclinical infections, 3) anorectic and metabolic properties that characterize immunological stress when various external inflammatory stimuli and recombinant cytokines injected into animals, 4) bona fide receptors for cytokines which are present in different non-immunological body tissues, and 5) a specific receptor antagonist which are blocked the anorectic properties of at least one cytokine (interleukin-1; IL-1) (Klasing, 1988, Klasing and Johnstone, 1991, Kelley et al., 1994).

The immune response promotes released cytokines, which are major mediators of intermediary metabolism in immunologically challenged mammals (Klasing, 1988). A number of experiments in chicks were based on this assumption that confirmed that reduced feed intake and efficiency of gain, increased plasma corticosterone, increased body temperature, and modified the distribution of zinc, iron, and copper caused by a various number of inflammatory agents (Klasing, 1984, Klasing et al., 1987, Klasing and Barnes, 1988). Decreased in voluntary feed intake was directly affected approximately 70% of the reduction in weight gain. Cell-free supernatant collected from stimulated macrophages for used to induce the same responses (Klasing et al., 1987), it was evident that the anorectic and metabolic effects occurred by an immunogenic component, instead of a pathogenic component which established earlier information on rats and mice (Beutler and Cerami, 1989) and strongly concerned a collection of cytokines released by mononuclear myeloid cells (e.g. macrophages). Nanogram amounts of lipopolysaccharide (LPS, a molecule present on the membrane of Gram-negative bacteria) injected directly into the central nervous system, CNS which caused many components of immunological stress, including anorexia, fever, secretion of corticosterone and hypercuppremia for confirmed this idea was advanced when number of scientific reports on chickens in

1993 (Johnson et al., 1993b, Johnson et al., 1993a). These data recommend that LPS stimulate cells in the CNS of chickens for producing cytokines, and eventually that the CNS plays a critical role to the overall response to immune challenge.

Macrophages secrete three known cytokines (IL-1, IL- 6, and TNF-α) that have profound metabolic effects. In a group, those three cytokines referred to as pro-inflammatory cytokines because they are secreted primarily by macrophages, which act as the first line of defence in the mammalian immune system. Therefore, blood concentrations of IL-1, IL-6, and TNF-α raise resulting acute challenge with LPS. Consequently, cytokines can perform locally to increase the cellular immune response, but they also can perform physiologic body systems to change behaviour, metabolism, and neuroendocrine secretions. This emphasizes two innate properties of the pro-inflammatory cytokines. Firstly, various functions in different organ systems owing to ability of extremely pleiotropic effect (Kroemer et al., 1993). Secondly, the pro-inflammatory cytokines demonstrate marked redundancy in that different cytokines exhibit the same biological function.

In spite of, the pleiotropy and redundancy effect of cytokines, the cytokine network has poses a widespread system for regulating and integrating itself (Table 1). For example, mononuclear myeloid cells synthesize an inactive 31-kDa precursor protein namely IL-1β (Mosley et al., 1987). The biologically active, secreted 17.5-kDa form of IL-1β generated through interleukin-1β converting enzyme (ICE) (Black et al., 1989). The ICE itself is an inactive 45-kDa precursor when it is synthesized (Ayala et al., 1994). Eventually it must undergo enzymatic processing to produce 20 kDa (p20) and 10 kDa (p10) of two biologically active polypeptides which process pro-IL-1β (Thornberry et al., 1992, Miller et al., 1993). IL-1β may interact with at least two known receptors after IL-1β is released in body systems. Type I IL receptor consists of 80-kDa and 68-kDa were originally cloned from the murine T cell thymoma EL4 6.1 C10 cell line and from B cells respectively (Sims et al., 1988, McMahan et al., 1991). Type II IL-1 receptor is structurally similar to the type I IL-1 receptor. Furthermore, type II IL-1 receptor composed of a signal peptide, an extracellular portion with three immunoglobulin-like domains, one transmembrane region, and a cytoplasmic domain. However, the cytoplasmic domain of types I and II IL-1 receptors are composed of 217 and 29 amino acids, respectively. Consequently, it is now understandable that the type I receptor, and the type II receptor serve as a biological actions of IL-1 and functional decoy, absorbing excess cytokine, respectively. Finally, a naturally occurring human IL-1 receptor antagonist (IL-1ra) has also been characterized and cloned (Carter et al., 1990, Eisenberg et al., 1990, Arend, 1991). IL-1ra binds to human and murine type I receptors with an affinity similar to IL-1. Moreover, it also binds poorly with the murine type II IL-1 receptor. The modulate activity of TNF-α and IL-6; the regulatory steps are similar as like as IL-1 (Table 1).

Ligand	Receptor	Comments
IL-1β	Type I IL-1 (80 kD)	Biologically active IL-1 receptor
	Type II IL-1 (68 kD)	Binds IL-1, but due to a truncated cytoplasmic domain, is biologically inactive
IL-1ra	Type I IL-1	Naturally occurring antagonist to the type I IL-1 receptor
TNF-α	TNF-R1 (55 kD)	Transduces signal for cytotoxicity
	TNF-R2 (75 kD)	Stimulates thymocyte proliferation
	Soluble TNF receptor	Generated by proteolytic removal of the type I or type II TNF receptors from cell surface
IL-6	IL-6	Comprises a cellular ligand-binding a-chain (gp80) and a non-ligand-binding b-chain (gp130) that associate upon binding of IL-6
	Soluble IL-6 receptor	Generated by proteolysis of the ligand-binding a-chain

Table 1. Pro-inflammatory cytokines and their receptors (Johnson, 1997)

How do cytokines antagonize growth?

Pro-inflammatory cytokines are likely to contribute to the poor growth condition via act on number of target organs in immunologically challenged animals. Immunologically challenged animals showed several metabolic, neuroendocrine, and behavioural effects of pro-inflammatory cytokines in the periphery and in the CNS (Figure 1). It is the direct and indirect actions of the pro-inflammatory cytokines act on various organ systems resulting immunological stress.

Cytokines in the periphery

An inflammatory response causes accelerated muscle protein degradation and increased hepatic acute phase protein synthesis. The stimulation of hepatic synthesis of acute phase proteins *in vivo* and *in vitro* occurs by the cytokines IL-1, IL-6, and TNF-α (Richards et al., 1991) and synthesis of some acute phase proteins may increase several hundredfold when inflammatory response occurred. IL-6 (once known as hepatocyte stimulating factor) enhances production of a wide arrange of acute phase proteins (Richards et al., 1991). It is also considered the primary mediator of this metabolic response to inflammation, on the other hands, IL-1 and TNF-α initiate production of several acute phase proteins in cultured hepatocytes. Interleukin-6 also stimulates the uptake of amino acids by hepatocytes. IL-6 alone initiates uptake of amino acids by hepatocytes *in vitro*, but IL-1 and TNF- α enhance hepatic uptake of amino acids *in vivo* (Andus et al., 1991).

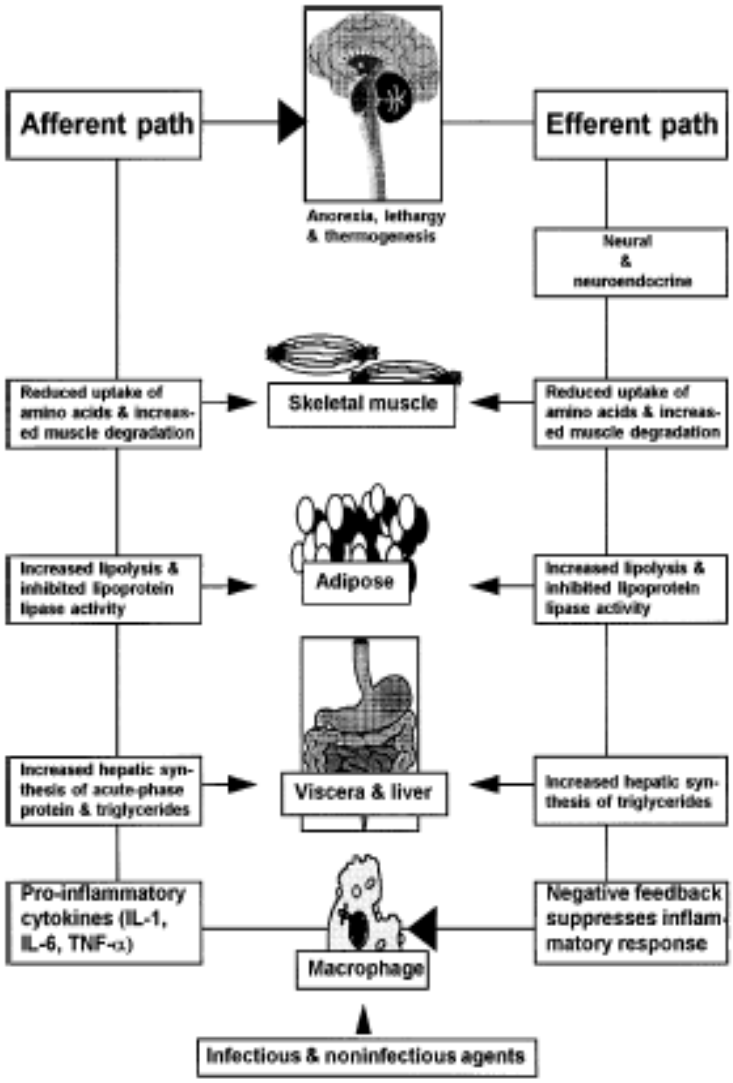

Figure 1. Schematic representation of the possible mechanisms by which pro-inflammatory cytokines inhibit growth. Cytokines act on peripheral and central targets. Cytokines in the brain reduce appetite, but they also alter the hypothalamic-pituitary axis and increase sympathetic nervous system outflow, which ultimately affect intermediary metabolism (Johnson, 1997).

It seems that liver synthesis protein by the effect of IL-1 and TNF-α with indirectly by stimulating the production of IL-6. Approximately 60% amino acids are originated from body protein degradation during the amino acids used in protein synthesis.

Enhanced muscle protein degradation releases amino acids and those amino acids provide as a fuel to use by liver for the synthesis of protein and that muscle proteolysis is mediated by IL-1, IL-6, and TNF-α. Additionally, IL-1 revealed to limit the anabolic effects of insulin on skeletal muscle cell (Klasing and Johnstone, 1991). Consequently, IL-1, IL-6, and TNF-α are part of a network system in body physiology which innately and integrated links muscle protein degradation with hepatic acute phase protein synthesis and this link evidently represents an integrated host response against to inflammatory stimuli. Increases in plasma levels of non-esterified fatty acids and with hypertriglyceridemia are associated with a variety of infections, which are also mediated by the cytokines IL-1, IL-6, and TNF-α. Increase plasma triglycerides through inflammatory stimuli (including cytokines) by at least two ways: 1) a reduction in lipoprotein lipase activity in adipose tissue due to decrease in triglyceride clearance and 2) increased *de novo* fatty acid synthesis and increased re-esterification of NEFA released from adipose tissue those effects an increase in the production of very-low-density lipoprotein. TNF-α is mostly ability to modulate lipid metabolism. Tumour necrosis factor-α enhances hepatic fatty acid synthesis, however in cultured fat cells it decreases the activity of lipoprotein lipase, inhibits *de novo* fatty acid synthesis, and stimulates lipolysis (Memon et al., 1994). Interestingly, TNF-α inhibits lipoprotein lipase activity and lipoprotein lipase mRNA in adipose tissue. On the other hand, it causes a marked increase in mRNA encoding for lipoprotein lipase in the liver hepatocyte (Grunfeld et al., 1989).

As in rodents, amino acid, protein, and fat metabolism have marked effects by cytokines in immunologically challenged pigs, cytokines also tentatively related to the depression in lean growth. For example, pigs housed under management schemes that apparently give smallest quantity of immunological challenges (e.g., medicated early weaning, and all-in, all-out) consume more feed, grow faster, and retain more nitrogen for proteineous tissue growth (Williams et al., 1997a). In these experiments, pigs reared in an atmosphere that most probably compulsory a high quantity of immunological initiation result high plasma levels of the acute phase protein, α1-acid glycoprotein. Cytokines are active in pigs in this regard, however, is indirect at best through by this confirmation.

A wasting syndrome in pigs as like as humans that are attributed to TNF-α has been described (Kyriakis and Andersson, 1989, Morrow-Tesch and Andersson, 1994). Clinical signs of wasting pigs are a chronic anorexia, a reduced weight gain and a number of metabolic disorders including hypozincemia and decreased plasma alkaline phosphatase (Kyriakis and Andersson, 1989). Furthermore, human TNF-α and sera from pigs infected with *Sarcocytis suicanis* resulted lipolytic activity on adipocytes *in vitro* (Jewell et al., 1988). Swine injected LPS to stimulate immunological stress and investigating the relationship between plasma cytokines and protein and lipid metabolism through this concept (Webel et al., 1997). The results from this experiment in figure 2 are indicated to note that after marked increase of TNF-α, IL-

6, and cortisol that affect an increase in plasma urea nitrogen due to the pigs fed to poor amount of feed on the beginning of 12 h before injection, and hepatic acute phase protein synthesis by the IL-6 as a potent stimulator (Andus et al., 1991). It is hypothesized that the threefold increase in plasma urea nitrogen owing to the end results of muscle protein degradation. However, α-1-acid glycoprotein, triglycerides and NEFA were not elevated by LPS in swine, although IL-6, which was increased more than 200-fold, has recently been shown to increase triglycerides in rats (Nonogaki et al., 1995). The differences in metabolic response to LPS in rodents and pigs emphasize the importance of studying the biological effects of cytokines directly in the applicable species.

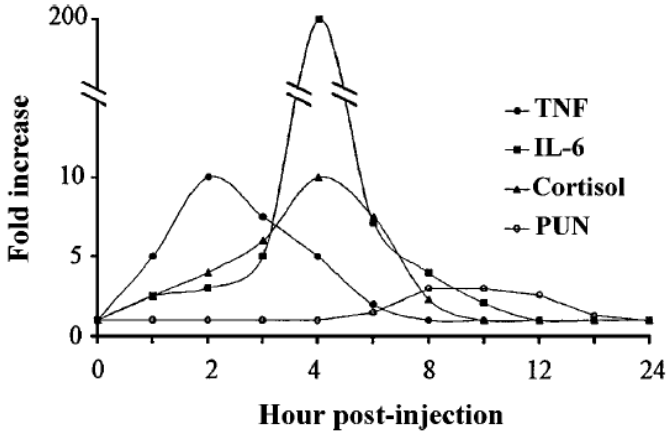

Figure 2. The chronology and magnitude of change in plasma levels of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), cortisol, and urea nitrogen (PUN) following intraperitoneal injection of lipopolysaccharide (LPS; 5 mg/kg BW) in pigs. Note the threefold increase in PUN after the marked increase in IL-6. Plasma triglycerides, nonesterified fatty acids, and a1-acid glycoprotein were not changed following injection of LPS (Webel et al., 1997).

Cytokines in the central nervous system

The first suggestion that experimentally recombinant cytokines were injected peripherally to potentiate immune responses or treat neoplastic disease and that affect the CNS by release of IL-1, IL-6, TNF-α and other products from activated leukocytes (Adams et al., 1984, Fent and Zbinden, 1987). As soon as patients receiving TNF-α and IFN-a showed flu-like symptoms for example fever, anorexia, fatigue, headache, and dementia, which indicate that, these efforts were completed. The administration of an immunogenic component rather than a pathogenic component can cause the central effects of acute infection and it is now confirmed by numerous independent studies involving administration of recombinant cytokines

(Kent et al., 1992). Recent evidence now indicates that are the actions of cytokines in the brain mediates a numerous number of the metabolic effects of peripheral immunological stress. First, it is noticeable that a central cytokine network complete with cytokine-producing cells and bona fide cytokine receptors, exists. The gene and protein level of type I and type II IL-1 receptors in brain tissue have been identified (Parnet et al., 1994). Most of the receptors for IL-1 are found in the hippocampus and choroid plexus by identified experiments using quantitative autoradiography (Farrar et al., 1987, Takao et al., 1990, Ban et al., 1991, Haour et al., 1991). The location and binding sites for TNF-α have been identified in the brainstem, cortex, cerebellum, thalamus, and basal ganglia of rat brains (Kinouchi et al., 1991). IL-1, TNF-α, and IL-6 *in vitro* and *in vivo* synthesize and secrete by microglia and astrocytes in CNS (Fontana et al., 1987, Benveniste, 1992, Hopkins and Rothwell, 1995). Second, immunological challenge causes a number of metabolic effects of peripheral, which are blocked by central administration of IL-1ra. For instance, chronic intracerebroventricular (ICV) infusion of IL-1ra was blocked the anorexia following acute colitis in the rats (McHugh et al., 1994). Furthermore, the expression of mRNA encoding corticotropin-releasing hormone in the paraventricular nucleus of the hypothalamus following i.p. injection of LPS, was entirely blocked by chronic ICV infusion of IL-1ra (Kakucska et al., 1993). The injection of LPS i.p. before microinjection of IL-1ra into the third ventricle of rats is complete blocking of the suppression in plasma growth hormone (Peisen et al., 1995). Abundant peripheral metabolic effects can be enhanced by injecting nanogram-amounts of cytokines directly way into the CNS which is not unexpected.

Many author suggested that inducing weight loss and depressing motivation for feed by IL-1β which is 100-500-fold more effective when administered into a lateral ventricle of the brain rather than into the periphery (Johnson et al., 1997, Segreti et al., 1997). Central IL-1β also provided to modulate intermediary metabolism of carbohydrates in the peripheral tissue (Stith and Templer, 1994). Specifically, central injection of IL-1 caused a marked decrease in hepatic glycogen and is associated with increase in plasma glucose. Even though, the glycolytic actions of glucocorticoids could be recognized by these effects, which are particularly increased by central IL-1 (Berkenbosch et al., 1987), also increases sympathetic nerve activity increased by central administration of IL-1 and increases plasma epinephrine and norepinephrine turnover in different peripheral organs including the liver, spleen, and pancreas (Vriend et al., 1993, Terao et al., 1994). These interpretations recommend that a peripheral target modulate by central IL-1b via efferent nerves, additionally through the neuroendocrine system.

Profound effects on gastrointestinal secretions and motility stimulate by central cytokines, with changes resembling those that occur in satiated animals (i.e., a decrease in gastric acid secretion and inhibition of gastric motility) (Saperas et al., 1990, Robert et al., 1991, McCarthy and Daun, 1992, Tache and Saperas, 1993). Moreover, the metabolically active cytokine, IL-6 in the periphery is to induce by IL-1 in the brain. High circulating levels of IL-6 increased either by centrally injected IL-1 or LPS (De Simoni et al., 1990, De Simoni et al., 1995) stimulates hepatic synthesis

of acute phase proteins and triglycerides (Andus et al., 1991, Memon et al., 1994, Nonogaki et al., 1995). Furthermore, secretion of IL-6 and hypertriglyceridemia by activating α_1- and α_2-adrenergic receptors induces increased by LPS in the brain (Finck et al., 1997). The effects of TNF-α and IL-1α on leukocyte trafficking in swine have investigated by a few studies (Binns et al., 1992, Woolley et al., 1995). For example, endothelial luminal surface expression of the leukocyte trafficking molecule, E-selectin induced by intravenous infusion of recombinant human IL-1α in pigs (Keelan et al., 1994). In addition to, neonatal pig astroglial cells induced prostaglandin synthesis through injecting human IL-1α (Nam et al., 1995) and vasoconstriction of pial microvessels occurred and the permeability of the blood-brain barrier increased to fluorescein-labelled sodium in neonatal pigs due to intracisternal injection of human TNF-α (Megyeri et al., 1992).

The effects of recombinant porcine TNF-α injected into the CNS of pigs on behaviour, feed intake, and plasma cortisol was investigated and caused marked reduction in feed intake, an increase in somnolence, and secretion of cortisol which induced transient (Warren et al., 1997). Furthermore, the anorectic properties of TNF-α were noticeable as a reduction in feed intake was manifest although pigs deprived of feed for 12 h. In the pigs, the dose of TNF-α needed to stimulate behavioural responses which is 50-1,000-fold less than dose require to induce comparable responses in rodents. Whether it is sure or not, pigs are more sensitive to cytokines in the CNS than are rodents; but, pigs have been selected for hyperphagia and may be more responsive to appetite-regulating factors (Denbow, 1989). Furthermore, most studies in rodents have used heterologous cytokines (i.e., human), which may partially explain the relative insensitivity of these species.

Cytokines and animal growth: an integrated view

Pleiotropic and redundant properties of cytokine

The major pro-inflammatory cytokines, for example, IL-1, TNF-α, and IL-6 that share significant biologic properties and are released by activated macrophages (as well as other cell types) (Dinarello, 1991b, Dinarello, 1996). All three cytokines stimulate release of hepatic acute phase proteins, stimulate T and B cells, stimulate the hypothalamic-pituitary-adrenal axis, and provoke fever. Moreover, IL-1 and TNF-α are helped to induce anorexia and hypersomnia and depress social behaviour. However, it is understandable that certain cytokines are better than others at inducing certain responses, in spite of the pleiotropic and redundant character of cytokines. For instance, IL-1 is more effective than TNF-α for reducing nutrient intake (Plata-Salaman et al., 1988, Sonti et al., 1996), changing glucose metabolism (Memon et al., 1994), and suggesting certain sickness-related behaviours (Bluthe et al., 1994). On the

other hand, IL-1, does not stimulate lipolysis as like as TNF-α, and that's why its effects on serum triglyceride levels are secondary to enhanced fatty acid synthesis and very-low-density lipoprotein secretion (Memon et al., 1994). But IL-1, TNF-α and IL-6 revealed to stimulate corticotropin-releasing hormone neurons in the hypothalamus (Rivier et al., 1989, Navarra et al., 1991, Kakucska et al., 1993, Perlstein et al., 1993), the cytokine involved is dependent on the level of immunological challenge (Ebisui et al., 1994). Furthermore, TNF-α with glucocorticoids enhance the muscle proteolysis, because some components are inhibited by surgical or chemical adrenalectomy, but the IL-1-induced proteolysis does not (Zamir et al., 1992, Zamir et al., 1993).

Mononuclear myeloid cells in the periphery (e.g., macrophages) and CNS (e.g., microglia) are released IL-1, IL-6, and TNF-α, individual cytokine have individual metabolic properties, the animal's overall metabolic state formulate by converge individual effects of the various cytokines (Figure 1). Therefore, synergism between cytokines action is just now commencement to be explored. For instance, when administered simultaneously IL-1 and TNF-α that do not induce anorexia or increase plasma ACTH individually (Perlstein et al., 1993, Yang et al., 1994, Van der Meer et al., 1995). Interestingly, LPS causes the release of increased ACTH or the combination of IL-1 and TNF-α was repressed by injecting with a monoclonal antibody to IL-6, indicating LPS induced the ACTH profile in serum is in fact the combine result of at least three different cytokines.

Several research studies indicate that cytokines also synergize in the CNS. Most recently, IL-6 and its soluble receptor, when injected ICV, have been shown to interact in a way that potentiates fever and anorexia (Schobitz et al., 1995). As a result, the presence (or absence) of agonistic soluble receptors that increases receptor capacity, which leads to the biological activity of cytokines. Additionally, it is important to note that there is a synergize relation between cytokines in the CNS and cytokines in the periphery. For instance, IL-1 acts straight way on skeletal muscle and stimulates proteolysis. On the other hand, effects of IL-1 in the brain with increased plasma concentration of glucocorticoids and thermogenesis are expected to speed up this process.

Cytokines inhibit growth directly and indirectly

Specified the pleiotropic effects of cytokines, it is not expecting that clinically identifiable diseased animals fail to grow, or even that immunologically challenged, although otherwise healthy, animals grow slowly. The cytokine-induced growth inhibitions are likely to involve through the different mechanism (Figure 1). Pro-inflammatory cytokines released by activated macrophages act direct way on peripheral somatic tissues according to this model and causes to stimulate an array of metabolic responses suggestive of immunological stress. Cytokines also activate neural mediated events neither by straightly accessing the CNS nor by activating the

synthesis of cytokines from the cells of CNS. In another case, modify the neuroendocrine system, reducing level of growth hormone secretion and increasing amount of plasma corticosteroids are affected by the cytokines in the CNS. Also increase activity of sympathetic outflow to increase catecholamine secretion activity due to cytokines in the CNS. Immunological and inflammatory responses are modulated by epinephrine and glucocorticoids as a part of an important negative feedback loop (Johnson and von Borell, 1994, Johnson et al., 1996) but also these two chemicals alter the gastrointestinal tract activity, induce lipolysis and muscle protein degradation.

Circulating cytokines as endogenous pyrogens

The role of pro-inflammatory cytokines acts as a pyrogenic property specially IL-1 by the purification of IL-1 which has been discovered at the end of the 1970s and at the beginning of the 1980s (Dinarello, 1999). TNF, IFNs, and IL-6 are also able to induce fever and can be considered independent EP which noticeable later (Dinarello et al., 1999, Saper and Breder, 1994, Luheshi and Rothwell, 1996) (Table 2).

IL-1α and IL-1β have EP properties. IL-1 injected into experimental animals resulted potent pyrogenic effects. But administration of an excess dose of IL-1 receptor antagonist (IL-1Ra) can prohibit fever (Chai et al., 1996b). The IL-1 receptor (IL-1R) type I and IL-1R accessory protein (IL-1RAcP) are controlled to the pyrogenic effects of IL-1 (Malinowsky et al., 1995, Zetterstrom et al., 1998b, Zetterstrom et al., 1998a). In humans, IL-1 appears to be the most potent pyrogenic cytokine by the injection of recombinant IL-1α and IL-1β into patients with solid tumours or patients, who had undergone bone marrow transplantation, chills (Tewari et al., 1990). Increasing doses induced the febrile response (Nemunaitis et al., 1991, Crown et al., 1993), and fever was decreased by administration of indomethacin (Iizumi et al., 1991). Lipopolysaccharide (LPS) enhances the role of IL-1 in mediating fever. Some authors reported that after treatment with IL-1Ra (Smith and Kluger, 1992) or anti–IL-1 antiserum decreased LPS-induced fever (Long et al., 1990) and inhibition of LPS-induced fever in IL-1$\beta^{-/-}$ mice (Kozak et al., 1995). But, IL-1β seems to be the major mediator of fever during infection of poxvirus or after injection of turpentine (Alcami and Smith, 1996, Horai et al., 1998).

Both IL-1 and TNF is a pro-inflammatory cytokine that shares many biologic properties (Dinarello, 1991a). In rabbit TNF injection induces a typical fever that is identical from IL-1 (Dinarello et al., 1986). Moreover, TNF increases a fever peak 3–4 h again after challenge, which is recommended to be mediated via stimulation of endogenous IL-1 production (Dinarello et al., 1999, Dinarello et al., 1986). In humans, recombinant human TNF is highly pyrogenic. And the induction of fever by this cytokine is rapid which associated with generalized malaise and joint pain (Dinarello, 1999).

Cytokine	EP activity	Comment	Reference
IL-1	+++	The most potent EP in humans; both IL-1a and IL-1b are EP	(Dinarello, 1999, Chai et al., 1996a, Tewari et al., 1990).
TNF-α	++	Possible role for both soluble and membrane-bound form	(Dinarello, 2005, Dinarello et al., 1986, Grell et al., 1995, Burger et al., 1997).
IL-6	++	IL-6 acts distally of TNF and IL-1 in the cytokine cascade	(Van Oers et al., 1988, Chai et al., 1996b, Zetterstrom et al., 1998a).
IFN	+/++	IFN-a is the most potent; IFN-b less pyrogenic; IFN-g may be pyrogenic through induction of IL-1 and TNF	(Dinarello, 1999, Dinarello et al., 1984, Horning et al., 1982, Lane and Fauci, 1985).

Table 2. Pro-inflammatory cytokines that act as endogenous pyrogens (Netea et al., 2000). NOTE. EP, endogenous pyrogen

Initially IL-6 stimulates potent acute-phase protein inducer (Gauldie et al., 1987). It is demonstrated that induction of fever in rabbits by injection of IL-6 which concentrations were correlated with fever in human patients with burns (Van Oers et al., 1988). The most potent pro-inflammatory properties of IL-6 is the stimulation of PG synthesis, but 50-100-fold higher amounts of IL-6 is required for the induction of fever in rabbits than of IL-1 (Dinarello, 1999). The induction of fever by IL-6 was demonstrated in mice deficient in IL-6, which were insensitive to the pyrogenic effects of LPS, IL- 1β, or TNF (Chai et al., 1996b, Zetterstrom et al., 1998b). This indicated that both TNF and IL-1 are control of the expression of IL-6 (Van Damme et al., 1987), it has been anticipated that IL-6 is a downstream mediator of fever from IL-1 and TNF (Zetterstrom et al., 1998b). The primary induction of IL-1 and TNF by bacterial products stimulates secondary synthesis of IL-6 resulting release of PG in the CNS and finally fever appearance (Figure 3).

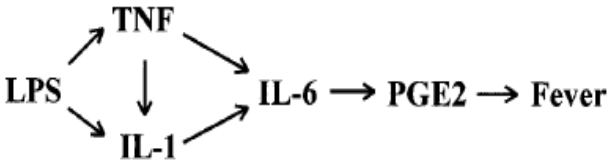

Figure 3. The cascade of cytokines acting as endogenous pyrogens in the pathogenesis of fever induced by bacterial products such as lipopolysaccharide (LPS). PGE2, prostaglandin E2 (Netea et al., 2000).

It has been recently invented that member of the Toll-like receptor (TLR) family act as the signalling chain of the LPS receptors. In case of humans, there are at least 5 TLRs (Rock et al., 1998), and it reports that the stimulation of intracellular signals to LPS is involved by TLR4 (Poltorak et al., 1998). Either membrane or soluble form of CD14 is greatly enhanced LPS to binding with low affinity TLR4.

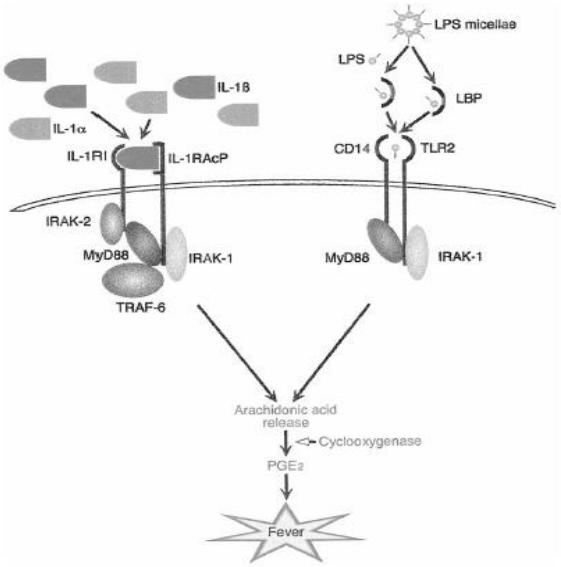

Figure 4. IL-1 (IL-1a and b) binds to its cellular receptor type I (IL-1RI) and the IL-1 receptor accessory protein (IL-1RacP). This leads to signal transduction via receptor associated proteins IRAK-1 and -2, MyD88 and TRAF-6 with release of arachidonic acid and prostaglandin E2 (PGE2) and finally induction of fever. Lipopolysaccharides (LPS) complex with LPS-binding protein, which enables binding of LPS to CD14 and Toll-like receptor 2 (TLR-2). Thereafter, the signal transduction pathway is very similar to that of the IL-1 receptor. Here too, MyD88 and IRAK- 1 are activated, arachidonic acid is released, and fever induced through PGE2 (Netea et al., 2000).

The ligand-receptor interaction is a plasma factor (LPS-binding protein) is a supplementary important factor which transfers LPS from the circulating micellae to the receptor complex (Ulevitch and Tobias, 1995). Interestingly, an intracytoplasmic domain of the TLRs and IL-IR type are homologous structurally (Heguy et al., 1992) which resulting in remarkable similarities in the intracellular signals induced by LPS and IL-1, respectively (Figure 4). Regarding fever, the observation of fever by IL-1 or TNF are identical with injection of a small amount of LPS into rabbits induces a monophasic fever (Cannon et al., 1989), probably through intermediary production of IL-6 (Chai et al., 1996b). These results give the theoretical basis for the hypothesis that certain bacterial products can circumvent the need of stimulating a circulating EP in order to be able to induce fever (Dinarello, 1999, Dinarello et al., 1999).

Acute phase protein

The acute phase proteins (APP) are composed of a group of blood proteins which help to restoring homeostasis and restricting microbial growth in an antibody-independent manner in animals subjected to infection, inflammation, surgical trauma or stress. Although nonspecific way, it induces as a core of the innate immune response linking physical and molecular barriers and responses that serve to prevent infection, clear potential pathogens, initiate inflammatory processes, and contribute to resolution and the healing process. Acute phase proteins, which related with the acute phase response as an integrated part, have been a focus of many potential applications in human diagnostic medicine. Recently it has been identified in common animal species. Potential applications of acute phase protein to make diagnosis, prognosis, assessment of animal health, and laboratory animal welfare are readily noticeable.

The acute phase response is induced by protein hormones called cytokines, which act as messengers between the local site of injury and the hepatocytes to synthesizing the acute phase proteins. Most cytokines have ability to secrete from multiple sources with multiple targets and multiple functions (Gabay and Kushner, 1999) (Table 1). Also they have been found in a large number of animal species including mammals, birds, fish, reptiles and starfish (Huang et al., 1999, Beck and Habicht, 1986, Bird et al., 2002, Murtaugh, 1994, Myers and Murtaugh, 1995, Schijns and Horzinek, 1997).

For acute phase protein induction, the pro-inflammatory cytokines can be divided into two major groups namely IL-1 type cytokines (including IL-1 and TNF-α) and IL-6 type cytokines (including IL-6). Two groups are acting via various receptors, which located on the cell membrane of the hepatocytes in liver (Suffredini et al., 1999, Mackiewicz, 1997). IL-1 type cytokines help to produce a primary auto-stimulatory signal (Nawroth et al., 1986, Content et al., 1985) (Dinarello et al., 1986) stimulating the release of a secondary cytokine signal, IL-6 type cytokines, in various cell types (Mackiewicz, 1997). These IL-6 type cytokines also seem to apply a negative

feedback mechanism on the production of IL-1 type cytokines (Jordan et al., 1995, Mizuhara et al., 1994, Aderka et al., 1989, Schindler et al., 1990).

Biological function of the acute phase protein response

The acute phase response is consideration to be part of a general defence-response against tissue injury (Gauldie et al., 1989). Fever and slow-wave sleep induced by the acute phase response which is some way beneficial to an organism under physical stress that is generally established (Kluger et al., 1975).

Cytokine	Common Functions	Reference
IL-1, IL-6, TNF- α	Induction of hepatic acute phase response Induction of fever Activation of T, B and NK cells Induction of IL-2 in T-cells	(Baumann and Gauldie, 1994, Dinarello, 1984, Steel and Whitehead, 1994)
	Specific Functions	
IL-1	Activation of stroma, chondrocytes and epithelium in response to localized tissue damage	(Murtaugh et al., 1996)
	Regulation of B-lymphopoiesis in bone marrow	(Fauteux and Osmond, 1996)
	Mediation of tissue infiltration of leucocytes (via IL-8)	(Sayers et al., 1988)
	Osteoblast activation, bone and cartilage degradation	(Saklatvala and Sarsfield, 1985)
IL-6	Participates in induction and differentiation of cytotoxic T lymphocytes	(Okada et al., 1988, Takai et al., 1988)
	Stimulates the differentiation of hematopoietic stem cells	(Koike et al., 1988)
	Modulates the production of IL-1 and TNF	(Schindler et al., 1990, Aderka et al., 1989)
TNF-α	Induction of IL-1 production	(Dinarello et al., 1986)
	Participates in cell destruction by suppressing protein synthesis with resulting cachexia	(Beutler et al., 1985)
	Elicits local endothelial damage	(von Asmuth et al., 1991)

Table 1. Major biological functions of the cytokines Interleukin-1 (IL-1), Interleukin-6 (IL-6) and Tumor necrosis factor- α (TNF-α) (Petersen et al., 2004).

The most common APP in most species is expected to take part directly in the protection of the host (Table 2). For example, Hp binds haemoglobin which is released by damaged erythrocytes, and, Hp-haemoglobin complex bind with haemopexin and transferrin helps to decrease the detrimental effects of free iron and to limit the availability of free iron to invading bacteria and bacterial multiplication (Putnam, 1975).

Selected acute phase proteins of veterinary importance

Acute phase proteins could be helpful to provide another means of diagnosis and monitoring animal health. For this type of purpose, the increased application of APP has been developed (Skinner et al., 1991). Owing to a comparatively short biological half-life and high concentration and increased response in diseased animals(Mackiewicz, 1997), APP serum responses constitute a valid measure of a systemic response to an initiating stimulus at the time of blood sampling. Like rectal temperature, for establishment of specific diagnosis of any infectious and stress condition of animals by measurement of APP levels which are not suitable however can make available purposive information regarding the degree of current lesions, disease and stress condition in individual animals.

At the level of herd health management system, APP might be constructive for determining where the spread, rate and level of the disease are taking place (age group, part of the production system), by providing information about high serum concentration of selected APP due to the prevalence of ongoing clinical and subclinical infections (Petersen et al., 2002b) and the magnitude and duration of the acute phase response reflecting the severity of infection which is helpful as a prognostic tool (Hirvonen et al., 1999, Hulten and Demmers, 2002, Peltola, 1982, Skinner et al., 1991). Prior to using APP as objective and non-specific markers of animal health, important points to be considered before are the possible influence of environmental factors, handling and other types of stress in the absence of disease. In human population, a high level of individual variation in acute phase proteins has been occurred (Salonen and Vaheri, 1981, Clark and Fraser, 1993). Also, in laboratory rodents, age, gender and strain specific differences in acute phase protein responses may occur (Pepys et al., 1979). In this thesis, we emphasized on porcine haptoglobin (Hp), which is among the strongly reacting acute phase proteins in porcine biological system.

Haptoglobin

Haptoglobin is composed of a α_2-globulin and a molecular weight of approximately 125 kDa (Putnam, 1975). It was first thought to be as a proteinaeous substance accompany with the capability to enhance the stability of the peroxidase activity of haemoglobin to low pH (Polonovski and Jayle, 1939). It consists of a four chain structure, $(\alpha\beta)_2$ which linked by disulfide bonds (Putnam, 1975). In case of humans, there are 16 different subtypes observed which creating Hp a useful genetic marker in man (Thymann et al., 1990). Porcine Hp has an ability of electrophoretic mobility which similar with human Hp phenotype 1-1 (Lockhart et al., 1972).

Acute phase protein	**Activities**	**References**
Haptoglobin	Binding hemoglobin	(Putnam, 1975)
	Bacteriostatic effect	(Delanghe et al., 1998, Eaton et al., 1982)
	Stimulation of angiogenesis	(Cid et al., 1993)
	Role in lipid metabolism/development of fatty liver in cattle	(Katoh and Nakagawa, 1999, Uchida et al., 1993)
	Immunomodulatory effect	(Murata and Miyamoto, 1993, El Ghmati et al., 1996)
	Inhibition of neutrophil respiratory burst activity	(Oh et al., 1990)
C-reactive protein	Complement activation and opsonisation	(Fiedel et al., 1982, Mold et al., 1982, Volanakis, 1982)
	Modulation of monocytes and macrophages, cytokine production	(Ballou and Lozanski, 1992, Cermak et al., 1993, Pue et al., 1996)
	Binding of chromatin	(Robey et al., 1984)
	Prevention of tissue migration of neutrophils	(Zouki et al., 1997)
Serum amyloid A	Transport of cholesterol from dying cells to hepatocytes	(Liang and Sipe, 1995)
	Inhibitory effect on fever	(Shainkin-Kestenbaum et al., 1991)
	Inhibitory effect on the oxidative burst of neutrophilic granulocytes	(Linke et al., 1991)
	Inhibitory effect on *in vitro* immune response	(Aldo-Benson and Benson, 1982, Benson and Aldo-Benson, 1979)
	Chemotaxic effect on monocytes, polymorphonuclear leucocytes and T cells	(Badolato et al., 1994, Xu et al., 1995)
	Induction of calcium mobilisation by monocytes	(Badolato et al., 1995)
	Inhibition of platelet activation	(Zimlichman et al., 1990)

Table 2. Biological activities of selected acute phase proteins (Petersen et al., 2004)

The molecular weight of porcine Hp estimated to be approximately 120 kDa (Lockhart et al., 1972, Shim et al., 1971, Heegaard et al., 1998, Toussaint et al., 1995). The monomers of bovine Hp estimated 16 to 23 kDa (α-chains) and 35 to 40 kDa (β-chains) (Eckersall and Conner, 1990, Morimatsu et al., 1991) and also has a polymer in association with albumin which estimated to be a molecular weight above 1000 kDa in cattle serum (Eckersall and Conner, 1990). Bovine acute phase serum composed of a macromolecular protein (MW-1000 to 2000 kDa) isolated and characterized as Hp (Morimatsu et al., 1991) which is not present in normal bovine serum. In case of Bovine Hp, various degrees of polymerization with large and heterogeneous molecular sizes also reported (Morimatsu et al., 1992). Equine Hp consisting of a pair of polypeptides which estimated molecular weights of 108 with consisted of a two identical subunits and 105 kDa with composed of a two different subunits (Taira et al., 1992).

Biological functions of haptoglobin

There are many functions of haptoglobin, which have been proposed (Tab. II). The most important functions of Hp are to prevent the loss of iron in circulation by the formation of very stable complexes with free haemoglobin in the blood by damaged of RBC (Keene and Jandl, 1965, Laurell and Nyman, 1957, Putnam, 1975) and to have a bacteriostatic effect by restricting the availability of iron which is necessary for bacterial growth and multiplication (Eaton et al., 1982, Bullen, 1981). For example, the growth of *Streptococcus pyogenes in vitro* culture media inhibit by human Hp (Delanghe et al., 1998). Renal excretion of free haemoglobin is seen when the total vascular binding capacity of Hp is saturated (Laurell and Nyman, 1957). The haptoglobin-haemoglobin-complexes are usually supposed to be circulated to the liver through the reticuloendothelial system and metabolized by Kuppfer-cells in liver (Putnam, 1975). In case of cattle, Hp supposed to be involved in the regulation of lipid metabolism (Nakagawa et al., 1997) and as an immunomodulator (Murata and Miyamoto, 1993).

Factors influencing serum haptoglobin concentration

Haptoglobin is an important APP in near about most species. But the serum concentration of Hp can be influenced by other factors than the acute phase response. Decreased serum concentrations of free Hp owing to increased levels of free haemoglobin in the serum were observed during babesiosis in cattle and post-surgical hematomas in horse (Kent and Goodall, 1991, Bremner, 1964). Renal disease and obstructive jaundice may cause hyperhaptoglobulinemia along with the acute phase response (Putnam, 1975). Reduce level of the measured concentration of Hp

also found by an effect of free haemoglobin in serum samples (Eckersall et al., 1999, Petersen et al., 2001).

Porcine haptoglobin

Among other APP, Haptoglobin is considered to be a helpful diagnostic tool in most species. In newborn piglets blood plasma concentration contained low level of Hp (Richter, 1974). In case of adult pigs, two to three weeks of age require reaching peak level of Hp and that reaches higher levels than that seen in slaughter pigs and sows was seen around 30 to 50 days of age. Normally the Hp concentration was not determined after 50 days of age and mature boars shown to have significantly lower Hp concentration than sows and castrated boars (Richter, 1974). There is no effect between different breeds (Lipperheide et al., 1998) on the other hand, a difference observed between herds (Lipperheide et al., 1998, Hall et al., 1992, Petersen et al., 2002b). A high concentration of serum Hp was affected by Clinical signs of lameness, respiratory disease, diarrhoea, tail bite and ear necrosis (Petersen et al., 2002a). At slaughter, Hp was established to indicate lesions defined as abscesses and chronic abnormalities (Toussaint et al., 1995). Experimentally induced inflammation (Eckersall et al., 1996, Lampreave et al., 1994, Richter, 1975), surgery (Jacobson et al., 2001) and a range of experimental and natural infections resulting increased concentration of Hp in porcine (Table 3). Moreover, different serotypes of *Actinobacillus pleuropneumoniae* (Agerso et al., 1998, Hall et al., 1992, Heegaard et al., 1998), *Mycoplasma hyorhinis* (Magnusson et al., 1999) or Porcine Reproductive and Respiratory Syndrome virus (Asai et al., 1999) causes porcine respiratory infections which are all reflected by increased haptoglobin concentration.

Animal	Cause	References
Pig	Experimental local aseptic inflammation	(Eckersall et al., 1996, Lampreave et al., 1994, Richter, 1975)
	Intramuscular injection of lipopolysaccharide (*Escherichia coli* serotype 0.55:B5)	(Dritz et al., 1996)
	Surgery	(Jacobson et al., 2001)
	Infection with *Actinobacillus pleuropneumoniae* (serotype 1, 2 and 5)	(Agerso et al., 1998, Hall et al., 1992, Heegaard et al., 1998, Lauritzen et al., 2003)
	Infection with *Mycoplasma hyorhinis*	(Magnusson et al., 1999)
	Infection with *Toxoplasma gondii*	(Jungersen et al., 1999)
	Infection with Porcine Reproductive and Respiratory Syndrome (PRRS) virus	(Asai et al., 1999)
	Intranasal inoculation of *Bordetella bronchiseptica* and toxigenic *Pasteurella multocida* type D	(Francisco et al., 1996)

Table 3. Summary of treatments and infections leading to increased haptoglobin concentration (Petersen et al., 2004)

Melon pulp concentrate

Background

Reactive oxygen species (ROS) are mainly primary free radicals continuously produced from oxygen during essential physiological processes, such as cell energy metabolism. They are also a powerful tool used by immune cells to kill pathogens (bacteria, viruses). ROS are converted by a set of primary antioxidant enzymes (SOD, CAT and GSH-Px) into less harmful molecules. When ROS are produced in excess, the antioxidant system is overwhelmed and ROS may react with cellular components (DNA, membrane lipids, proteins) to produce a chain reaction involving secondary free radicals ($ROO^{\cdot}$), which may be potentially scavenged by secondary antioxidants (vitamin E, C, A, polyphenols, carotenoids, selenium etc). Deficiencies in primary antioxidant enzymes and secondary antioxidants (AOX) or excess exposure to stimulators of ROS production (physiological or environmental stress, pathogen invasion, higher metabolic needs) may result in oxidative stress, defined as an impaired homeostasis between oxidants and antioxidants. The molecular damage to DNA, lipids membrane and proteins impacts cellular functioning, then results in physiological and tissue damage to end up with farm level damage and economic losses (Lallès et al., 2011).

Melon pulp concentrate is a melon freeze-dried juice concentrate naturally rich in antioxidant enzymes, SOD and CAT, coated with vegetable fat (palm oil) and then adsorbed on a mineral matrix to facilitate the blending into animal food and to protect SOD from gastric acid degradation during its passage through the stomach. Coating with palm oil brings to SOD a gastric protection which allows its gradual release into intestinal tract. This product is manufactured by Lallemand (France) from a proprietary variety of melon (*Cucumis melon* L.) not GMO, containing guaranteed high levels of SOD (2.6 UI SOD/mg) and exclusively distributed by Lallemand for animal nutrition applications.

Suggested mechanism of action

The main reasons to think that dietary SOD could stimulate the expression of endogenous SOD is that the increase in plasma SOD has higher value than added SOD ingested: even if all the SOD ingested was absorbed through the intestinal wall, it could not explain the sharp rise in plasma SOD (Lallès et al., 2011). Dietary SOD could be able to stimulate Nrf2, a nuclear transcription factor that controls the expression and coordinated induction of a battery of defensive genes encoding detoxifying enzymes and antioxidant proteins. In absence of stress, Nrf2 sequestered into the cell cytoplasm by an inhibitor Keap1, forming an Nrf2-Keap1 complex. In presence of stress, Nrf2 is released from Keap1, enters into the cell nucleus, binds to Maf protein and then to ARE (Antioxidant Response Element), which stimulates transcription of downstream genes (and potentially SOD gene). It takes less than 15 min from the time of stress exposure to induce import of Nrf2. The nutritional values and microbiological specifications are indicated in table below.

Nutritional values	**Ranges**
Crude protein	9-11%
Crude ash	37-39%
Crude fat	16-19%
Crude fibre	4-6%
Dry matter	Minimum 95%
Ca	11-15%
Na	0.05-0.15%
P	0.25-0.40%
Mg	0.15-0.25%
Lys	2.4-2.7g
Met	1.5-1.8g
SOD activity	Minimum 2600000 UI/kg
Microbiological specifications	
Total plate counts	Maximun 5000 CFU/g
Yeast and moulds	Maximun 100 CFU/g
Enterobacteria	Absence/g
Escherichia coli	Absence/g
Salmonella sp	Absence/25g
Staphylococcus aureus	Absence/g

Table 4. Nutritional values and microbiological specifications of melon pulp concentrate.

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CHAPTER 2

Objectives

The objective of this thesis was to study the effects of melon pulp rich in superoxide dismutase on piglets and poultry health.

To reach this aim three trials were organized:

1. Effects of melon pulp concentrate rich in superoxide dismutase on antioxidant status and growth performance of piglets exposed to chronic LPS challenge (Chapter 3).

2. Effects of melon pulp concentrate rich in superoxide dismutase on broiler growth performance, pododermatitis and cellulitis (Chapter 4).

3. Effects of melon pulp concentrate rich in superoxide dismutase on hepatic gene expression of antioxidant proteins in piglets and poultry (Chapter 5).

CHAPTER 3

Effects of melon pulp concentrate rich in superoxide dismutase on antioxidant status and growth performance of piglets exposed to chronic LPS challenge

Effects of melon pulp concentrate rich in superoxide dismutase on antioxidant status and growth performance of piglets exposed to chronic LPS challenge

Abstract

The aim of the trial was to study the effects of an antioxidant feed supplement (melon pulp concentrate, MPC) that contains high levels of superoxide dismutase (SOD) as a primary antioxidant on antioxidant status and growth performance of LPS challenged weaned piglets. A total of 48 weaned piglets were individually allocated to 4 experimental groups in 2×2 factorial design for 29 days. Two different dietary treatments were adopted a) C, control fed a basal diet, b) MPC, treated fed the basal diet plus 30g/ton fed of MPC. On day 19, 21, 23 and 25 half of the animals within C and MPC groups were subjected to a challenge with an intramuscular injection of an increasing dosage of LPS (*E. coli* serotype 0.55:B5) to mimic a chronic inflammation (+) or were injected with an equal amount of PBS solution (-). Growing performances were evaluated weekly and blood samples were collected on day 0, 19, 21, 23, 25, 27 and 29 for total antioxidant activity (TAOC), SOD activity and reactive oxygen species (ROS). On days, 0, 19, 21, 23, 25 and 29, serum content of Haptoglobin (Hp) and interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) were determined. On days, 19, 25 and 29, blood resistance to haemolysis and 8-hydroxy-2'deoxyguanosine content were evaluated. A positive effect of MPC was evidenced on feed intake (FI) and average daily gain (ADG) ($P<0.01$; $P=0.05$ respectively), while LPS challenge significantly reduced both parameters in injected animals ($P<0.01$). Antioxidant status of MPC fed piglets was improved by higher TAOC levels (MPC=7.22 mM Trolox equivalent vs. C=4.54 mM Trolox equivalent; $P<0.01$) and RBC resistance to haemolysis (MPC=70.71 HT50, min vs. C=66.41 HT50, min; $P\leq0.01$). The challenge increased TNF-α, IL-1β, IL-6 ($P<0.01$) and Hp ($P=0.03$) levels in blood, but no differences were found for MPC administration. These results suggest that oral SOD supplementation by MPC increase some aspects of antioxidant status of post weaning piglets with positive results on growing performance.

Introduction

Weaning stress acts as a crucial factor in terms of potential changes and modification of the immune system (Kick et al., 2012), intestinal physiological functions (Wijtten et al., 2011) and endocrine system (Zhu et al., 2012). Furthermore, a current research indicated that weaning can also induce oxidative stress and free radical production (Zhu et al., 2012). Normally, high concentrations of reactive oxygen species (ROS) are the cause of oxidative injuries in bodies' tissues, the oxidative damages affecting the main cell components: lipids of the cell membrane, proteins and DNA. The DNA damage occurs by base modification, strand breaks, and loss of purines (Beckman and Ames, 1997). On the other hand, a low or transient concentration of ROS acts to trigger cellular proliferation or survival signalling pathways (Buetler et al., 2004, Finkel, 2003), demonstrating that ROS are important signalling molecules and that it is only their production in excess which is an issue, the objective of primary antioxidant supplementation is therefore to control their production, but not completely supress them. Increased production of ROS and reactive-nitrogen species can induce an imbalance in oxidant/antioxidant equilibrium, overwhelming antioxidant enzymes, superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) (Han et al., 2009, Valko et al., 2007, Han et al., 2011). Oral supplementation of natural antioxidants has been used to prevent oxidative stress in livestock husbandry system (Bonnette et al., 1990, Eicher et al., 2006, West et al., 2008, Deng et al., 2010, Zhang et al., 2014, Bontempo et al., 2014).

Some pro-inflammatory cytokines (namely TNF-α, IL-1β, and IL-6) are able to trigger the acute phase response of the immune system. Those cytokines stimulate hepatocytes to synthesize and release acute phase proteins such as haptoglobin (Hp) among others (Baumann and Gauldie, 1994). Low-dose endotoxin or lipopolysaccharide (LPS) challenges have been effectively used to study the time response of TNF-α, and IL-6 in pigs (Warren et al., 1997, Webel et al., 1997). *In vivo* challenges with low doses of LPS can simulate sub-acute inflammation/infection and therefore give valuable information regarding the use of these acute phase response products as indicators of subtle conditions where the health and welfare of pigs may be also compromised (Llamas Moya et al., 2006).

Contrary to the use of live pathogens, dosages of non-pathogenic immunogens such as *Escherichia coli* lipopolysaccharide (LPS) is easily controlled and can induce reproducible responses (Fink and Heard, 1990, Deitch, 1998). In addition, limited biosecurity is needed when they are administered to animals. Different studies (Goode and Webster, 1993, Novelli, 1997, Luster, 1998) outlined that lipopolysaccharide can enhance the formation of ROS and lipid peroxidation products such as superoxide anions and peroxides and their secondary products. The most important form of the free-oxygen radical is superoxide (O_2^-) (Bautista and Spitzer, 1990) that represents the source for a second active, but more stable and less toxic, form such as H_2O_2. The combination of both oxygen species leads to a more active and aggressive form of oxygen, hydroxyl radical ($HO^\cdot$) (Sewerynek et al., 1996).

Moreover, LPS also generates free radicals intracellularly through the ischemia reperfusion syndrome secondary to the decrease in tissue blood flow and by altering the activity of the major physiological sources of free radicals in the heart-microsomes, peroxisomes and mitochondria (Portoles et al., 1993; Cadenas et al.,1998). All these mechanism of actions promoted oxidative stress in the rat model (Ben-Shaul et al., 1999).

The administration of large single doses of these immunogens can cause severe responses, including high mortality and organ failure (Fink and Heard, 1990). Reducing the dose of LPS antigen improves survival rates; however, animal recovery often occurs within 48 h after the challenge (Deitch, 1998). Prolonged repeated administration (Van Heugten et al., 1996) or continued infusion (Fish and Spitzer, 1983) of LPS leads to endotoxin tolerance (Ash and Griffin, 1989, Deitch, 1998).

The *Cucurbitaceae* family includes numerous species of cultivated plants of great economic importance, including watermelon (*Citrullus lanatus L.*), squash (*Cucurbita maxima L.*), cucumber (*Cucumis sativus L.*) and cantaloupe (*Cucumis melo L.*) (Ritschel et al., 2004). Cantaloupe is one of the most consumed fruit crops in the world owing to its pleasant flavour. SOD is found in several crop plants (Perl-Treves and Galun, 1991, Sandalio et al., 1997), however its use as a diet supplement is limited due to its inactivation by the digestive enzymes in the gastrointestinal tract and its low concentration in vegetables and fruits. Trials performed in various experimental models showed positive correlations between the maintenance of cellular integrity (i.e. a delayed senescence) (Lacan and Baccou, 1998) and the effect of oral administration of encapsulated melon extract rich in SOD. Moreover, the antioxidant and anti-inflammatory properties of melon concentrate *in vitro* and *in vivo* models were confirmed by measuring its effects on redox status and its capacity to stimulate the production of anti-inflammatory cytokines (Vouldoukis et al., 2004b). Furthermore, it was established that an oral supplementation with this melon extract could increase the resistance of red blood cells to oxidative stress-induced haemolysis in mice (Vouldoukis et al., 2004a). Muth et al., (2004) demonstrated the efficacy of oral supplemented SOD on hyperbaric oxygen-related cell damage. Lastly, Kick et al., (2007) concluded that oral melon extract may be a therapeutic option to reduce oxidative cell injury with a model of induced ischaemia/reperfusion (aortic cross-clamping) injury in pigs.

Data on the effect of feeding melon pulp concentrate rich in SOD on piglets are at present not available, with the exception of a study by Lallès et al. (2011) on stress proteins along the gastrointestinal tract. Therefore, our aim of the study was to evaluate the effects of oral supplementation of a SOD-rich MPC on growth performance, antioxidant status and inflammation responses in weaning piglets when chronically challenged with LPS.

Materials and Methods

The protocol for care, handling, and sampling of animals defined in the present study was reviewed and approved by the Università degli Studi di Milano Animal Care and Use Committee (Protocol No 82/14). The trial was performed at the Animal Production Research and Teaching Centre of the Polo Universitario di Lodi, Università degli Studi di Milano.

Animals, housing and experimental design

A total of Forty-eight 24-d old weaned piglets (Topigs 40 x Topdelta; 7.79±0.17 kg of initial BW) were selected from the same herd and were divided in four homogeneous experimental groups of twelve animals each in a 2 x 2 factorial design. The piglets were placed in individual pens (0.47 m^2 for each piglet) and allocated in the same environmentally-controlled post-weaning room on slatted floor. Each pen was equipped with one standard nursery pig bite-style nipple drinker and a self-feeder to allow for ad libitum access to water and feed. Room temperature and ventilation were electronically controlled over a 24 h period, providing thermal comfort to piglets throughout the experiment. Starting temperature was 28°C with a ventilation of 10m^3/h/head, and the temperature programme decreased values by 1°C/week until 25°C were reached at the end of the trial.

The first factorial arrangement consisted on the administration of a basal diet without any antimicrobial growth promoter (C) or the same basal diet plus 30g/ton fed of MPC (Melofeed®, Lallemand, Blagnac, France) (MPC).

The second factorial arrangement consisted on a LPS challenge with repeated increasing intramuscular injections of low levels of *E. coli* (serotype 055:B5, Sigma-aldrich Canada Ltd, Oakville, ON, Canada; cat. no. L2880) (+) to mimic chronic inflammation, or the injection of an equivalent amount of PBS solution (-). LPS challenge was performed starting on day 19 of the trial. Subsequent injections were performed on days 21, 23 and 25. On day 19 challenged piglets were inoculated with 60 μg/kg of BW with LPS. Lipopolysaccharide dosage was increased by 12% at each subsequent injection to reduce endotoxin tolerance (Rakhshandeh and de Lange, 2012). The basal diets were calculated to be isonutritive, and to meet the nutrient requirements of weaned piglets recommended by NRC (2012).

The chemical composition of the basal diet was analysed at the beginning of the trial in order to determine dry matter (DM), crude protein (CP), ether extract (EE) and ash content (Table 1). Individual piglet BW was recorded on 0, 8, 15, 19, 21, 23, 25, 26 and 29 days by an electronic scale (Ohaus ES100L). Individual feed intake was calculated by subtracting the relative orts to the total daily-administered feed. Subsequently, individual average daily gain (ADG) and gain:feed ratio (G:F) were calculated. The health status of the piglets was checked daily and any sanitary treatment was recorded.

Morbidity, medications and mortality were eventually recorded. As described by Ribeiro et al., (2010), rectal temperature was measured before LPS challenge as a baseline measurement and after two hours from each LPS injection. The body temperature of piglets was also measured at the last day of trial (day 29).

All samples were collected early in the morning, before feeding, to reduce biological variation. Piglets were placed in a recumbent position on a V shaped table to restrict their movement and blood was collected from the cranial venacava with a 10 ml Lithum Heparin tube (VF-109SHL, Terumo Venosafe®, Italy) and a clot activator tube (VF-109SP, Terumo Venosafe®, Italy) to yield blood plasma and serum on 0, 19, 21, 23, 25, 27 and 29 days of experiment, respectively. Following collection, blood samples were stored at 4°C for biochemical analysis and blood resistance to haemolysis (KRL test). Blood samples for the determination of biochemical profiles were stood for 15 minutes to clot prior to centrifugation at 1300 g at 4°C for 10 minutes. Aliquots of plasma and serum from each piglet were stored at -20°C. 8-oxodGuo, SOD activity, TAOC, cytokines (IL-1β, IL-6 and TNF-α), ROS and Hp were measured in the sera.

Oxidative biomarkers status

Serum SOD activity was measured using a commercial available kit in accordance with the manufacturer's instructions (Sigma-Aldrich, Cat. No. 19160). As the absorbance at 440 nm is proportional to the amount of superoxide anion produced, SOD inhibitory activity can be quantified by measuring the decrease in the colour development at 440 nm. ROS were evaluated by Cyt C kit (Sigma Aldrich, cat. No. C2506). The modulation of serum oxidative burst activity was studied by cytochrome C reduction assay as reported by (Sartorelli et al., 2000). Briefly, 20 µl of Cytochrome C (1 mM) and 80 µl of serum were added in a total volume of 100 µl in a 96-well plates. Absorbance was measured on a plate reader (Bio-Tec Instruments Inc., Winooski, VT, USA). Optical density (OD) was measured after 40 min at a wavelength of 550 nm. Background values, calculated from wells with 100 µl of H_2O supplemented with Cytochrome C, were subtracted from all values.

TAOC, used as an overall measure of serum antioxidant capacity, was assessed with a commercially available kit (Sigma Aldrich Antioxidant assay kit, Cat. No. CS0790). 8-oxodGuo levels in serum were determined using an ELISA kit based on monoclonal antibody (8-OH-dG Check, Japan Institute for the Control of Aging, Fukuroi City, Japan).

The total antiradical activity of whole blood, plasma and RBC for each piglet was evaluated using the KRL biological test (Rossi et al., 2013). After sampling on heparinized lithium tubes, the blood, diluted to 1/50 in isotonic saline solution, was submitted to organic free radicals produced at 37°C under air atmosphere from the thermal decomposition of a 27 mmol/l solution of 2,2' azobis (2-aminido-propane) dihydrochloride (Spiral, Dijon, France). For each well, absorbance measurements

were performed 75 times, once every 150s. Hemolysis was recorded using a 96 well microplate reader by measuring the optical density decay at 450 nm. Results were expressed as the time that is required to reach 50% of maximal hemolysis. Half hemolysis time for total blood cells (HT50 WB), expressed in minutes, refers to the whole blood resistance to free radical attack. Half hemolysis time for red blood cells (HT50 RBC), expressed in minutes, refers to the red cell resistance to free radical attack. The plasma resistance to hemolysis (HT50 Plasma contribution), also expressed in minutes, was calculated by subtracting HT50 RBC from HT50 WB. Intra-assay and inter-assays CVs of KRL test were less than 2.5 and less than 4%, respectively.

Inflammatory biomarkers status

TNF-α, IL-6 and IL-1β were measured by porcine-specific ELISA according to the recommendation of the manufacturer (R and D Systems Inc., Abingdon Science Park, UK). Briefly, standard, control and samples were added to the wells with a coated monoclonal antibody specific for each cytokine. After incubation for 2 h, the unbound substances were washed away, and an enzyme-linked polyclonal antibody specific for the cytokine was added to the wells to sandwich the cytokine immobilized during the first incubation. A further 2 h of incubation was followed by a wash to remove any unbound antibody-enzyme reagent, and then a substrate solution was added to the wells and colour developed in proportion to the amount of the cytokine bound in the initial step. The colour development was stopped by adding the stop solution, and the intensity of the colour was measured at 450 nm with the correction wavelength set at 540 nm. The results of TNF-α were expressed in pg/mL and the results of IL-1β, IL-6 were expressed in ng/mL on the basis of a standard curve for cytokines.
The serum concentrations of the Hp were determined by ELISA (Tridelta PHASERANGE serum Haptoglobin assay, Cat. No. TP-801). Hp concentration was quantified as reported by Cooke and Arthington (2013). The result of Hp was expressed in μg/mL on the basis of a standard curve for Hp.

Statistical analysis

Body weight, body temperature, Hp, TAOC, ROS, SOD, KRL in blood, KRL in RBC, plasma contribution in KRL test, 8-oxo-dGuo, IL-1β, IL-6 and TNF-α were performed by a MIXED procedure for repeated measurements with the piglet as the experimental unit. The statistical model included effects of LPS challenge, MPC, time and their interaction as fixed effects. The effect of the piglets was nested with the MPC x challenge group. Moreover a GLM procedure using body weight at day 19

(before LPS challenge) as covariate was used to detect any differences in the final body weight within the experimental groups. ADG, FI, and G:F considered two different trial periods corresponding to day 0-19 (pre-challenge) and 19-29 (challenge and post challenge) of the trial. Statistical analyses over these parameters were performed by a GLM procedure with the piglet as the experimental unit. The effect of the piglets was nested with the MPC x challenge group. For ADG, FI, and G:F, the corresponding data relative to the pre-challenge period (0-19 d) were used as covariate. For total feed intake, average daily gain and G:F for the whole trial period, and body temperature on day 29, a GLM procedure was adopted taking into account the piglet as the experimental unit and considering the effect of MPC, challenge and MPC x challenge interaction. Significance level was fixed for $^{A,B}P \leq 0.01$ and $^{a,b}P \leq 0.05$, $0.05 < P \leq 0.1$ was considered as a trend.

Results

Growth performance

No significant effects of MPC supplementation (P=0.70) and LPS challenge (P=0.47) or any interactions were found on BW with the exception of time (P<0.01) (Table 2). However, when BW before LPS challenge (day 19) was used as covariate, MPC fed animals were significantly heavier (P=0.05) and LPS challenge determined lower BW both in C and MPC (P<0.01). ADG and FI were not influenced by MPC until day 19 and overall the experimental period. ADG and FI were not significantly different between the four experimental groups during the period before LPS challenge, but when introducing the respective values in the first 19 days as covariate in the statistical model, ADG and FI were increased by MPC supplementation under the challenge and post-challenge period (P=0.05 and P<0.01, respectively).

No differences were found for G:F considering MPC effect. The LPS challenge effect lead to lower values for ADG (P<0.01), FI (P<0.01) and G:F (P<0.01), but no significant effect for MPC x challenge interaction was found.

From day 19 to day 29 of the trial, LPS challenge reduced FI in C and MPC groups with respect to the relative no-challenged animals. Within the challenge groups, MPC animals displayed higher FI than C over 19-29 days: FI was increased in no challenge and LPS challenge groups with MPC. LPS challenge induces a decrease in ADG but at a lesser extent during and after the challenge in C and MPC groups. Furthermore, dietary supplementation with MPC significantly increased ADG over 19-29 days: treated groups showed +12% and 25% ADG than no challenge and LPS challenge control groups groups.

Finally G:F was negatively affected by LPS challenge (P<0.01) from day 19 to day 29 of the trial, while MPC and MPC x challenge interaction did not show any differences between the groups (P=0.64 and P=0.72, respectively).

Relative to pre-challenge values, body temperature (BT) was increased in piglets treated with LPS (Table 3). There was no effects of dietary MPC supplementation on temperature for the first 3 LPS injections at days 19, 21 and 23, but the body temperature of the experimental piglets two hours after the fourth challenge injection was significantly reduced by the MPC (C= 40.03 °C vs. MPC= 39.89 °C, P=0.02). A significant effect was evidenced for time (P<0.01), challenge (P<0.01) and challenge x time interaction (P<0.01) during LPS challenged period. The body temperature of the experimental piglets at the end of the trial (day 29) was not significantly influenced by the MPC (P=0.97) and the challenge (P=0.38), and the MPC x challenge (P=0.34) interaction was not significant.

Oxidative biomarkers status

Oxidative biomarkers activities and serum 8-oxodGuo values are reported in table 4. No significant effects of LPS challenge were evidenced for all the considered parameters during the trial, while MPC significantly increased TAOC levels (C=4.54mM Trolox equivalent vs. MPC=7.22 mM Trolox equivalent, $P<0.01$), the effect of time was always significant, but no differences were found accounting for the tested interactions between MPC, challenge and time. Trends for LPS challenge effect (6.46mM Trolox equivalent vs. 5.30 mM Trolox equivalent respectively for no challenge and LPS challenged piglets, $P=0.08$), and MPC x time ($P=0.08$) were also detected for TAOC.

No effects were showed in SOD activity and ROS levels during the trial for MPC (P=0.66 and P=0.73 respectively), challenge (P=0.77 and P=0.62 respectively), time (P=0.22 for SOD), MPC x time (P=0.81 and P=0.90 respectively), challenge x time (P=0.89 and P=0.95 respectively), MPC x challenge (P=0.65 and P= 0.74 respectively). In the same way we did not find significant effects for 8-oxo-dGuo level during the trial for MPC (P=0.99), challenge (P=0.40), MPC x time (P=0.83), MPC x challenge (P=0.26), challenge x time (P=0.17), and MPC x challenge x time (P=0.45), while the effect of time was significant (P<0.01).

Blood resistance to haemolysis is reported in table 5**.** Melon pulp concentrate supplementation significantly affected KRL in RBC ($P<0.01$), while LPS challenge tended to increase whole blood and plasma contribution to blood resistance ($P= 0.09$ and $P=0.08$ respectively). A significant effect of time ($P<0.01$) was evidenced for KRL in whole blood and KRL in RBC, while no significant effect for interactions was found.

Inflammatory biomarkers status

LPS challenge determined a huge increase in pro-inflammatory cytokines (IL-1β: 0.21 ng/mL vs. 0.53 ng/mL; IL-6: 3.10 ng/mL vs. 6.99 ng/mL; TNF-α: 14.40 pg/mL vs. 25.55 pg/mL, respectively for C and MPC) and Hp levels (782.21 μg/mL vs. 896.39 μg/mL; $P=0.03$) during the trial (Table 6), while MPC supplementation did not determine any significant variation. Time effect was always significant as the interaction among LPS challenge and time.

Discussions

In the present trial we decided to adopt a chronic challenge procedure as reported by Rakhshandeh and de Lange (2012), to mimic subclinical or mild clinical disease conditions that frequently occur in the field. Lipopolysaccharide challenge significantly increased pro-inflammatory interleukins levels and Hp concentration in blood with impaired piglet performance in both in C and MPC groups from 19 to 29 days of the trial, but without any effects on the oxidative status of the piglets. The lack of increased level of oxidative biomarkers when LPS challenge was performed can be attribute to a number of factors. Beside the low dosage applied to mimic a chronic inflammation, the LPS effects on oxidative stress biomarkers are often reported to be organ-specific, where the preferential target organ seems to be the liver rather than the heart (Giralt et al., 1993, Nowak et al., 1993, Portolés et al., 1993, Goode and Webster, 1993, Novelli, 1997, Luster, 1998, Ben-Shaul et al., 1999) sometimes outlining no significant increases in oxidative markers in blood (van de Crommenacker et al., 2010).

On the contrary, the melon pulp concentrate supplementation in the diet of weaned piglets caused an improvement in total antioxidant status and blood resistance to haemolysis together with some positive results on growth performance, independently from the LPS challenge applied.

The pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, are important inducers of the synthesis of acute phase proteins, such as Hp, by hepatocytes (Carroll et al., 2004). According with this general mechanism of action, in our trial higher ILs levels were translated in higher Hp serum concentrations during LPS challenge period. In this view the impaired growth performance in both LPS challenged groups can be attributed to a decreased appetite, as observed in the present trial, and a competition for supplied nutrients between the immune system and growth processes (Doeschl-Wilson et al., 2009) together with a direct effect of high concentrations of inflammatory mediators on metabolic systems (Toepfer-Berg et al., 2004, Gomez-Laguna et al., 2010, Che et al., 2011).

The exposure of animals to pathogenic or non-pathogenic antigens results in activated immune system and subsequently cytokine release. Metabolic shifts are characterized by the redistribution of nutrients away from the growth processes toward immune system function (Beisel, 1977), and subsequently result in decreased feed efficiency for growth (Daiwen et al., 2008), as found in the present trial. Specifically, in agreement with our results, a number of works (Carroll et al., 2001, Frank et al., 2003, Frank et al., 2005) showed that immune challenge with LPS acts on the immune system through increased levels of serum TNF-α, IL-1β and IL-6. In our trial we did not observe significant interactions among the dietary treatment and the challenge applied over almost all considered parameters, including pro-inflammatory cytokines. Differences between the experimental groups were only evidenced for the main effect of melon pulp concentrate administration.

To the best author knowledge, at the moment no information are available on the effects of oral melon pulp concentrate administration when a chronic LPS

challenged is performed in piglets. Lallés et al., (2011) administered melon pulp concentrate for 5 and 12 days to postweaning piglets after an initial 2-days period of fasting to induce higher stress levels; animals were then slaughtered on day 7 and 14 after weaning to detect any variation in stress proteins level along the gastrointestinal tract: the authors did not find increased performance or higher feed efficiency, differently to us. Although it was not the main aim of the trial, the lack of improved performance in the work of Lallés et al., (2011), can be probably due to the too short duration of the trial to translate any positive effect in the gastrointestinal tract or on immune response to improved growth performance.

Although we did not find a significant diet effect on pro-inflammatory cytokines levels and serum Hp concentration, in the present trial we found increased TAOC levels and blood resistance to haemolysis for the main effect of melon pulp concentrate oral administration that confirms an improvement in the antioxidant defence system in treated piglets; this anyway did not turn into any significant variation in blood levels of SOD, ROS, or 8-oxo-dGuo.

If it is generally recognized that exogenous administration of SOD can be effective on the antioxidant status of animals (Carillon et al., 2013), the antioxidant mechanism of action of SOD administered with the diet opens a wide discussion that still has some lacking points.

In facts, if Vouldoukis et al., (2004a) and Notin et al., (2010) outlined improved resistance to the induced haemolysis of red blood cells respectively in mice and horse fed melon extract, some concerns still remain on its efficacy/mechanism of action mainly due to: a) the capacity of such high molecular weight proteins like SODs to pass the cell wall, b) the high degradation of SOD in the gastrointestinal tract before reaching the intestinal barrier, c) the high passage rate of oral SOD in the faeces, and d) the short half life of SOD in the blood stream.

As regards the molecular properties of SOD, in mammals three isoforms are present: i) a homodimer copper/zinc (Cu/Zn-SOD) of 32 kDa that is localized in the cytosol or in the mitochondrial inter-membrane space, ii) a homotetramer manganese-SOD (Mn-SOD) of 88 kDa, that is localized in the matrix and inner membrane of mitochondria, and iii) an extracellular tetrameric glycoprotein Cu/Zn form (EC-SOD) of 135 kDa (Fridovich, 1995, Faraci and Didion, 2004). While a fourth isoform of SOD has been found in bacteria coupled with iron SOD (Fe-SOD) (Beyer et al., 1990), plants seems to be somewhat in between mammalians and bacteria: still 3 isoforms are present, but these are a mitochondrial Mn-SOD, a Cu/Zn- SOD in the cytosol and the chloroplasts and a Fe-SOD in the chloroplasts (Hassan and Scandalios, 1990, Scandalios, 1997). Different studies in humans administered SOD by subcutaneous (Segui et al., 2004), intravenous (Jubeh et al., 2006), intraperitoneal (Jadot and Michelson, 1987), intramuscular (Lefaix et al., 1996) and local (Bartsch et al., 1980) injections or directly to the damaged tissue (Campana et al., 2004), but it is difficult to accept that such a high molecular weight proteins can enter directly to the cell (Carillon et al., 2013).

On the other side, the efficacy of SOD through its oral administration seems to be affected by the high degradation of the molecule in the gastrointestinal tract at

a comparable level as the other dietary proteins supplied, if not protected with some biopolymers such as wheat gliadin (Zidenberg-Cherr et al., 1983, Giri and Misra, 1984, Vouldoukis et al., 2004b).

The presence of orally administered SOD in the faeces was reported by Giri and Misra, (1984) who used a labelled form of SOD with Zn+ tracing both the fate of the metal ion and the changes in SOD enzyme activity in various tissues after oral administration in human. In this study almost ninety percent of the label came out in the faeces, and the remainder was almost certainly free metal separated from the protein, while no increased blood or hepatic SOD activity were evidenced.

Finally, it is believed that therapeutic levels of SOD cannot be maintained in plasma or non-renal tissues due to the short half-life (few minutes) of the protein (Huber et al., 1980) before being eliminated by the kidneys as the preferred organ for SOD accumulation (Swart et al., 1999).

Oxidative stress and inflammation are reported to be closely linked each other (Reuter et al., 2010), being inflammation an effect of increased oxidative stress, and oxidative stress a factor inducing the mediators of inflammation (Carillon et al., 2013). With respect to this, lower inflammatory response should be expected when high antioxidant effects of SOD are found, partially due to the chelating properties of the protein on metal ions. It is in fact recognized that transition metal ions participate in a variety of antioxidant defence systems. For example, Cu^{2+} and Mn^{2+} are essential cofactors in the enzyme activity of the SOD enzymes. SOD1 also contains Zn^{2+} in a structural (non-catalytic) role and employs the catalytic Cu^{2+}/Cu^{+} couple, i.e., copper cycles between its +2 and +1 oxidation states as it first reduces superoxide (O_2) to hydrogen peroxide (H_2O_2) and then oxidises it to dioxygen. It "dismutates" superoxide radicals in that the first radical is reduced while the second is oxidised and subsequently the product H_2O_2 is converted by catalase enzymes to water and O_2. Oxidative stress often includes diminished (Cu/Zn SOD or Mn-SOD2) activity or SOD-like activity (Thackray et al., 2002) and elevated radical-mediated lipid peroxidation: in such way increased reactive oxygen and nitrogen species are generated during pathogenesis (Brazier et al., 2014, Cristiana et al., 2014).

The short half-life of SOD in circulating blood and the lack of absorption by the gastrointestinal tract, together with its long-term effects when orally administered, suggest additional mechanism/s of action besides the chelating properties. On the basis of different *in vivo* and *in vitro* trials (Vouldoukis et al., 2004a, Izumi et al., 2002, Gonzalez et al., 1995, Yang et al., 2004), one further proposed mechanism of action is that exogenous SODs can someway induce increased global antioxidant defence as outlined by TAOC, but not by SOD, ROS or 8-oxo-dGuo levels in the present trial. In this view, exogenous SODs could regulate the induction of antioxidant enzymes at transcriptional level through the antioxidant response element (ARE) and the nuclear-transcription-factor-E2-related factor (Nrf2) (Carillon et al., 2013) activating the immune system locally and leading to the activation of macrophages in the entire body (Vouldoukis et al., 2004b, Lallès et al., 2011).

Unfortunately in our trial both ARE and Nrf2 expression level were not tested, but the generally increased total antioxidant capacity in blood after SOD oral administration, together with higher RBC blood resistance to haemolysis can be included in this suggested hypothesis although at the moment some mechanism of action of orally administered SOD must still be elucidated.

Conclusions

The present study demonstrated that oral supplementation with melon pulp concentrate can enhance the antioxidant status and growth rate of weaned pigs. These findings suggest that piglets can benefit the inclusion of melon pulp concentrate when oxidative stress and/or inflammation are increased, although the mechanism by which it exerts its biological effect remains to be clarified.

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Tables

Table 1. Diet composition, expected and calculated analyses of basal diet (% as fed)

Ingredients	
Wheat meal	29.465
Barley meal	23.120
Wheat flaked	14.000
Soybean meal 48%	17.000
Sweet whey powder	6.000
Soybean oil	3.000
Corn gluten meal	2.500
Dextrose	1.500
Dicalcium phospate	1.300
L-Lysine	0.570
Calcium carbonate	0.500
Sodium chloride	0.300
Vitamins + trace elements[x]	0.250
L-Threonine	0.230
DL-Methionine	0.180
Flavour	0.050
Optisweet SD	0.015
Zinc oxide	0.010
Cu sulphate	0.010
Chemical composition	
DM	89.44
CP	18.91
EE	4.76
ASH	5.58
NDF	14.59
Ca	0.75
P	0.57
Calculated composition	
DE (Mcal/kg)	3.44
NE (Mcal/kg)	2.45
Lys total (%)	1.25
Met+Cyst total (%)	0.80
Threonine total (%)	0.85
Trytophane (%)	0.21

[x] Vit E was contained in the form of all rac-alpha-tocopheryl acetate at 100 mg/kg of complete feed; selenium was included as sodium selenite (0.66 mg/kg of complete feed, providing 0.3 mg/kg of selenium)

Table 2. Effects of melon pulp concentrate supplementation on growth performance of LPS challenged (+) and non-challenged (-) post-weaning piglets

Item	Day	Challenge				SEM	*P* value						
		-		+									
		Group											
		C	MPC	C	MPC		MPC	Challenge	Time	MPC X Time	MPC X Challenge	Challenge X Time	MPC X Challenge X Time
BW(kg)	0	7.79	7.79	7.78	7.79	0.71	0.70	0.47	<0.01	1.00	0.73	0.50	1.00
	8	8.85	8.69	9.24	8.98								
	15	11.22	11.10	11.96	11.65								
	19	13.41	13.42	14.30	14.04								
	21	14.23	14.32	14.14	14.23								
	23	15.43	15.79	15.15	15.17								
	25	16.49	16.86	15.82	15.96								
	26	16.98	17.43	16.12	16.41								
	29	17.94	18.48	16.98	17.37								
ADG (g/d)	0-19	298	297	343	328	0.05	0.79	0.13			0.78		
	19-29	464	517	255	327	0.06	0.05	<0.01			0.75		
	0-29	350	368	317	331	0.05	0.50	0.14			0.91		
FI (kg)	0-19	8.56	8.55	9.50	9.31	1.24	0.87	0.18			0.89		
	19-29	7.31	8.22	4.99	6.15	0.67	<0.01	<0.01			0.73		
	0-29	15.74	16.63	14.65	15.56	1.75	0.31	0.22			0.10		
G:F	0-19	0.64	0.65	0.69	0.66	0.04	0.71	0.12			0.32		
	19-29	0.62	0.63	0.51	0.53	0.05	0.64	<0.01			0.72		
	0-29	0.64	0.64	0.63	0.61	0.03	0.69	0.18			0.41		

LPS= lipopolysaccharide; C-= control without LPS challenged; C+= control with LPS challenged; MPC-=melon pulp concentrate without LPS challenged; MPC+= melon pulp concentrate with LPS challenged; BW=body weight; ADG=average daily gain; FI=feed intake; G:F=gain:feed. Dose of MPC=30g/ton fed. $^{A,B}P \leq 0.01$, $^{a,b}P \leq 0.05$.

Table 3. Effects of melon pulp concentrate supplementation on body temperature of LPS challenged (+) and non-challenged (-) post-weaning piglets

Item		Challenge - Group		Challenge + Group		SEM	*P* value						
		C	MPC	C	MPC		MPC	Challenge	Time	MPC X Time	MPC X Challenge	Challenge X Time	MPC X Challenge X Time
Body Temperature, Challenge Days													
Day 1 (19)	Pre-Ch	39.36	39.52	39.65	39.36	0.10	0.23	<0.01	<0.01	0.72	0.05	<0.01	0.34
	Post-Ch	39.77	39.72	40.97	40.77								
Day 2 (21)	Pre-Ch	39.73	40.12	39.73	39.61	0.15	0.40	0.10	<0.01	0.04	0.06	<0.01	0.59
	Post-Ch	40.24	40.07	40.99	40.54								
Day 3 (23)	Pre-Ch	40.08	40.12	39.87	39.72	0.11	0.25	<0.01	<0.01	0.65	0.92	<0.01	0.19
	Post-Ch	40.11	39.87	41.02	41.01								
Day 4 (25)	Pre-Ch	39.88	39.75	39.75	39.58	0.08	0.02	<0.01	<0.01	0.89	0.78	<0.01	0.52
	Post-Ch	39.98	39.79	40.52	40.44								
Day 29		39.50	39.38	39.28	39.39	0.24	0.97	0.38	---	---	0.34	---	---

Pre-Ch= Pre-Challenge; Post-Ch= Post-Challenge; LPS= lipopolysaccharide; C-= control without LPS challenged; C+= control with LPS challenged; MPC-=melon pulp concentrate without LPS challenged; MPC+= melon pulp concentrate with LPS challenged. Dose of MPC=30g/ton fed. $^{A,B}P \leq 0.01$, $^{a,b}P \leq 0.05$.

Table 4. Effects of melon pulp concentrate supplementation on SOD, TAOC, ROS and 8-oxo-dGuo of LPS challenged (+) and non-challenged (-) post-weaning piglets

Item	Day	Challenge - Group C	Challenge - Group MPC	Challenge + Group C	Challenge + Group MPC	SEM	*P* value MPC	Challenge	Time	MPC X Time	MPC X Challenge	Challenge X Time	MPC X Challenge X Time
SOD (U/mL)	0	49.87	55.62	53.88	53.33	3.69	0.66	0.77	0.22	0.81	0.65	0.89	0.56
	19	54.87	56.77	57.47	57.90								
	21	51.14	54.90	54.92	59.02								
	23	53.90	47.84	49.39	55.12								
	25	53.72	55.81	53.37	54.69								
	27	54.32	55.30	54.54	51.73								
	29	63.70	55.24	56.41	56.99								
TAOC (mM Trolox equivalent)	0	4.69	3.47	3.78	4.91	1.71	<0.01	0.08	<0.01	0.08	0.66	0.61	0.91
	19	4.51	8.65	3.96	7.07								
	21	7.32	12.73	5.21	6.42								
	23	5.96	7.44	4.63	6.26								
	25	5.90	10.22	5.26	8.17								
	27	2.97	9.54	2.67	9.40								
	29	3.47	3.50	3.26	3.23								
ROS (Abs)	0	0.31	0.36	0.36	0.30	0.03	0.73	0.62	<0.01	0.90	0.74	0.95	0.36
	19	0.14	0.12	0.14	0.13								
	21	0.12	0.11	0.14	0.12								
	23	0.12	0.12	0.12	0.11								
	25	0.14	0.12	0.10	0.11								
	27	0.12	0.12	0.07	0.13								
	29	0.15	0.12	0.11	0.13								
8-oxo-dGuo (ng/mL)	19	0.77	0.70	0.68	0.69	0.06	0.99	0.40	<0.01	0.83	0.26	0.17	0.45
	25	0.82	0.85	0.85	0.85								
	29	1.07	0.98	1.09	1.20								

SOD= superoxide dismutase; TAOC= total antioxidant activity; ROS= reactive oxygen species; 8-oxo-dGuo=8-hydroxy-2'deoxyguanosine; LPS= lipopolysaccharide; C-= control without LPS challenged; C+= control with LPS challenged; MPC-=melon pulp concentrate without LPS challenged; MPC+= melon pulp concentrate with LPS challenged. Dose of MPC=30g/ton fed. $^{A,B}P \leq 0.01$, $^{a,b}P \leq 0.05$.

Table 5. Effects of melon pulp concentrate supplementation on KRL in blood, plasma contribution and RBC of LPS challenged (+) and non-challenged (-) post-weaning piglets

Item	Day	Challenge - Group C	Challenge - Group MPC	Challenge + Group C	Challenge + Group MPC	SEM	*P* value MPC	*P* value Challenge	*P* value Time	*P* value MPC X Time	*P* value MPC X Challenge	*P* value Challenge X Time	*P* value MPC X Challenge X Time
KRL in blood (HT50, min).	19	103.99	109.09	100.95	107.76	4.60	0.20	0.09	<0.01	0.67	0.32	0.17	0.64
	25	101.54	109.98	115.53	115.57								
	29	112.61	117.62	123.67	119.19								
KRL in RBC (HT50, min).	19	60.19	67.77	60.39	67.66	2.40	<0.01	0.66	<0.01	0.28	0.26	0.96	0.67
	25	65.28	69.69	67.70	69.31								
	29	70.47	76.12	74.45	73.73								
KRL plasma contribution	19	43.81	41.31	40.55	40.10	3.89	0.72	0.08	0.51	0.81	0.62	0.13	0.77
	25	36.26	40.28	47.84	46.25								
	29	42.14	41.51	49.22	45.46								

KRL=Kit Radicaux Libres; LPS= lipopolysaccharide; C-= control without LPS challenged; C+= control with LPS challenged; MPC-=melon pulp concentrate without LPS challenged; MPC+= melon pulp concentrate with LPS challenged. Dose of MPC=30g/ton fed. $^{A,B}P \leq 0.01$, $^{a,b}P \leq 0.05$.

Table 6. Effects of melon pulp concentrate supplementation on pro-inflammatory cytokines and Hp of LPS challenged (+) and non-challenged (-) post-weaning piglets

Item	Day	Challenge				SEM	*P* value						
		-		+									
		Group											
		C	MPC	C	MPC		MPC	Challenge	Time	MPC X Time	MPC X Challenge	Challenge X Time	MPC X Challenge X Time
IL1β (ng/mL)	0	0.23	0.26	0.27	0.30	0.03	0.71	<0.01	<0.01	0.75	0.63	<0.01	0.81
	19	0.18	0.15	0.17	0.19								
	21	0.20	0.24	0.73	0.75								
	23	0.22	0.20	0.80	0.79								
	25	0.19	0.21	0.84	0.80								
	29	0.23	0.24	0.36	0.34								
IL6 (ng/mL)	0	5.52	5.75	5.79	5.93	0.47	0.89	<0.01	<0.01	0.95	0.40	<0.01	0.79
	19	2.63	2.81	2.65	2.44								
	21	2.63	2.94	10.48	9.38								
	23	2.24	2.57	10.59	10.26								
	25	2.68	2.79	10.32	10.63								
	29	2.51	2.18	2.70	2.74								
TNFα (pg/mL)	0	33.13	34.30	33.90	31.94	2.29	0.41	<0.01	<0.01	0.92	0.15	<0.01	0.98
	19	6.69	9.40	9.37	9.42								
	21	10.95	14.03	32.37	33.95								
	23	12.11	12.30	34.23	32.48								
	25	8.48	13.13	32.14	30.74								
	29	8.54	9.67	13.07	12.97								
Hp (μg/mL)	0	902.09	997.38	504.66	619.58	123.78	0.25	0.03	<0.01	0.52	0.37	<0.01	0.93
	19	712.64	705.06	594.60	584.08								
	21	1090.14	856.57	1641.78	1424.43								
	23	811.54	694.94	1209.25	1255.71								
	25	917.91	612.02	900.15	921.86								
	29	575.14	511.15	567.57	532.97								

IL-1β= interleukin-1 beta; IL-6=interleukin-6; TNF-α= tumor necrosis factor-alpha; Hp=Haptoglobin; LPS= lipopolysaccharide; C-= control without LPS challenged; C+= control with LPS challenged; MPC-=melon pulp concentrate without LPS challenged; MPC+= melon pulp concentrate with LPS challenged. Dose of MPC=30g/ton fed. $^{A,B}P \leq 0.01$, $^{a,b}P \leq 0.05$.

CHAPTER 4

Effects of melon pulp concentrate rich in superoxide dismutase on broiler growth performance, pododermatitis and cellulitis

Effects of melon pulp concentrate rich in superoxide dismutase on broiler growth performance, pododermatitis and cellulitis

Abstract

The aim of the trial was to study the effects of an antioxidant feed supplement (melon pulp concentrate, MPC that contains high level of SOD as a primary antioxidant) on growth performance, pododermatitis and cellulitis of broilers. A total of 1104 broilers were allocated to 4 experimental groups for 35 days with four different dietary treatments: a) basal diet (C: control), b) basal diet plus melon pulp concentrate (MPC1, MPC2 and MPC3) as described below. The feeding regimen consisted of starter (0-10 d), grower (11-24 d) and finisher diet (25-35 d). During starter phase, dietary treatments were corn-soybean meal based diets supplemented with 0 g/ton (C), 30 g/ton (MPC1), 15 g/ton (MPC2), 15 g/ton (MPC3) of melon pulp concentrate (MPC) (Melofeed®, Lallemand, Blagnac, France). In grower phase, C, MPC1 and MPC2 received same basal diet but MPC3 received basal diet supplemented with 15 g/ton of MPC. During 24-35 days, the same basal diet without MPC was supplied to four experimental groups. 12 pens per group with 23 broilers per pen were used. On day 24 and 25, pododermatitis was evaluated on all animals using a scoring system that ranged from 0 to 2. On day 35, cellulitis was evaluated on all slaughtered birds. Litter score and litter DM was assessed on day 35. Body weight (BW) of birds per pen was recorded on 0, 10, 24, 35 experimental days from the beginning of the experiment. Individual body weight, slaughter live body weight, carcass weight, dressing percentage and organs weight (liver, heart, intestine, Bursa of Fabricius, spleen, pancreas and gizzard) percentage were also assessed at day 35. Pen feed intake was recorded on 0, 10, 24, 35 experimental days. Pen feed residues were determined at the end of each feeding period to estimate mean ADG, ADFI and gain: feed (G:F) ratio for each pen. Treatment (MPC) was able to affect body weight ($P<0.05$), ADG ($P<0.05$) and G:F ($P=0.05$) during experimental period. Final BW was higher in MPC1 and MPC2 than C and MPC3 ($P<0.01$). Carcass weight tended to be higher in MPC2 than C and MPC3 ($0.05<P\leq0.1$). Percentage of the Bursa of Fabricius weight tended to be higher ($0.05<P\leq0.1$) in MPC1 and MPC3. Incidence and severity of pododermatitis varied within each feeding period. Significant treatment, time and their interaction ($P<0.01$) were found in pododermatitis lesions analysis. At 24 d of age, MPC3 scores were the highest ($P<0.01$) between diets. At 35 d of age, scores in MPC3 were still higher than MPC1 ($P<0.05$). Pearson correlation between pododermatitis and body weight was not significant, by the way it was highly significant ($P<0.01$) between pododermatitis and

litter dry matter (-30%). No cellulitis was detected in experimental animals. MPC supplemented at 15 or 30 g/ton for the first 10 days of lifecycle in female commercial broilers grown in high stock density conditions improved performance. Longer (24 days) supplementation increased pododermatitis score.

Introduction

Antioxidant supplementation has beneficial effects in several situations where oxidative stress is involved. Besides treatment with dietary antioxidants such as selenium or vitamins, an original way to increase antioxidant capacity could be by supplying antioxidant enzymes, which have longer lasting effects because of their lower rate of exhaustion than mere metabolites. Numerous animal and human studies have suggested the potential therapeutic role of the superoxide dismutase (SOD) enzyme supplementation in several pathological situations, such as carcinogenesis (Okada et al., 2006), inflammatory (Salvemini et al., 2001, Segui et al., 2004, Watterlot et al., 2010), infectious (Webb et al., 2008), respiratory (Gonzalez et al., 1995, Tanaka et al., 2011) and vascular diseases (Fukai and Ushio-Fukai, 2011). Sources of SOD are numerous, but its poor bioavailability requires formulation improvements (Arangoa et al., 2001, Dugas, 2002, Vouldoukis et al., 2004). In this context, SOD-rich melon pulp concentrate (Melofeed®, Lallemand Company, France) is gastro-resistant, being coated with hydrogenated vegetable oil. Carillon et al. (2012) previously demonstrated *in vitro* that the antioxidant capacity of this melon concentrate is due to its high SOD content rather than to the other compounds present.

In today's commercial broiler production, lesions on footpads are a common finding, leading to economic and production losses and raising concern about animal welfare (Breuer et al., 2006). The skin of footpads is considered a specialized epidermal type with reticular scales that protect birds against environmental hazards (Sawyer et al., 1984, Pass, 1989). Footpad dermatitis (FPD) condition is most likely to have multifactorial origin (Shepherd and Fairchild, 2010, De Jong et al., 2012). It has been shown that wet litter, together with nutrition, are the major causative agents of FPD (Shepherd and Fairchild, 2010). Skin strength, bird weight, sex, and breed may also be related to the development of these lesions (Mayne, 2005).

Foot pad dermatitis (FPD), also called pododermatitis or footpad lesion, ranks among the most frequent occurring medical conditions in poultry and characterizes an inflammatory to necrotic state of the plantar skin of the metatarsal and in severe cases of the digital foot pads (Hafez et al., 2005). Foot pad alterations appear in turkeys and broilers as well as laying hens in a frequency that requires arrangements for prevention and attenuation (Kamphues et al., 2011). Multiple factors, for example, poor litter condition, especially high litter moisture (Martland, 1984, Clark et al., 2002, Spindler, 2007, Mayne et al., 2007a, Youssef et al., 2010, El-Wahab et al., 2011, Wu and Hocking, 2011) and chemical irritants bound to litter (Martland, 1984), exposition durance (Berk, 2007, Krautwald-Junghanns et al., 2011, Schumacher et al., 2012), stocking density (Clark et al., 2002, Hafez et al., 2005), nutritional deficiencies in biotin (B-complex vitamin) or zinc (Youssef et al., 2012), and genetic factors (Ask, 2010), have been linked to FPD. Within 24 h after birds were initially exposed to wet litter, the first signs of FPD appeared, possibly due to a rapid inflammatory reaction

(Mayne et al., 2007b). Several authors state that FPD, especially in severe cases, may cause discomfort and pain for the birds (Ekstrand et al., 1997, Buda et al., 2002, Mayne et al., 2006, Berk, 2007, Mirza, 2011) and therefore has the potential to influence health and well-being of animal (Shepherd and Fairchild, 2010, Youssef et al., 2010).

Wet litter is the most important factor causing FPD, and many factors may determine litter quality (Shepherd and Fairchild, 2010). It is known that there may be a seasonal effect on prevalence of FPD (Ekstrand and Carpenter, 1998a). Elevated humidity levels may result in wet litter and increase the incidence of FPD (Shepherd and Fairchild, 2010). Mainly the winter season has been associated with higher levels of FPD (Ekstrand and Carpenter, 1998b, Dawkins et al., 2004, Shepherd and Fairchild, 2010). Other potentially influencing factors are breed, feed manufacturer, and stocking density (Haslam et al., 2007, Shepherd and Fairchild, 2010) and slaughter plant (Pagazaurtundua and Warriss, 2006).

Presently, broiler welfare is receiving increasing attention across Europe, which is illustrated by the European Council Directive laying down minimum rules for the protection of chickens raised for meat production (Directive, 2007). In addition to requirements on administration, light intensity and light schedule, air quality, and training of the farmer, the Council Directive also places a restriction on the stocking density for broiler chickens to a maximum of 42 kg/m2 (if all requirements are fulfilled and the mortality rate is kept below the maximum level stated in the Directive, and if national legislation permits). Denmark and Sweden included FPD as an additional welfare indicator in their own broiler welfare legislation (Berg and Algers, 2004).

Cellulitis in broiler chickens is only detectable at slaughter, once the carcass has been plucked and scalded. Cellulitis, known as infectious process or inflammatory process (IP), is of increasing concern for broiler integrators. In the U.S., an estimated $20 million annually is lost to whole carcass condemnation due to cellulitis (Norton, 1997, Norton and Bilgili, 1996). In addition, when excessive trimming, parts condemnation, and slowed line speeds are included in this estimate, losses may exceed $50 million annually (Downs et al., 2000). Cellulitis results from a dermal/subdermal colonization of bacteria (primarily *Escherichia coli*) when the skin is scratched or punctured. To combat the invading bacteria, the bird mounts an immune response (i.e., immune cell accumulation at wound entrance). The resulting plaque-like deposit must be trimmed or it can result in part or whole carcass condemnation, depending on the severity of the cellulitis lesion.

Several management factors contributing to cellulitis incidences have been identified (Downs et al., 2000, Elfadil et al., 1996a, Elfadil et al., 1996b). Among these are overcrowding, feed restriction, or feed outages (resulting in high bird activity when feed is re-introduced), inadequate water and feeder space, and excessive bird activity, all of which contribute to increased skin scratches.

Supplementation of vitamins and minerals influences human and animal health (Downs et al., 2000). More specifically, vitamin E and zinc play crucial roles in immune response (cellular and humoral), wound healing, and maintenance of skin

quality (Finch and Turner, 1996, Kidd et al., 1994, Pimentel et al., 1991, Colnago et al., 1984, Gross et al., 1979, Pekarek et al., 1979). Consequently, cellulitis, which is caused primarily by skin damage that elicits an immune response against invading bacteria, may be reduced by increasing the dietary level of vitamin E and zinc in broiler diets.

Bioavailability of certain mineral supplements can be low; however, complexing them with more readily available compounds (e.g., amino acids, proteins, carbohydrates, or organic acids) can substantially enhance mineral absorption, thereby enhancing use efficiency. Therefore, an amino acid complexed zinc product may contribute more to cellulitis reduction than an inorganic zinc source.

The aim of this study was to evaluate the effects of oral supplementation of a SOD-rich melon pulp concentrate (MPC) on broiler growth performances, pododermatitis and cellulitis.

Materials and Methods

The protocol for care, handling, and sampling of birds defined in the present study was reviewed and approved by the Università degli Studi di Milano Animal Care and Use Committee (Protocol No 25/15 06.05.15). The trial was performed at the Animal Production Research and Teaching Centre of the Faculty of Veterinary Medicine of the Università degli Studi di Milano.

Animals, housing and experimental design

One-day-old Ross 308 female broilers were obtained from a commercial hatchery. Birds were vaccinated against Marek's disease, infectious bronchitis and New Castle disease at the hatchery. 1104 broiler chicks with similar average weights (39.19±4.70 g) were raised from 1 to 35 d of age and randomly distributed into 48 floor pens with 23 birds per pen. Birds were divided into four homogenous experimental groups of 276 birds (12 replicates) each in a randomized block design. Each floor pen had 1.3 m^2 size and each pen was equipped with 1 drinker and 1 feeder. A 2-3 cm new wood shavings layer was added on top of a previous 35 days broiler growing cycle litter dried for one week before brooding as a sanitary challenge. The feeding regimen consisted of starter (0-10 d), grower (11-24 d) and finisher diet (25-35 d). During starter phase, dietary treatments were corn-soybean meal based diets supplemented with 0 g/ton (C), 30 g/ton (MPC1), 15 g/ton (MPC2), 15 g/ton (MPC3) of melon pulp concentrate (MPC) (Melofeed®, Lallemand, Blagnac, France). In grower phase, C, MPC1 and MPC2 received same basal diet but MPC3 received basal diet supplemented with 15 g/ton of MPC. During 24-35 days, the same basal diet without MPC was supplied to four experiment groups. The basal diet was mash feed and prepared with the same batch of ingredients for starter, grower, and finisher periods and was formulated to meet the nutrient requirements according to Ross-308 rearing guidelines (Aviagen, 2007). All birds had free access to feed and water. The ingredients and chemical composition of the basal diets are shown in table 1. Temperature was initially set at 34.5^{0}C on d 1 and decreased linearly by 0.5^{0}C per day to a temperature of 25^{0}C. During the study, the birds received a lighting regimen of 23L:1D from 1 to d 7 and afterward 20L: 4D until d 35.

Pen body weight (BW) of birds was recorded on 0, 10, 24, 35 experimental days from the beginning of the experiment. Individual body weight was also assessed at day 35. Pen feed intake was recorded on 0, 10, 24, 35 experimental days. Pen feed residues were determined at the end of each feeding period to estimate mean average daily gain (ADG), average daily feed intake (ADFI) and gain:feed (G:F) ratio for each

pen. ADG and ADFI were determined by measuring the BW of the birds and their feed consumption per cage (sum of feed offered - feed leftover at the weighing time).

Slaughter data collection and litter score

On day 35, two birds from each pen were selected as previously described; one bird was used to determine weight of liver, pancreas, spleen, Bursa of Fabricius, gizzard, heart and small intestine. Organs weight was expressed as a percentage of body weight. The second bird was used to assess live body weight at slaughter (SLBW), carcass weight and dressing percentage. Morbidity, medications and mortality were eventually recorded on a daily basis in each pen. Litter score and litter DM was assessed on day 35. Litter score was evaluated by visual inspection and received a score between 0 (highest quality/new bedding) and 5 (as lowest/severely caked and wet).

Pododermatitis and cellulitis

Pododermatitis on all birds was scored when birds were weighed at day 24 and day 35. The birds were scored using a scoring system that ranged from 0 to 2 (Ekstrand et al., 1998) as following: 0= no lesions; no or very small superficial lesions, slight discoloration on a limited area, mild hyperkeratosis, 1= Mild lesion; discoloration of the foot pad, superficial lesions, dark papillae and 2= Severe lesion; ulcers or scabs, signs of haemorrhages or swollen foot pads. All birds, except 96 selected for organs weight and gene expression evaluation, were slaughtered and checked for cellulitis lesion scoring (Olkowski et al., 2005).

Statistical analysis

Data were analysed using a MIXED procedure of SAS (SAS Inst. Cary, NC) with a randomized complete block design. Body weight, ADG, ADFI and gain:feed (G:F) and pododermatitis were performed by a MIXED procedure for repeated measurements with the pen as the experimental unit. ADG, ADFI and G:F referred to 0-35 days of trial data, were analysed by MIXED procedure as well for slaughter live body weight (SLBW), dressing percentage, carcass weight and organ weight. Pearson correlation was performed on pododermatitis score, BW, litter dry matter and litter score. Significance level was stated for $P \leq 0.05$. $0.05 < P \leq 0.1$ was considered as a tendency.

Results

Growth Performance

The effects of MPC on growth performance in the pen are shown in table 2. Initial body weight of the four experimental groups was not statistically different, confirming the homogenous status of the birds at the beginning of the trial. Time ($P<0.01$) and treatment ($P<0.05$) effects were significant during trial period. Significantly higher ($P<0.05$) mean body weight was observed in MPC2 in comparison with C and MPC3. Treatment x time interaction was statistically significant ($P<0.01$). On day 24 of the trial, body weight in MPC2 was significantly higher than MPC3 ($P<0.01$) and a tendency ($0.05<P\leq0.1$) was observed in MPC2 body weight compared to MPC1. At the end of trial (on day 35), birds were heavier ($P<0.01$) in MPC1 and MPC2 than C and MPC3.

During trial period, time ($P<0.01$) and treatment ($P<0.05$) effects on ADG were statistically significant. Considering the overall trial period, MPC2 ADG values were higher ($P<0.05$) than C and MPC3; MPC1 gain tended also to be higher ($0.05<P\leq0.1$) compared to MPC3. Treatment x time interaction effect was significant ($P=0.01$). During grower phase (10-24 days), MPC2 had higher statistically significant ADG values ($P<0.01$) than MPC3; also C ADG tended to be higher than MPC3 ($0.05<P\leq0.1$). In the last feeding phase, higher significant gain values ($P<0.01$) were registered in MPC1 and MPC2 in comparison with C and MPC3.

Time ($P<0.01$) effect on ADFI was statistically significant during trial period. Treatment effect and treatment x time interaction were not significant. Time and treatment x time interaction ($P<0.01$) effects on G:F were significant during the trial period. MPC1 and MPC2 tended ($0.05<P\leq0.1$) to have higher G:F compared to MPC3 considering the 0-35 days period. On day 0-10 of the trial, G:F in MPC2 and MPC3 were significantly higher than C ($P<0.05$) and tended to be higher also than MPC1 ($0.05<P\leq0.1$). Considering 10-24 days period, MPC3 had the lowest value of G:F ($P<0.01$). During finisher phase, MPC1 had higher G:F values than C ($P<0.05$).

Slaughter data collection and litter score

The melon pulp concentrate did not significantly affect Slaughter Live Body Weight (SLBW), dressing percentage, litter score and visceral organs percentage (liver, pancreas, gizzard, heart, spleen and small intestine) as presented in table 3. Broilers in MPC2 tended to have higher ($0.05<P\leq0.1$) carcass yield than MPC3 and C at 35 d of experiment. Furthermore, litter dry matter tended to be higher in MPC2 than MPC3 and C ($0.05<P\leq0.1$). The Bursa of Fabricius percentage of birds in MPC1 and MPC3 tended to be greater than in C ($0.05<P\leq0.1$).

Pododermatitis and cellulitis

Scores of pododermatitis are presented in table 4. Incidence and severity of pododermatitis varied within each feeding period. Pododermatitis score increased in all treatment between 24 to 35 days. On average, pododermatitis severity was significantly higher in MPC3 than in MPC1 and MPC2 ($P<0.01$), and in C ($P<0.05$). Significant treatment, time and their interaction effects ($P<0.01$), were found in pododermatitis lesions analysis. At 24 d of age, MPC3 scores were the highest ($P<0.01$). Birds reared on MPC3 diet showed higher scores than those reared on C, MPC1 and MPC2 diets. At 35 d of age, scores in MPC3 were still higher than MPC1 ($P<0.05$). Pearson correlation between pododermatitis and body weight was not significant but it was highly significant ($P<0.01$) between pododermatitis and litter dry matter (-30%). No cellulitis was observed in birds involved in the present trial.

Discussions

Recently antioxidant and anti-inflammatory properties of some compounds containing high levels of superoxide dismutase (SOD) became more interesting because of their therapeutic use during stress condition in animals (Yasui and Baba, 2006). When oxidative stress occurs, SOD is able to convert superoxide ions to oxygen and hydrogen peroxide with a protective effect on the cells. A second function of SOD is to regulate neutrophil apoptosis that can positively decrease inflammatory disorders (Yasui and Baba, 2006), especially if SOD is preserved from digestion by protection (Jubeh et al., 2006, Segui et al., 2004). Moreover endogenous SOD and stress proteins are known to be positively correlated, as demonstrated in mice (Lallès et al., 2011). Due to these mechanisms of action, the use of SOD is expected to improve the health status and the immunity of animals contributing to improve the overall growth performance. At the present few studies are available on poultry, especially if related to the use of melon concentrate as a high SOD content compound (Barbé et al., 2015). However, referring to monogastric animals, Lallès et al., (2011) found a positive effect of the administration of a melon concentrate rich in SOD in post-weaning piglets over stress proteins along the gastrointestinal tract that was not, however, traduced in increased growth performance. During the study these authors also found that plasma SOD was increased only with the higher amount supplied and at the end of the trial (14 day post weaning). Lallès et al., (2011) concluded that the administration of exogenous sources of SOD is in agreement with the general concept of a positive link between oxidative stress and tissue stress protein expression and of their reduction by natural antioxidant substances. In laying hens, melon juice concentrate increased shelf life of eggs, yolk percentage and vitamin E transfer in yolk. It also stimulated SOD, CAT and GPx production in the reproductive tract.

Some other researchers found that the supplementation with a melon juice concentrate rich in SOD (140 IU SOD per capsule/day) may have a positive effect on several signs and symptoms of perceived stress and fatigue linked to performance, pain, cognition or behaviour in healthy humans compared to a placebo (Milesi et al., 2009). The present trial supports the hypothesis that MPC can have a positive effect on performance in contrast with the results obtained on pigs by Lallès et al., (2011). The reason could be the size of the experimental sample that, for the pig study, was not enough to reach a statistical significance, or interspecies differences. Indeed, the performances obtained in MPC3 are supportive of the idea that, either specific growing period either length of supplementation of the diet with MPC can modulate the response of the animals.

The current study was designed to evaluate the effects of feeding melon pulp concentrate in broiler performance, pododermatitis and cellulitis. Performance obtained by broilers in the present trial were lower than objectives for genetic type

across treatments, this results could be explained mainly by the high density of the animals within pens and the mash diet offered to chicken all over the trial.

Carillon et al., (2013) observed that highest dose of melon pulp concentrate reduced significantly BW gain with no differences in feed and water consumption in 83 weeks-old rat fed diets containing 3 levels of melon pulp concentrate (10, 40, and 160 U SOD/day) for 28 days proposing a diuretic activity as main reason. Results of the current study showed that dietary supplementation with MPC in growing chicks improved BW, ADG, and G:F of MPC1 (30 g/ton MPC during starter phase) and MPC2 (15 g/ton MPC during starter phase) compared with MPC3 (15 g/ton during starter and growing phases) and C. These results support the idea that MPC supplementation could improve performances in specific productive phases (early life stage) and the prolonged use could be detrimental. The obtained results do not show difference between the two levels of MPC used suggesting the lower could be sufficient to obtain positive results. It has to be taken in account that also genotype could be crucial to decide proficient levels of use of this supplement. Indeed, it has been observed that the enzymatic antioxidative system in the erythrocytes of Cobb strain chickens is not very susceptible to stress (heat) compared with the one of Ross (Altan et al., 2003). At the end of trial (on day 35), statistically significant greater body weight were observed in MPC1 and MPC2 than C and MPC3, but no significant effect were noted on slaughter live body weight, even if the results display a similar pattern between groups. Birds selected for dressing percentage assessment were in fact chosen for average weight within each pen. The statistical model used for BW, considering a larger number of animals was more powerful and so able to detect significances. Although MPC had no effect on dressing percentage for the four groups, dietary treatment tended to have an effect on carcass weight. All together, positive results just described, could be due to improved general bird health, resulting in fewer nutrients being directed to immune function and more being available for muscle deposition.

The Bursa of Fabricius is an important immunological organ in poultry, indeed, is a central lymphoid organ (Cooper et al., 1966), and is involved in cellular and humoral immunity. This organ is, actually, commonly weighed when studying immune function in birds (Pope, 1991). Furthermore, it has been observed that bursa weight is a very good marker for stress related to housing density, being the two parameters inversely proportional (Heckert et al., 2002). Inclusion of MPC SOD in the diet tended to increase the Bursa of Fabricius of broilers (MPC1 and MPC3) compared with the C. Similar results were observed in birds fed Forsythia suspense extract (FSE) and berberine (BE), herbal extract with powerful antioxidant activity, in fact FSE + BE treated animals had greater bursa weight than the birds in control group, alleviating the stress induced by high stock density (Zhang et al., 2013). Our stock density was at the upper limit of EU legislation and was close to the normal one used by Zhang et al. (2013).

Pododermatitis is associated with a range of welfare issues (Martland, 1984, Martland, 1985, Kestin et al., 1999) and high prevalence may be indicators for poor welfare conditions (Haslam et al., 2007). Studies have shown that a genetic

background for pododermatitis in broilers exists (Kjaer et al., 2006, Ask, 2010); therefore, genetic selection against the disposition to develop pododermatitis may contribute to the reduction of the prevalence. Pododermatitis is also linked to poor litter quality (Berg, 2004), our results confirm this association in particular in the MPC3 group. The negative correlation observed between litter dry matter and pododermatitis score is clearly related to this point. Referring to the performance data obtained in rats (Carillon et al., 2013) the diuretic activity suggested to explain those results, could be in agreement with lower litter dry matter and consequently higher pododermatitis score observed in our trial on broilers. However, no data on litter dry matter are available at day 24 to fully support this hypothesis, since pododermatitis in MPC3 was already higher at that time of the experiment In fact, a general increase of pododermatitis score was recorded from day 24 to day 35, most probably due to worse litter conditions, increased weight of the animals and time of exposure to wet litter.

Conclusions

Results obtained evidenced that MPC supplemented at 15 or 30 g/ton for the first 10 days of lifecycle, improved performance in female commercial broilers grown under high stock density condition. Longer (24 days) supplementation increased pododermatitis score. MPC tended also to increase the percentage of weight of Bursa of Fabricius, indicating its influence on the immune status and stress response of the animals.

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Tables and pictures

Table 1. Diet composition and calculated chemical analyses of the basal diets

Ingredients	Starter	Grower	Finisher
Corn	55.09	57.42	61.69
Soybean Meal 48	37.30	34.10	29.20
Soybean Oil	3.00	4.30	5.30
Dicalcium Phosphate	2.50	2.50	2.10
Calcium Carbonate	0.70	0.45	0.50
MinVit Premix[1]	0.50	0.50	0.50
Salt	0.40	0.40	0.40
Methionine DL	0.28	0.16	0.14
L-Lysine HCL	0.23	0.17	0.17
Total	100.00	100.00	100.00
Chemical composition (% as fed basis)			
DM	87.77	87.82	87.80
PG	22.61	21.15	19.13
EE	5.63	6.94	7.98
CF	2.67	2.59	2.50
Ashes	6.82	6.40	5.86
Ca	1.01	0.91	0.81
P	0.87	0.85	0.76
Lys Content (%) SID	1.28	1.16	1.04
Met+Cys Content (%) SID	0.87	0.73	0.66
Met Content (%) SID	0.58	0.44	0.40
Lys Content (%) total	1.40	1.27	1.13
Met+Cys Content (%) total	0.96	0.81	0.74
Met Content (%) total	0.60	0.47	0.42
ME (Kcal/kg)	3003	3100	3200

[1]composition per kg of premix: 2,000,000 IU vitamin A, 400,000 IU vitamin D3, 4,000 IU vitamin E, 400 mg vitamin B1, 1000 mg vitamin B2, 2000 mg calcium D-pantothenate, 600 mg vitamin B6, 2 mg vitamin B12, 5000 mg niacin, 100 mg folate, 10,000 mg iron carbonate, 200 mg calcium iodine, 100 mg cupric oxide, 12,000 mg manganese oxide, 18,850 mg zinc oxide, 30 mg sodium selenite.

Table 2. Effects of melon pulp concentrate supplementation on growth performance of broilers

Item	Days	C	MPC1	MPC2	MPC3	SEM	Treatment	Time	Treatment x Time
BW (g)						9.40	0.03	<0.01	<0.01
	0	39.38	39.00	39.24	39.13				
	10	184.47	186.57	193.10	193.11				
	24	754.21	750.91$^{(b)}$	774.80$^{A(a)}$	736.12^{B}				
	35	1484.25^{B}	1521.88^{A}	1538.25^{A}	1477.65^{B}				
	Average	615.58^{b}	624.59	636.35^{a}	611.5^{b}				
ADG (g)						0.71	0.04	<0.01	0.01
	0-10	14.51	14.76	15.39	15.40				
	10-24	40.70$^{(a)}$	40.31	41.55^{A}	38.79$^{B(b)}$				
	24-35	66.39^{B}	70.12^{A}	69.41^{Aa}	67.41^{Bb}				
	0-35	41.29^{b}	42.38$^{(a)}$	42.83^{a}	41.1$^{b(b)}$	0.48	0.04		
ADFI (g)						0.86	0.22	<0.01	0.26
	0-10	21.44	21.66	21.97	22.02				
	10-24	61.56	62.10	63.60	62.92				
	24-35	114.93	116.95	118.01	115.04				
	0-35	66.87	67.78	68.80	67.61	0.64	0.22		
G:F						0.01	0.20	<0.01	<0.01
	0-10	0.68^{b}	0.68$^{(b)}$	0.70$^{a(a)}$	0.70$^{a(a)}$				
	10-24	0.66^{A}	0.65^{A}	0.65^{A}	0.62^{B}				
	24-35	0.58^{b}	0.60^{a}	0.59	0.59				
	0-35	0.62	0.63$^{(a)}$	0.62$^{(a)}$	0.61$^{(b)}$	0.01	0.05		

BW= body weight; ADG= average daily gain; ADFI= average daily feed intake; G:F= gain:feed; C= control; MPC=melon pulp concentrate. Starter phase (0-10 d)-0 g/ton MPC (C), 30 g/ton MPC (MPC1), 15 g/ton MPC (MPC2), 15 g/ton MPC (MPC3). Grower phase (11-24 d) - C, MPC1 and MPC2 received the same basal diet, only MPC3 received basal diet supplemented with 15 g/ton of MPC. Finisher diet (25-35 d) – Four experimental groups received the same basal diet. $^{A,B}P\leq0.01$, $^{a,b}P\leq0.05$ and $^{()}0.05<P\leq0.1$.

Table 3. Effects of melon pulp concentrate supplementation on SLBW, carcass weight, dressing percentage, litter score and organs weight of broilers

Item	C	MPC1	MPC2	MPC3	SEM	Treatment
SLBW (g)	1505.08	1536.58	1563.67	1505.33	19.98	0.13
Carcass weight (g)	1066.75$^{(b)}$	1084.08	1113.67$^{(a)}$	1066.92$^{(b)}$	14.59	0.09
Dressing %	70.86	70.55	71.26	70.88	0.34	0.53
Litter Dry Matter %	39.09$^{(b)}$	41.60	43.09$^{(a)}$	39.50$^{(b)}$	1.22	0.08
Litter Score (0-5)	3.33	3.17	3.42	3.42	0.24	0.87
Organs %						
Liver %	2.24	2.27	2.42	2.40	0.08	0.27
Pancreas %	0.24	0.21	0.24	0.23	0.01	0.23
Spleen %	0.101	0.103	0.105	0.104	0.01	0.98
Bursa of Fabricius%	0.16$^{(b)}$	0.20$^{(a)}$	0.19	0.21$^{(a)}$	0.01	0.06
Gizzard %	1.46	1.47	1.38	1.49	0.05	0.48
Heart %	0.52	0.49	0.49	0.52	0.02	0.34
Small Intestine %	2.99	2.98	3.01	2.95	0.08	0.97

SLBW=Slaughter live body weight; C= control; MPC= melon pulp concentrate. Starter phase (0-10 d) – 0 g/ton MPC (C), 30 g/ton MPC (MPC1), 15 g/ton MPC (MPC2), 15 g/ton MPC (MPC3). Grower phase (11-24 d) - C, MPC1 and MPC2 received the same basal diet, only MPC3 received basal diet supplemented with 15 g/ton of MPC. Finisher diet (25-35 d) – Four experimental groups received the same basal diet.$^{A,B}P\leq0.01$, $^{a,b}P\leq0.05$ and $^{\emptyset}0.05<P\leq0.1$.

Table 4. Effects of melon pulp concentrate supplementation on Pododermatitis score of broilers

Item	Days	C	MPC1	MPC2	MPC3	SEM	Treatment	Time	Treatment x Time
Pododermatitis	**24**	0.41^{B}	0.36^{B}	0.30^{B}	0.72^{A}	0.06	<0.01	<0.01	<0.01
	35	1.08	0.95^{b}	1.03	1.13^{a}				
	Average	0.75^{b}	0.65^{B}	0.67^{B}	0.92^{Aa}				

C= control; MPC= melon pulp concentrate. Starter phase (0-10 d) - 0g/ton MPC (C), 30g/ton MPC (MPC1), 15g/ton MPC (MPC2), 15 g/ton MPC (MPC3). Grower phase (11-24 d) - C, MPC1 and MPC2 received the same basal diet, only MPC3 received basal diet supplemented with 15 g/ton of MPC. Finisher diet (25-35 d) – Four experimental groups received the same basal diet. $^{A,B}P \leq 0.01$, $^{a,b}P \leq 0.05$ and $^{0}0.05 < P \leq 0.1$.

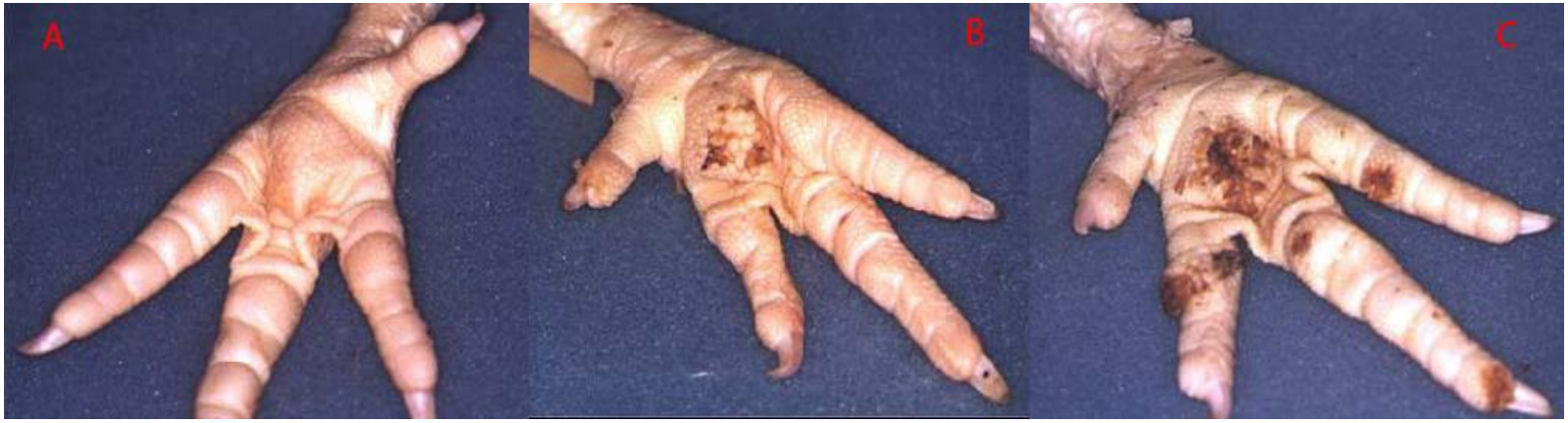

Figure 1. Examples of three tier scoring system used to assess pododermatitis in the experimental trial. 0=no lesions (A); 1=mild lesion (B); 2=Severe lesion (C) (Dakessian, 2008)

CHAPTER 5

Effects of melon pulp concentrate rich in superoxide dismutase on hepatic gene expression of antioxidant proteins in piglets and poultry

Effects of melon pulp concentrate rich in superoxide dismutase on hepatic gene expression of antioxidant proteins in piglets and poultry

Abstract

The aim of the trial was to study the effects of an antioxidant feed supplement (melon pulp concentrate, MPC) that contains high levels of superoxide dismutase (SOD) as a primary antioxidant on antioxidant gene expression in the liver of both piglets under LPS challenge and poultry. Liver tissue was collected from six piglets per treatment at the end of the trial 1 and 12 poultry per treatment at the end of grower phase (day 24) from trial 2 respectively. Collected samples were stored in liquid nitrogen for subsequent gene expression analysis by RT-PCR. β-actin and GAPDH of *S. scrofa* or *Gallus gallus* respectively were used as internal reference genes. *GPx1*, *CAT* and *SOD1* were determined on piglet liver samples, while *NFEL2L, CAT* and *SOD1* were determined on poultry hepatic tissue samples. Results from piglets' trial showed that MPC, LPS challenge and MPC x LPS challenge interaction did not significantly affect liver gene expression of *GPX1*, *CAT* and *SOD1* ($P>0.05$), with the exception of trend ($P=0.09$) for lower down-regulation of *CAT* for MPC effect. Results from poultry trial showed that MPC did not significantly affect liver gene expression of *NFEL2L* in treated groups, while *SOD1* expression showed a trend to reduce the down-regulation in MPC3 vs. C ($P=0.09$). *CAT* was significantly up-regulated in MPC3 than C and MPC1 ($P=0.02$). The obtained results show that MPC supplementation can directly affect *CAT* expression in the liver of poultry and challenged piglets and, to a lesser extent *SOD1* in poultry.

Introduction

Weaning stress has the potential to change and modify the immune system (Kick et al., 2012) and induce oxidative stress and free radicals production (Zhu et al., 2012), but recent studies by different authors found that the inclusion of SOD in the diet as an exogenous source contained in melon pulp concentrate, can be effective in improving the antioxidant status of animals (Carillon et al., 2013). Anyway, in most of these researches, the specific effects of SOD-rich melon were limited to mice or pigs (Notin et al., 2010, Vouldoukis et al., 2004a, Vouldoukis et al., 2004b), with very few information on poultry. Besides this limited knowledge on the different animal species, still the general antioxidant mechanism of action of exogenous SOD administered with the diet is debated, mainly due to the physical characteristics of SOD and its ability to pass the intestinal barrier (Zidenberg-Cherr et al., 1983, Giri and Misra, 1983, Vouldoukis et al., 2004b) or its degradation in the gastrointestinal tract before reaching the intestinal barrier or, at least, the elimination in the faeces (Greenwald, 1990). Of course SOD has a chelating effect (Brazier et al., 2014), but this can be considered anyway only partially related to the antioxidant persistent effect observed when orally administered, mainly due its short half-life in circulating blood (Carillon et al., 2013). On the basis of different *in vivo* and *in vitro* trials one further proposed mechanism of action is that exogenous SODs can someway induce increased global antioxidant defence, regulating the induction of antioxidant enzymes at transcriptional level (Gonzalez et al., 1995, Izumi et al., 2002, Vouldoukis et al., 2004a, Yang et al., 2004). The main protein that is supposed to be influenced by SOD nowadays is the nuclear-transcription-factor-E2-related factor (Nrf2) (Carillon et al., 2013) together with classical, well-known, genes involved in antioxidant function such as catalase (CAT), superoxide dismutase (SOD) itself, and glutathione peroxidase (GPx).

The complex system of antioxidant enzymes (e.g., SOD, GSH-Px, and CAT) serves to protect the organism against the harmful prooxidants (Minelli et al., 2009). Of course the antioxidant properties of SOD, removing superoxide anion, are well known (Abreu and Cabelli, 2010, Fridovich, 1995, Fukai and Ushio-Fukai, 2011). However, SOD can also have a pro-oxidant effect because the dissociation of the superoxide anion produces peroxide, which is toxic to cells particularly if other antioxidant defence system, such as catalase (CAT) or glutathione peroxidase (GPx), are insufficient (Fukai and Ushio-Fukai, 2011).

Superoxide dismutase converts superoxide radicals into H_2O_2, and, like CAT and GPx, it is not normally expressed at its maximum capacity but is highly inducible in response to environmental stress by the keap1-Nrf2-ARE pathway (Kelch-like ECH associating protein1-Nuclear-factor erythroid 2-related factor 2-Antioxidant Regulatory Element) (Kobayashi and Yamamoto, 2005).

Specifically, nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a transcription factor and is known as a master regulator of the antioxidant response (Li and Kong, 2009, Nguyen et al., 2009) that plays important roles in oxidative stress

and initiate large numbers of antioxidant genes after translocation into the nucleus (Yin et al., 2013).

Under the thermoneutral or non-stress conditions condition, Nrf2 is is sequestered in the cytosol being bounded to another protein called kelch-like ECH-associated protein 1 (Keap1) in the cytosol (Itoh et al., 1999). Anyway, the cell exposition to oxidative stress, excessive ROS production, oxidation, conjugation, or phosphorylation of key cysteine residues in the inhibitory proteins leads to the disruption of cysteine residues in Keap1 with a consequent accumulation of free Nrf2 in the cytosol (Yamamoto et al., 2008). The result is an increased translocation of Nrf2 into the nucleus (Hayden and Ghosh, 2004) that regulates the expression of multiple antioxidant target genes (Hayden and Ghosh, 2004) binding to the antioxidant response element (ARE) in the promoter region (Itoh et al., 1997).

Some recent works focused on the gene expression of such *CAT, SOD1, GPX1* or *NFE2L2* in the intestine of piglets and poultry in different stress conditions and/or following a dietary treatments with antioxidant plant extracts (Yin et al., 2014, Zhu et al., 2012, Emadi and Kermanshahi, 2007, Yarru et al., 2009, Rastogi et al., 2001) but, to the best knowledge, none at the moment focused on the variations in the expression of such genes in the liver as the main detoxing organ that represents one of the primary target tissue when a challenge is applied such as with lipopolysaccharide (LPS) (Giralt et al., 1993, Nowak et al., 1993, Portolés et al., 1993, Goode and Webster, 1993, Novelli, 1997, Luster, 1998, Ben-Shaul et al., 1999) although no significant increases in oxidative markers in blood can be detected (van de Crommenacker et al., 2010). For these reasons, the aim of the present study was to determine the effects of oral administration of SOD-rich melon pulp concentrate on antioxidant gene expression in the liver of both piglets under LPS challenge and poultry.

Materials and methods

The protocol for care, handling, and sampling of animals defined in the present study was reviewed and approved by the Università degli Studi di Milano Animal Care and Use Committee (Protocol No 82/14 and Protocol No 25/15 06.05.15). The trial was performed at the Animal Production Research and Teaching Centre of the Polo Universitario di Lodi, Università degli Studi di Milano.

Animals, housing and experimental design

Gene expression analyses were performed on representative piglets and poultry involved in trials 1 and 2 described earlier in the thesis. The experimental designs of both the trials and the description of the collected liver samples are reported below.

Trial 1. A total of Forty-eight 24-d old weaned piglets (Topigs 40 x Topdelta; 7.79±0.17 kg of initial BW) were selected from the same herd and were divided in four homogeneous experimental groups of twelve animals each in a 2 x 2 factorial design. The piglets were placed in individual pens (0.47 m^2 for each piglet) and allocated in the same environmentally-controlled post-weaning room on slatted floor.

The first factorial arrangement consisted on the administration of a basal diet without any antimicrobial growth promoter (C) or the same basal diet plus 30 g/ton fed of MPC (Melofeed®, Lallemand, Blagnac, France) (MPC).

The second factorial arrangement consisted on a LPS challenge with repeated increasing intramuscular injections of low levels of *E. coli* (serotype 055:B5, Sigma-Aldrich Canada Ltd, Oakville, ON, Canada; cat. no. L2880) (**+**) to mimic chronic inflammation, or the injection of an equivalent amount of PBS solution (**-**).

LPS challenge was performed starting on day 19 of the trial. Subsequent injections were performed on days 21, 23 and 25. On day 19 challenged piglets were inoculated with 60 μg/kg of BW with LPS. Lipopolysaccharide dosage was increased by 12% at each subsequent injection to reduce endotoxin tolerance (Rakhshandeh and de Lange, 2012).

The basal diets were calculated to be isonutritive, and to meet the nutrient requirements of weaned piglets recommended by NRC (2012).

At the end of the trial period, liver tissue was collected from six animals per treatment, selected upon average live body weight, to perform gene expression of antioxidant enzymes and transcription factors implicated in oxidative stress. All collected tissue samples were immediately placed in 0.9-mL cryovials under liquid nitrogen and subsequently stored at -80°C.

Trial 2. One-day-old Ross 308 female broilers were obtained from a commercial hatchery. Birds were vaccinated against Marek's disease, infectious bronchitis and New Castle disease at the hatchery. 1104 broiler chicks with similar average weights were raised from 1 to 35 d of age and randomly distributed into 48 floor pens with 23 birds per pen. Birds were divided into four homogenous experimental groups 276 birds (12 replicates) each in a randomized block design.

The feeding regimen consisted of starter (0-10 d), grower (11-24 d) and finisher diet (25-35 d). During starter phase, dietary treatments were corn-soybean meal based diets supplemented with 0 g/ton (control), 30 g/ton (MPC1), 15 g/ton (MPC2), 15 g/ton (MPC3) of melon pulp concentrate (MPC) (Melofeed®, Lallemand, Blagnac, France). In grower phase, control, MPC1 and MPC2 received same basal diet, but MPC3 received basal diet supplemented with 15 g/ton of MPC. During 24-35 days, the same basal diet was supplied to four experiment groups. The basal diet was formulated to meet the nutrient requirements according to Ross-308 rearing guidelines (Aviagen, 2007). All birds had free access to feed and water.

At the end of grower phase (day 24), liver tissue (12 per treatment) was collected from a bird per pen, selected upon average live body weight, to perform gene expression of antioxidant enzymes and transcription factors implicated in oxidative stress. All collected tissue samples were immediately placed in 0.9-mL cryovials under liquid nitrogen and subsequently stored at -80°C.

GPX1, NFEL2L, CAT and *SOD1* gene expression in the liver of piglets and broilers

Total RNA from piglets and chicken liver samples was extracted with TRIzol Reagent® (Invitrogen), purified with commercial kit (Macherey-Nagel, Milano, Italia), and quantitated with Nanodrop (Thermo Scientific, Wilmington, DE). Specific mRNA was amplified and quantitated by real time PCR, using iScriptTM One Step RT-PCR for Probes (Bio-Rad, Milano, Italia), following the manufacturer's instructions. The RT-qPCR analysis was performed with a CFX384 Real-Time System (Bio-Rad, CA, USA).

Piglets liver samples were analysed for *GPX1, CAT* and *SOD1* gene expression, while on poultry *NFEL2L*, *CAT* and *SOD1* were performed.
The relative expression levels of the target genes were assessed using a standard four-point (100, 200, 40 and 8) five-fold-diluted curve. The standard curve was generated with increasing amounts of cDNA. All PCR relative quantities were normalised to geometric means of internal control genes *ACTB* and *GAPDH* of *S. scrofa* and *G. gallus* respectively.

Experiments were performed in triplicate. Primers for real-time PCRs were designed by IDT software available on line optimized to work in a one-step protocol (10 min at 50°C for reverse transcription, 40 cycles of amplifications each consisting of a denaturation step at 95° C for 10 s and an annealing/extension step at 60° C for

30 s). The oligonucleotides used for real-time PCR were synthesized by Eurofin MWG Operon (Ebersberg, Germany).

Primers and probes for piglets were provided by Applied Biosystems and were (F, forward; R, reverse; P, probe)

ACTB F: 5'-ACTCGATCATGAAGTGCGAC-3';
R: 5'-GTGATCTCCTTCTGCATCCTG-3';
P: 5'-CGTGTTGGCGTAGAGGTCCTTCC-3',

for *SOD1, CAT*, *GPX1* and *GAPDH* the TaqMan® Gene Expression Assays Ss03375614_u1, Ss04323022_m1, Ss03383336_u1 and Ss03375435_u1 were respectively used.

Primers and probes for chicken were provided by Applied Biosystems and were

GAPDH, F: 5'- TCTCTGGCAAAGTCCAAGTG-3';
R: 5'-TCACAAGTTTCCCGTTCTCAG-3';
P: 5'- AGTGCCCTTGAAGTGTCCGTGT-3',
CAT, F: 5'-GATTCCTGAAAGAGTTGTGCATG-3';
R: 5'-GCAACAGTGGAGAACCGTATAG-3';
P: 5'-ACCAAGTACTGCAAGGCGAAAGTGT-3',

for *SOD1*, *NFEL2L,* and *ACTB* the TaqMan® Gene Expression Assays Gg03348481_m1, Gg03366567_m1 and Gg03815934_s1 were respectively used.

Statistical analysis

Normalised data were log (base 2) transformed before performing statistical analysis. Gene expression data were analysed by MIXED procedure of SAS (SAS Inst. Cary, NC) with a randomized complete block design. Animal was considered as the experimental unit in the model statement. Significance level was stated for $P \leq 0.05$. $0.05 < P \leq 0.1$ was considered as a tendency.

Results

Trial 1. Melon pulp concentrate in postweaning piglets diet.
The results on hepatic gene expression in LPS challenged postweaning piglets fed melon pulp concentrate are reported in table 1.

The dietary treatment did not significantly affect liver gene expression of both *GPX1* (-0.44 *vs.* -0.26 respectively for C and MPC; *P*=0.21), and *SOD1* (-0.31 *vs.* -0.12 respectively for C and MPC; *P*=0.19), while a trend for lower down-regulation of *CAT* was evidenced (-0.29 *vs.* -0.03, respectively for C and MPC groups; *P*=0.09). The challenge effect was not significant over the three considered genes (*GPX1*: -0.38 *vs.* -0.32, *P*=0.68; *CAT*: -0.01 *vs.* -0.25, *P*=0.20; *SOD1*: -0.32 *vs.* -0.11, *P*=0.24 respectively for no-challenged or challenged piglets). As a result, the MPC x challenge interaction did not evidence any significant variation in the expression level of *GPX, CAT,* and *SOD1* (*P*=0.42, *P*=0.14, and *P*=0.59 respectively).

Trial 2. Melon pulp concentrate in poultry diet.
The results on hepatic gene expression in poultry fed melon pulp concentrate are reported in table 2.

Dietary treatments did not significantly affect liver gene expression of *NFEL2L* (0.05 *vs.* -0.08 respectively for C and MPC1; *P*=0.25), while a tendency was observed in *SOD1* differential expression in MPC3 compared with C (-0.19 *vs.* -0.01 respectively for C and MPC3; *P*=0.09), and a significant up-regulation of *CAT* was evidenced in MPC3 (-0.19, -0.15 *vs.* 0.14, respectively for C, MPC1 and MPC3 groups; *P*=0.02). *CAT* mRNA expression tended to be higher also in MPC3 compared to MPC2 (*P*=0.09).

Discussions

In the present studies *SOD1* (poultry) and *CAT* (poultry and piglets) expression in the liver tissue of piglets and poultry were affected by the administration of melon pulp concentrate. On the other side the LPS challenge in piglets trial did not negatively change the oxidant status, contrary to what was hypothesized.

Very few information are available at the moment on the effects and the mechanisms of action as an antioxidant of SOD-rich melon pulp concentrate, especially if we account for piglets and poultry species. Several animal and human studies have suggested the potential therapeutic role of SOD supplementation in inflammatory (Salvemini et al., 2001, Segui et al., 2004, Watterlot et al., 2010), and infectious (Webb et al., 2008) diseases, but the efficacy of melon pulp concentrate has been extensively evaluated just by Lallès et al., (2011) with respect to stress proteins levels in the gastrointestinal tract and oxidative biomarkers in blood of postweaning piglets.

Different authors found positive results supplementing natural antioxidant additives, such as plant extracts, on oxidative status biomarkers in the liver. Phytochemicals showed to exert positive health benefits by directly affect specific molecular targets, such as genes (Kelloff et al., 2000, Aggarwal and Shishodia, 2006). At this very moment anyway the aim of most of the studies is still to understand and formulate mechanistic pathways of these naturally-derived substances (Sahin et al., 2013), where the antioxidant effect is the most investigated factor (Sahin et al., 2008, Sahin et al., 2011, Tuzcu et al., 2008). In recent years, as an example, some active principles as epigallocatechin-3-gallate (EGCG; green tea), lycopene and resveratrol have become the subject of more intensive investigations in poultry with positive results on oxidation (Sahin et al., 2008, Sahin et al., 2010, Sahin et al., 2012). Rastogi et al., (2001) found that the supplementation of root extracts of *Picrorhiza kurroa* and seeds of *Silybum marianum* prevented the effects of AFB1 by improving the performance of chicks, reducing the formation of peroxides, and returning antioxidant enzymes to control levels. Similar results were obtained by Renzulli et al., (2004) using rosmarinic acid from the *Boraginaceae* species of plants that lead to reduced free radical production in human hepatoma cells induced by AFB1. Further, earlier reports suggested that curcumin inhibits superoxide anion generation (Iqbal et al., 2003). Recently, Emadi and Kermanshahi (2007) found that the inclusion of turmeric in the diet of broiler (0.25, 0.5, 0.75%) from hatch to 49 d might have some positive effects on liver enzymes that directly or indirectly reflect a healthier liver. Thus, at the present moment, consistent evidence outline as plant-derived antioxidant extracts/concentrates can have beneficial effects on oxidative status also at hepatic level, due to their active principles or antioxidant molecules contained.

In the present trial, evaluating the expression of different genes involved in the antioxidant metabolism in the liver, minor changes in *SOD1* gene expression were observed in poultry, but a higher *CAT* expression was found, while we did not observe any effect on *GPX1* in piglets and *NFE2L2* in poultry.

Conversely in piglets we did not find any variation in *SOD1* expression, but a trend for a lower down-regulation of *CAT* was observed. In this view it can be speculated that exogenous SOD administration with the diet is not able to significantly improve endogenous production of SOD in the liver (as previously found in the blood in trial 1), but can increase the expression of other antioxidant genes such as *CAT*. The mechanism of action for this pathway still remains unclear but it can be supposed that exogenous SOD can lead to higher production of hydrogen peroxide produced from oxygen during essential physiological processes, such as cell energy metabolism, activating the conversion of H_2O_2 to water and O_2 by CAT. Unfortunately in the present trials we did not quantify the production of such intermediates of oxidative metabolism to confirm this hypothesis.

In piglets' trial, LPS challenge was performed to provide an oxidative stress on the animals in addition to weaning. It is in fact recognized that weaning represent a stressful period and a critical factor accounting for the potential changes in the immune system (Kick et al., 2012), intestinal barrier function and absorption (Wijtten et al., 2011) and both endocrine system and oxidative stress (Zhu et al., 2012).

The negative effects of LPS on oxidative stress biomarkers are generally recognized and often reported to be organ-specific. One of the main target organs is of course the liver together with the brain (Giralt et al., 1993, Nowak et al., 1993, Portolés et al., 1993, Goode and Webster, 1993, Novelli, 1997, Luster, 1998, Ben-Shaul et al., 1999). It must be, however, outlined as increased oxidative stress in the target tissue due to LPS is not strictly correlated with significant variations in blood oxidative markers (van de Crommenacker et al., 2010).

In the same way during the trial on piglets we did not observe any significant change in the oxidative biomarkers in blood, but the lack of a significant effect of LPS challenge still remains unclear. At the present moment two hypothesis can be done: a) the mimic of a chronic inflammation by the use of relatively low dosage of LPS is not able to negatively affect the oxidative status of animals, but can provide a good inflammation response; b) the tissue samples collection was performed after two days from the last LPS injection in piglets, and this could have negatively affected someway the pro-oxidant effect of LPS.

The first hypothesis seems the most interesting since we had the evidence that the LPS challenge was effective on inflammation markers such as interleukins in blood, and some positive results were evidenced on TAOC and blood RBC resistance to haemolysis. It is recognized that oxidative status is linked with inflammation and vice versa, thus the increasing level of oxidation lead to higher level of inflammation, but higher liver of inflammation also lead to an improvement of oxidative status. In fact LPS, as a component of the outer membrane of Gram-negative bacteria, it first acts by the stimulation of Kupffer cells, followed by the subsequent release of inflammatory mediators (such as IL-1, IL-6 and TNF-α (He et al., 2001) and radical oxygen intermediates (Su, 2002): these mediators further stimulate the immune system to protect the body.

Actually, most of the LPS challenges adopted are based on an acute exposition rather than a chronic inflammation: in this way the authors found higher oxidative stress in animals. In our trial the exposure to low and repeated LPS dosages could have outlined a different mechanism of action where primary the increase of inflammation processes and biomarkers in blood was the main response that did not interfere with the oxidative status of piglets neither at blood or liver level.

It must be anyway took in account that soon after challenge (day 29), most of the inflammation biomarkers in blood of challenged piglets decreased to comparable levels as in no-challenge animals with the exception of TNF α. This leads to the second hypothesis, but unfortunately no liver samples were performed during the challenge that could confirm its efficacy on oxidative status. Of course the applied chronic LPS challenge with two-days interval injection of increasing dosage was designed accounting for both contrasting an effect of animal addiction to the endotoxin tolerance(Van Heugten et al., 1996, Fish and Spitzer, 1983, Ash and Griffin, 1989, Deitch, 1998) and the relatively short duration of the negative effect itself due to the low dosage applied: it is reported that with a low dosage of LPS animal recovery often occurs within 48 h (Deitch, 1998), thus it can be someway speculated that collecting tissue samples after few days from the last LPS injection most of the adverse effect could not be visible anymore.

Conclusions

The present trial evidenced that dietary SOD-rich melon pulp concentrate is able to directly affect *CAT* expression in the liver of poultry and challenged piglets and, to a lesser extent *SOD1* in poultry.

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Tables

Table 1. Relative expression (Log-transformed mRNA abundance) of *GPX1, CAT* and *SOD1* in liver of LPS challenged (+) and non-challenged (-) post-weaning piglets supplemented with melon pulp concentrate (MPC).

Item	Challenge							
	-		+					
	Group				SEM	*P*-value		
	C	MPC	C	MPC		MPC	Challenge	MPC x Challenge
GPX1	-0.46	-0.18	-0.41	-0.35	0.13	0.21	0.68	0.42
CAT	-0.55	0.05	-0.03	0.01	0.19	0.09	0.20	0.14
SOD1	-0.25	0.02	-0.37	-0.27	0.16	0.19	0.24	0.59

LPS= lipopolysaccharide; C-= control without LPS challenged; C+= control with LPS challenged; MPC-=melon pulp concentrate without LPS challenged; MPC+= melon pulp concentrate with LPS challenged; GPX1= glutathione peroxidase 1; SOD1=superoxide dismutase 1; CAT=catalase. Dose of MPC=30g/ton fed. $^{A,B}P \leq 0.01$, $^{a,b}P \leq 0.05$

Table 2. Relative expression (Log-transformed mRNA abundance) of *NFE2L2, CAT* and *SOD1* in liver of female broilers supplemented with melon pulp concentrate (MPC).

Item	**Group**				**SEM**	***P*-value**					
	C	**MPC1**	**MPC2**	**MPC3**		**C *vs.* MPC1**	**C *vs.* MPC2**	**C *vs.* MPC3**	**MPC1 *vs.* MPC2**	**MPC1 *vs.* MPC3**	**MPC2 *vs.* MPC3**
NFE2L2	0.05	-0.08	0.07	0.05	0.11	0.25	0.92	0.99	0.38	0.32	0.92
CAT	-0.19	-0.15	-0.15	0.14	0.10	0.70	0.77	0.02	0.99	0.02	0.09
SOD1	-0.19	-0.10	-0.14	-0.01	0.08	0.32	0.71	0.09	0.76	0.41	0.34

SOD1=superoxide dismutase 1; CAT=catalase; NFE2L2=nuclear-transcription-factor-E2-related factor. C= control; MPC1 = melon pulp concentrate at 30 g/ton during starter phase (0-10d); MPC2 = melon pulp concentrate at 15 g/ton during starter phase (0-10d); MPC3 = melon pulp concentrate at 15 g/ton during starter and grower phases (0-24d). $^{A,B}P \leq 0.01$, $^{a,b}P \leq 0.05$

CHAPTER 6

General discussions

The aim of the present thesis was to assess the effects of the dietary supplementation of SOD-rich melon pulp concentrate (MPC) on oxidative status, inflammation and growth performance of post-weaning piglets and poultry. Results from the three run trials showed positive effects of MPC on growth performance and some parameters of the oxidative status at blood and hepatic gene expression level, with less evidence on inflammation biomarkers.

Recently antioxidant and anti-inflammatory properties of some compounds containing high levels of superoxide dismutase (SOD) became more interesting because of their therapeutic use during stress condition in animals (Yasui and Baba, 2006). When oxidative stress occurs, SOD is able to convert superoxide ions to oxygen and hydrogen peroxide with a protective effect on the cells. A second function of SOD is to regulate neutrophil apoptosis that can positively decrease inflammatory disorders (Yasui and Baba, 2006), especially if SOD is preserved from digestion by protection (Jubeh et al., 2006, Segui et al., 2004). Due to these mechanisms of action, the use of SOD is expected to improve the health status and the immunity of animals, contributing to higher overall growth performance.

As regards the molecular properties of SOD, in mammals three isoforms are present: i) a homodimer copper/zinc (Cu/Zn-SOD) of 32 kDa that is localized in the cytosol or in the mitochondrial inter-membrane space, ii) a homotetramer manganese-SOD (Mn-SOD) of 88 kDa, that is localized in the matrix and inner membrane of mitochondria, and iii) an extracellular tetrameric glycoprotein Cu/Zn form (EC-SOD) of 135 kDa (Fridovich, 1995, Faraci and Didion, 2004). While a fourth isoform of SOD has been found in bacteria coupled with iron SOD (Fe-SOD) (Beyer et al., 1990), plants seems to be somewhat in between mammalians and bacteria: still 3 isoforms are present, but these are a mitochondrial Mn-SOD, a Cu/Zn- SOD in the cytosol and the chloroplasts and a Fe-SOD in the chloroplasts (Hassan and Scandalios, 1990, Scandalios, 1997). Different studies in humans administered SOD by subcutaneous (Segui et al., 2004), intravenous (Jubeh et al., 2006), intraperitoneal (Jadot and Michelson, 1987), intramuscular (Lefaix et al., 1996) and local (Bartsch et al., 1980) injections or directly to the damaged tissue (Campana et al., 2004), but it is difficult to accept that such a high molecular weight proteins can enter directly to the cell (Carillon et al., 2013b).

On the other side, the efficacy of SOD through its oral administration seems to be affected by the high degradation of the molecule in the gastrointestinal tract at a comparable level as the other dietary proteins supplied, if not protected with some biopolymers such as wheat gliadin (Zidenberg-Cherr et al., 1983, Giri and Misra, 1984, Vouldoukis et al., 2004b). The presence of orally administered SOD in the faeces was reported by Giri and Misra (1984) who used a labelled form of SOD with Zn+ tracing both the fate of the metal ion and the changes in SOD enzyme activity in various tissues after oral administration in human. In this study almost ninety percent of the label came out in the faeces, and the remainder was almost certainly free metal separated from the protein, while no increased blood or hepatic SOD activity were evidenced. Finally, it is believed that therapeutic levels of SOD cannot be maintained in plasma or non-renal tissues due to the short half-life (few minutes)

of the protein (Huber et al., 1980) before being eliminated by the kidneys as the preferred organ for SOD accumulation (Swart et al., 1999). Anyway, the short half-life of SOD in circulating blood and the lack of absorption by the gastrointestinal tract, together with its long-term effects when orally administered, suggest additional mechanism/s of action beside the chelating properties. On the basis of different *in vivo* and *in vitro* trials (Vouldoukis et al., 2004a, Izumi et al., 2002, Gonzalez et al., 1995, Yang et al., 2004), one further proposed mechanism of action is that exogenous SODs can someway induce increased global antioxidant defence. In this view, exogenous SODs could regulate the induction of antioxidant enzymes at transcriptional level through the antioxidant response element (ARE) and the nuclear-transcription-factor-E2-related factor (Nrf2) (Carillon et al., 2013b) activating the immune system locally and leading to the activation of macrophages in the entire body (Vouldoukis et al., 2004b, Lallès et al., 2011).

Different authors found positive results supplementing natural antioxidant additives, such as plant extracts, on oxidative status biomarkers in the liver. Phytochemicals showed to exert positive health benefits by directly affect specific molecular targets, such as genes (Kelloff et al., 2000, Aggarwal and Shishodia, 2006). At this very moment, anyway, the aim of most of the studies is still to understand and formulate mechanistic pathways of these naturally-derived substances (Sahin et al., 2013), where the antioxidant effect is the most investigated factor (Sahin et al., 2008, Sahin et al., 2011, Tuzcu et al., 2008). In recent years, as an example, some active principles as epigallocatechin-3-gallate (EGCG; green tea), lycopene and resveratrol have become the subject of more intensive investigations in poultry with positive results on oxidation (Sahin et al., 2008, Sahin et al., 2010, Sahin et al., 2012). Rastogi et al., (2001) found that the supplementation of root extracts of *Picrorhiza kurroa* and seeds of *Silybum marianum* prevented the effects of AFB1 by improving the performance of chicks, reducing the formation of peroxides, and returning antioxidant enzymes to control levels. Similar results were obtained by Renzulli et al., (2004) using rosmarinic acid from the *Boraginaceae* species of plants that lead to reduced free radical production in human hepatoma cells induced by AFB1. Further, earlier reports suggested that curcumin inhibits superoxide anion generation (Iqbal et al., 2003). Recently, Emadi and Kermanshahi (2007) found that the inclusion of turmeric in the diet of broiler (0.25, 0.5, 0.75%) from hatch to 49 d might have some positive effects on liver enzymes that directly or indirectly reflect a healthier liver. Thus, at the present moment, consistent evidence outlines that plant-derived antioxidant extracts/concentrates can have beneficial effects on oxidative status also at hepatic level, due to their active principles or antioxidant molecules contained.

At the present, few studies are available on poultry and piglets, especially if related to the use of melon concentrate as a high SOD content compound (Barbé et al., 2015), also considering gene expression in target organs. However, referring to monogastric animals, Lallès et al., (2011) found a positive effect of the administration of a melon concentrate rich in SOD in post-weaning piglets over stress proteins along the gastrointestinal tract that was not, however, traduced in increased growth performance. During the study these authors also found that plasma SOD was

increased only with the higher amount supplied and at the end of the trial (14 day post weaning), but concluding that the administration of exogenous sources of SOD is in agreement with the general concept of a positive link between oxidative stress and tissue stress protein expression and of their reduction by natural antioxidant substances. In laying hens, melon juice concentrate increased shelf life of eggs, yolk percentage and vitamin E transfer in yolk. It also stimulated SOD, CAT and GPx production in the reproductive tract.

Oxidative stress and inflammation are reported to be closely linked each other (Reuter et al., 2010), being inflammation an effect of increased oxidative stress, and oxidative stress a factor inducing the mediators of inflammation (Carillon et al., 2013b). With respect to this, lower inflammatory response should be expected when high antioxidant effects of SOD are found, partially due to the chelating properties of the protein on metal ions. It is in fact recognized that transition metal ions participate in a variety of antioxidant defence systems. For example, Cu^{2+} and Mn^{2+} are essential cofactors in the enzymatic activity of the SOD enzymes. SOD1 also contains Zn^{2+} in a structural (non-catalytic) role and employs the catalytic Cu^{2+}/Cu^{+} couple, i.e., copper cycles between its +2 and +1 oxidation states as it first reduces superoxide (O_2) to hydrogen peroxide (H_2O_2) and then oxidises it to dioxygen. It "dismutates" superoxide radicals in that the first radical is reduced while the second is oxidised and subsequently the product H_2O_2 is converted by catalase enzymes to water and O_2. Oxidative stress often includes diminished (Cu/Zn SOD or Mn-SOD2) activity or SOD-like activity (Thackray et al., 2002) and elevated radical-mediated lipid peroxidation: in such way increased reactive oxygen and nitrogen species are generated during pathogenesis (Brazier et al., 2014, Cristiana et al., 2014).

Contrary to Lallès et al., (2001), in trial 1 (piglets) MPC supplementation in the diet lead increased ADG and FI during the challenge period, while poultry trial (trial 2) evidenced better performance when feeding MPC during the first ten days. This discrepancy from Lallès et al., (2011) can be attributed to the too short duration of the trial to translate into any positive effect in the gastrointestinal tract or on immune response to improved growth performance. In fact, Lallès et al., (2011) administered melon pulp concentrate for 5 and 12 days to post-weaning piglets after an initial 2-days period of fasting to induce higher stress levels; animals were then slaughtered on day 7 and 14 after weaning to detect any variation in stress proteins level along the gastrointestinal tract.

Carillon et al., (2013a) observed that highest dose of melon pulp concentrate reduced significantly BW gain with no differences in feed and water consumption in 83 weeks-old rat fed diets containing 3 levels of melon pulp concentrate (10, 40, and 160 U SOD/day) for 28 days proposing a diuretic activity as main reason. On the contrary, results from different MPC levels of inclusion in the diet of poultry showed improved BW, ADG, and G:F when MPC was fed in the first ten days of life. These findings support the idea that MPC supplementation could improve performances in specific productive phases (early life stage) and the prolonged use could be detrimental. Moreover, it has to be taken in account that also genotype of poultry could be crucial to decide proficient levels of use of this supplement. Indeed, it has

been observed that the enzymatic antioxidative system in the erythrocytes of Cobb strain chickens is not very susceptible to stress (heat) compared with the one of Ross (Altan et al., 2003).

Accounting for inflammation, the pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, are important inducers of the synthesis of acute phase proteins, such as Hp, by hepatocytes (Carroll et al., 2004). Moreover, the exposure of animals to pathogenic or non-pathogenic antigens results in activated immune system and subsequently cytokine release. Metabolic shifts are characterized by the redistribution of nutrients away from the growth processes toward immune system function (Beisel, 1977), and subsequently result in decreased feed efficiency for growth (Daiwen et al., 2008), as found in trial 1. Specifically, in agreement with our results, a number of works (Carroll et al., 2001, Frank et al., 2003, Frank et al., 2005) showed that immune challenge with LPS acts on the immune system through increased levels of serum TNF-α, IL-1β and IL-6.

In trial 1 we decided to adopt a chronic challenge procedure as reported by Rakhshandeh and de Lange (2012), to mimic subclinical or mild clinical disease conditions that frequently occur in the field. Lipopolysaccharide challenge significantly increased pro-inflammatory interleukins levels and Hp concentration in blood with impaired piglet performance in both in C and MPC groups from 19 to 29 days of the trial, but we did not observe significant interactions among the dietary treatment and the challenge applied over almost all considered parameters, including pro-inflammatory cytokines. Differences between the experimental groups were only evidenced for the main effect of melon pulp concentrate administration. It must be, nevertheless outlined, that the LPS effects on oxidative stress biomarkers are often reported to be organ-specific, where the preferential target organ seems to be the liver rather than the heart (Giralt et al., 1993, Nowak et al., 1993, Portolés et al., 1993, Goode and Webster, 1993, Novelli, 1997, Luster, 1998, Ben-Shaul et al., 1999) sometimes outlining no significant increases in oxidative markers in blood (van de Crommenacker et al., 2010). Besides, we did not find a significant diet effect on pro-inflammatory cytokines levels and serum Hp concentration, in the trial 1 we found increased TAOC levels and blood resistance to haemolysis for the main effect of melon pulp concentrate oral administration according with previous researches by Vouldoukis et al. (2004a) and Notin et al. (2010) who outlined improved resistance to the induced haemolysis of red blood cells respectively in mice and horse fed melon extract. These results confirm an improvement in the antioxidant defence system in piglets' trial that however did not turn into any significant variation in blood levels of SOD, ROS, or 8-oxo-dGuo.

In trial 2, we evaluated the weight of the Bursa of Fabricius as an important immunological organ in poultry, in fact, is a central lymphoid organ (Cooper et al., 1966), and is involved in cellular and humoral immunity. This organ is, indeed, commonly weighted when studying immune function in birds (Pope, 1991). Furthermore, it has been observed that Bursa weight is a very good marker for stress related to housing density, being the two parameters inversely proportional (Heckert

et al., 2002); the stock density in our trial was at the upper limit of EU legislation and was close to the normal one used by Zhang et al. (2013).

The inclusion of MPC SOD in the diet tended to increase the Bursa of Fabricius of poultry (MPC1 and MPC3) compared with C. Similar results were observed in birds fed Forsythia suspense extract (FSE) and berberine (BE), herbal extract with powerful antioxidant activity, in fact FSE + BE treated animals had greater bursa weight than the birds in control group, alleviating the stress induced by high stock density (Zhang et al., 2013).

Pododermatitis is associated with a range of welfare issues (Martland, 1984, Martland, 1985, Kestin et al., 1999) and high prevalence may be indicators for poor welfare conditions (Haslam et al., 2007). Studies have shown that a genetic background for pododermatitis in broilers exists (Kjaer et al., 2006, Ask, 2010); therefore, genetic selection against the disposition to develop pododermatitis may contribute to the reduction of the prevalence. Pododermatitis is also linked to poor litter quality (Berg, 2004), our results confirm this association in particular in the MPC3 group. The negative correlation observed between litter dry matter and pododermatitis score is clearly related to this point. Referring to the performance data obtained in rats (Carillon et al., 2013a) the diuretic activity suggested to explain those results, could be in agreement with lower litter dry matter and consequently higher pododermatitis score observed in our trial on broilers. However, no data on litter dry matter are available at day 24 to fully support this hypothesis, since pododermatitis in MPC3 was already higher at that time of the experiment.

Evaluating the expression of different genes involved in the antioxidant metabolism in the liver, minor changes in *SOD1* gene expression were observed in poultry, but a higher *CAT* expression was found, while we did not observe any effect on *GPX1* in piglets and *NFE2L2* in broiler. Conversely in piglets we did not find any variation in *SOD1* expression, but a trend for a lower down-regulation of *CAT* was observed. In this view it can be speculated that exogenous SOD administration with the diet is not able to significantly improve endogenous production of SOD in the liver (as previously found in the blood in trial 1), but can increase the expression of other antioxidant genes such as *CAT*. The mechanism of action for this pathway still remains unclear but it can be supposed that exogenous SOD can lead to higher production of hydrogen peroxide produced from oxygen during essential physiological processes, such as cell energy metabolism, activating the conversion of H_2O_2 to water and O_2 by CAT. Unfortunately, in the present trials we did not quantify the production of such intermediates of oxidative metabolism to confirm this hypothesis.

In piglets' trial, LPS challenge was performed to provide an oxidative stress on the animals in addition to weaning. It is in fact recognized that weaning represents a stressful period and a critical factor accounting for the potential changes in the immune system (Kick et al., 2012), intestinal barrier function and absorption (Wijtten et al., 2011) and both endocrine system and oxidative stress (Zhu et al., 2012).

The negative effects of LPS on oxidative stress biomarkers are generally recognized and often reported to be organ-specific. One of the main target organs is of course the liver together with the brain (Giralt et al., 1993, Nowak et al., 1993, Portolés et al., 1993, Goode and Webster, 1993, Novelli, 1997, Luster, 1998, Ben-Shaul et al., 1999). It must be anyway outlined that increased oxidative stress in the target tissue due to LPS is not strictly correlated with significant variations in blood oxidative markers (van de Crommenacker et al., 2010). In the same way during the trial on piglets we did not observe any significant change in the oxidative biomarkers in blood, but the lack of a significant effect of LPS challenge still remains unclear. At the present moment two hypothesis can be proposed: a) the mimic of a chronic inflammation by the use of relatively low dosage of LPS is not able to negatively affect the oxidative status of animals, but can provide a good inflammation response; b) the tissue samples collection was performed after two days from the last LPS injection in piglets, and this could have negatively affected somehow the pro-oxidant effect of LPS.

The first hypothesis seems the most interesting since we had the evidence that the LPS challenge was effective on inflammation markers such as interleukins in blood, and some positive results were evidenced on TAOC and blood RBC resistance to haemolysis. It is recognized that oxidative status is linked with inflammation and vice versa, thus the increasing level of oxidation lead to higher level of inflammation, but higher level of inflammation also lead to an improvement of oxidative status. In fact LPS, as a component of the outer membrane of Gram-negative bacteria, it first acts by the stimulation of Kupffer cells, followed by the subsequent release of inflammatory mediators (such as IL-1, IL-6 and TNF-α (He et al., 2001) and radical oxygen intermediates (Su, 2002): these mediators further stimulate the immune system to protect the body.

Actually, most of the LPS challenges adopted are based on an acute exposition rather than a chronic inflammation: in this way the authors found higher oxidative stress in animals. In our trial the exposure to low and repeated LPS dosages could have outlined a different mechanism of action where primarily the increase of inflammation processes and biomarkers in blood was the main response that did not interfere with the oxidative status of piglets neither at blood or liver level.

It must be however take in account that soon after challenge (day 29), most of the inflammation biomarkers in blood of challenged piglets decreased to comparable levels as in no-challenge animals with the exception of TNF-α. This leads to the second hypothesis, but unfortunately no liver samples were performed during the challenge that could confirm its efficacy on oxidative status. Of course the applied chronic LPS challenge with two-days interval injection of increasing dosage was designed accounting for both contrasting an effect of animal addiction to the endotoxin tolerance (Van Heugten et al., 1996, Fish and Spitzer, 1983, Ash and Griffin, 1989, Deitch, 1998) and the relatively short duration of the negative effect itself due to the low dosage applied. It is reported that with a low dosage of LPS animal recovery often occurs within 48 h (Deitch, 1998), thus it can be someway

speculated that collecting tissue samples after few days from the last LPS injection most of the adverse effect could not be visible anymore.

The results obtained in the present trials show that MPC supplementation in the diet of both piglets and broilers can positively affects growth performance, independently from the challenge and oxidative status. Besides LPS chronic challenge showed to primarily affect immune response in post-weaning piglets with no significant effects on the oxidative status of the animal, MPC could affect this parameter by increasing the global antioxidant defence through higher levels of TAOC and RBC blood resistance to haemolysis, rather than increased plasma SOD levels. This hypothesis is supported also by a slight increase of *CAT* gene expression in the liver of both piglets and chickens, not accompanied by an increase in *SOD1* expression.

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CHAPTER 7

Summary

The aim of the present thesis was to assess the effect of the dietary supplementation of SOD-rich melon pulp concentrate (MPC) on oxidative status, inflammation and growth performance of post-weaning piglets and poultry. With this purpose three different trials were conducted: 1) MPC administration to no challenge or LPS-challenged post-weaning piglets to evaluate changes in the oxidative status and inflammation markers in blood together with growth performance; 2) MPC administration to broilers to evaluate growth performance and the incidence of both pododermatitis and cellulitis; 3) expression of oxidative status genes in the liver in both piglets and chickens.

During trial 1, forty-eight female piglets weaned at 24 days of age were divided in four homogeneous experimental groups of twelve animals each in a randomized block design and fed a basal diet (C, n. 24) or a basal diet plus 30 g/ton melon pulp concentrate (Melofeed®, Lallemand, Blagnac, France) (MPC, n. 24). The experimental trial lasted 29 days from weaning, performing a LPS challenge by injecting increased dosages four times every two days starting from 19th day of the experiment (19, 21, 23, 25 days) in half of C (n.12) and half of MPC (n.12) groups. Initial LPS dosage of 60 μg/kg of body weight was increased by 12% at each subsequent injection to reduce endotoxin tolerance. Growth performance including live body weight, feed intake, average daily gain and G:F were monitored from the beginning of the trial until the end of the experiment. Blood samples were collected to evaluate the antioxidant status of experimental animals by SOD activity (Superoxide dismutase), TAOC (Total Anti-Oxidant Capacity), ROS (Reactive Oxygen Species), Kit Radicaux Libres (KRL) test on blood, plasma and red blood cell (RBC), and 8-oxodGuo (8-oxo-7, 8-dihydro-2'-deoxyguanosine). Moreover blood samples were analysed for immune and inflammatory response by haptoglobin (Hp) and Cytokines IL-6, IL-1β, TNFα in serum.

A positive effect of SOD-rich MPC was evidenced on FI and ADG ($P<0.01$; $P=0.05$ respectively), while LPS challenge significantly reduced both parameters in injected animals ($P<0.01$). Antioxidant status of MPC fed piglets was improved by higher TAOC levels (MPC=7.22 mM Trolox equivalent vs. C=4.54 mM Trolox equivalent; $P<0.01$) and RBC resistance to haemolysis (MPC=70.71 HT50, min vs. C=66.41 HT50, min; $P\leq0.01$). No significant differences were evidenced for ROS, SOD activity and 8-oxodGuo in the four experimental groups. The challenge with LPS increased TNF-α, IL-1β, IL-6 ($P<0.01$) and Hp ($P=0.03$) levels in blood, but no differences were found for MPC administration. These results suggest that oral SOD supplementation by MPC increase some aspects of antioxidant status of post-weaning piglets with positive results on growing performance.

In trial 2 a total of 1104 broilers were allocated to 4 experimental groups for 35 days with four different dietary treatments: a) basal diet (C: control), b) basal diet plus melon pulp concentrate (MPC1, MPC2 and MPC3) as described below. The feeding regimen consisted of starter (0-10 d), grower (11-24 d) and finisher diet (25-35 d). During starter phase, dietary treatments were corn-soybean meal based diets supplemented with 0 g/ton (C), 30 g/ton (MPC1), 15 g/ton (MPC2), 15 g/ton (MPC3) of melon pulp concentrate (MPC) (Melofeed®, Lallemand, Blagnac,

France). In grower phase, C, MPC1 and MPC2 received same basal diet but MPC3 received basal diet supplemented with 15 g/ton of MPC. During 24-35 days, the same basal diet without MPC was supplied to four experimental groups. 12 pens per group with 23 broilers per pen were used. On day 24 and 25, pododermatitis was evaluated on all animals using a scoring system that ranged from 0 to 2. On day 35, cellulitis was evaluated on all slaughtered birds. Litter score and litter DM was assessed on day 35. Body weight (BW) of birds per pen was recorded on 0, 10, 24, 35 experimental days from the beginning of the experiment. Individual body weight, slaughter live body weight, carcass weight, dressing percentage and organs weight (liver, heart, intestine, Bursa of Fabricius, spleen and pancreas and gizzard) percentage were also assessed at day 35. Pen feed intake was recorded on 0, 10, 24, 35 experimental days. Pen feed residues were determined at the end of each feeding period to estimate mean ADG, ADFI and gain: feed (G:F) ratio for each pen.

Treatment (MPC) was able to affect body weight ($P<0.05$), ADG ($P<0.05$) and G:F ($P=0.05$) during experimental period. Final BW was higher in MPC1 and MPC2 than C and MPC3 ($P<0.01$). Carcass weight tended to be higher in MPC2 than C and MPC3 ($0.05<P\leq0.1$). Percentage of the Bursa of Fabricius weight tended to be higher ($0.05<P\leq0.1$) in MPC1 and MPC3. Incidence and severity of pododermatitis varied within each feeding period. Significant treatment, time and their interaction ($P<0.01$) were found in pododermatitis lesions analysis. At 24 d of age, MPC3 scores were the highest ($P<0.01$) between diets. At 35 d of age, scores in MPC3 were still higher than MPC1 ($P<0.05$). Pearson correlation between pododermatitis and body weight was not significant, by the way it was highly significant ($P<0.01$) between pododermatitis and litter dry matter (-30%). No cellulitis was detected in experimental animals. These results suggest that oral SOD supplementation by MPC increases performance of poultry with a dose-dependent increase in pododermatitis incidence, but not significant effect on cellulitis occurrence.

In the third trial liver tissue was collected from six piglets per treatment at the end of the trial 1 and 12 poultry per treatment at the end of grower phase (day 24) from trial 2 respectively. Collected samples were stored in liquid nitrogen for subsequent gene expression analysis by RT-PCR. β-actin and GAPDH of *S. scrofa* or *G. gallus* respectively were used as internal reference genes. *GPx1*, *CAT* and *SOD1* were determined on piglet liver samples, while *NFEL2L, CAT* and *SOD1* were determined on poultry hepatic tissue samples. Results from piglets' trial showed that MPC, LPS challenge and MPC x LPS challenge interaction did not significantly affect liver gene expression of *GPX1*, *CAT* and *SOD1* ($P>0.05$), with the exception of trend ($P=0.09$) for a down-regulation of *CAT* for MPC effect. Results from poultry trial showed that MPC did not significantly affect liver gene expression of *NFEL2L* in treated groups, while *SOD1* expression showed a trend to reduce the down-regulation in MPC3 vs. C ($P=0.09$). *CAT* was significantly up-regulated in MPC3 than C and MPC1 ($P=0.02$). The obtained results show that MPC supplementation can directly affect *CAT* expression in the liver of poultry and challenged piglets and, to a lesser extent *SOD1* in poultry. Results from the present

thesis show that the inclusion of SOD-rich melon pulp concentrate in the diet of post-weaning piglets and poultry can significantly increase the blood levels of some antioxidant biomarkers and the expression of genes implicated in the regulation of the oxidative status in the liver.

CHAPTER 8

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