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Class XXXI

ANIMAL NUTRITION: A TOOL TO MITIGATE OXIDATIVE STRESS AND INFLAMMATION

AGR18

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Abstract

Nutrition plays an important role in maintaining a good health status of animals as nutrients can modulate oxidative status, immune and inflammatory response. Moreover, there is a need of innovative additives and feeds that can improve organism defences against diseases.
The aim of this work was to monitor different markers of oxidative stress and inflammation in production animals in response to different feed additives.

In this study, first, the activation of Nuclear factor-E2 related factor 2 in transgenic mice fed different sources and amount of fatty acids was monitored. Inducing the transcription of genes involved in oxidative stress response, Nrf_2 represents one of the main actor in the organism defence against oxidative stress. In this phase, we used an innovative technique of *in vivo* imaging that allowed us to follow the experimental subject in time and *in vivo*. reducing significantly animal stress.

Secondly, an evaluation of different oxidative and inflammatory markers was carried on post-weaning piglets fed with melon pulp concentrate. The double aim of this trial was to validate melon pulp concentrate as a feed additives to improve immune response in challenged pigs and to monitor the trends of commonly used markers during LPS challenge.

Then, as the effectiveness of new feed additives are under investigation and their effect on intestinal health need to be demonstrated, is essential to turn attention to intestinal health biomarkers, non- or minimally invasive. For this reason, the main aim of the last study was to develop and validate a new sandwich ELISA test for the quantification of Pacreatitis Associated Protein in pig faecal samples.

Finally, an observational study on a new Oxidative Stress Index (OSi) was conducted on dairy cows during the transition period. The driving hypothesis was that a composed index, as OSi, predicts more accurately the oxidative status than the evaluation of reactive oxygen species or serum antioxidant capacity separately. Furthermore, the relationship between indexes of oxidative status and markers of energy balance as blood free fatty acids and β-hydroxybutyrate, was determined.

Riassunto

Il mantenimento di un buono stato sanitario negli animali da reddito è garantito anche da una corretta gestione della nutrizione. È ampiamente dimostrato, infatti, che i nutrienti possono modulare lo stress ossidativo, l'immunità e la risposta infiammatoria. Inoltre, ad oggi, c'è la necessità di implementare le conoscenze riguardo nuovi additivi alimentari che possano migliorare le difese immunitarie degli animali.

Lo scopo di questo studio era quindi quello di monitorare diversi marker ossidativi e infiammatori anche in risposta a differenti additivi alimentari introdotti nella dieta.

Per prima cosa abbiamo condotto uno studio sull'attivazione del fattore di trascrizione Nuclear factor-E2 related factor 2 (Nrf2) in topi reporter alimentati con differenti fonti e quantità di acidi grassi. Nrf_2 induce la trascrizione di più di 200 geni coinvolti nella risposta ossidativa ed è quindi considerato uno dei fattori più importanti nel ripristino di un corretto equilibrio ossidativo. In questa parte, abbiamo utilizzato una tecnica innovativa di *in vivo* imaging che ci ha permesso di seguire i soggetti sperimentali nel tempo e *in vivo*, riducendo notevolmente lo stress per l'animale.

In secondo luogo, è stato effettuato uno studio sui marker ossidativi e dell'infiammazione in suinetti nella fase di post-svezzamento alimentati con concentrato di polpa di melone e sottoposti a challenge con LPS da E. coli. Il doppio scopo di questo studio era di validare il concentrato di polpa di melone come promotore del sistema immunitario e monitorare l'andamento dei marker più comunemente utilizzati in funzione del challenge effettuato per mimare uno stato di infiammazione cronica che spesso si può verificare nell'allevamento suino durante il post-svezzamento.

Ad oggi risulta di fondamentale importanza trovare nuovi biomarkers dell'infiammazione che siano non, o minimamente, invasivi. Per questo motivo, lo scopo del lavoro successivo era quello di sviluppare e validare un nuovo sandwich ELISA test per la quantificazione della Pacreatitis Associated Protein (PAP) in campioni di feci suine.

Infine, è stato condotto uno studio osservazionale su un nuovo indice di stress ossidativo (OSi) nel periodo di transizione della bovina da latte. L'ipotesi era quella che un indice composto quale l'OSi potesse definire più accuratamente lo status ossidativo dell'animale rispetto alle specie reattive dell'ossigeno o alla capacità sierica antiossidante totale. Inoltre, è stata valutata la correlazione fra i sopradetti markers dell'ossidazione e quelli del bilancio energetico negativo (i.e. acidi grassi liberi e β-idrossibutirrato).

Chapter 1

General Introduction

1.1 Oxidative stress and Inflammation

Oxidative stress is usually defined as imbalance between oxidants and antioxidants. It plays a key role in several pathological conditions and can cause decreased health status, growth rates and reproduction performance in farm animals (Lykkesfeldt and Svendsen, 2007). A common classification of oxidants is based on their chemical nature: they can be free radicals or not. Free radicals are atoms or molecules bearing one or more unpaired electrons in the outer orbit (Phaniendra et al., 2015).

Hydroxyl radical ($^{\bullet}OH$), nitric oxide ($NO^{\bullet}$) and superoxide ($^{\bullet}O_2^-$) are included in the radical group, whereas peroxynitrite ($ONOO^-$), hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) are part of the non-radical oxidants.

Reactive species can be divided in two categories, Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) (Bolisetty et al., 2013). Due to their biological role and produced amount, ROS are the most important. Under physiological conditions, molecular oxygen is usually reduced to water; ROS are the results of an incomplete reduction of oxygen (Buonocore et al., 2010). Hydroxyl radical is, among ROS, the most unstable and can react with surrounding molecules just after being synthetized (Tan et al., 1998). The high reactivity of hydroxyl radical, however, is also responsible for its short half-life (Draganic and Draganic, 1971).

Oxidative stress leads to a disrupt of redox signalling and could cause an excessive ROS formation, with a consequent damage on proteins and nucleic acids, DNA mutations, and a variety of degenerative processes and diseases (Rahal et al., 2014).

An excess in the generation of free radicals at mitochondria level could be due to different endogenous and exogenous factors.

Mitochondrial electron transport chain, nitric oxide synthase reaction, peroxisomal β-oxidation and respiratory burst of phagocytic cells are the main endogenous sources of free radicals (Poljsak and Milisav, 2013).

Electron leaking from mitochondrial electron transport chain is the main source of ROS and can be due to inhibition of complex I and complex III (Figure 1) that lead to a higher rate of ROS production in the mitochondria (Lenaz, 2001; Murphy, 2009).

The decrease in mitochondrial membrane potential and the inhibition of respiration due to hypoxia or anoxia can stimulate ROS production too, but only for short time. (Nohl et al., 1993; Abramov et al., 2007)

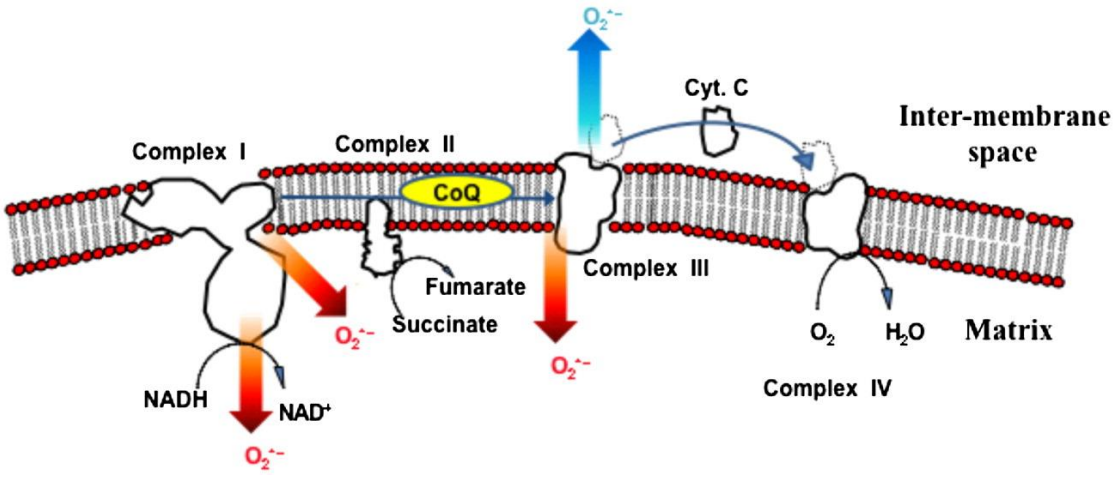

Figure 1: The electron transport chain. (Matsuzaki et al., 2009)

The exogenous causes of oxidative stress can be summarized (Birben et al., 2012) in:

- decreased intake of exogenous antioxidants from feed,
- increased metal ion intake (e.g., Fe, Cu, Cr) and
- ROS from ionizing radiation, air pollution, smoking and chronic inflammation.

Oxidative stress plays a crucial role in the development and perpetuation of inflammation (Lugrin et al., 2014). Inflammation is an adaptive response caused by infection or tissue injury that attempts to restore homeostasis. Commonly, a controlled inflammatory response is considered useful because provide protection against infectious disease but can be detrimental if excessive or dysregulated (Medzhitov, 2008). The inflammatory response is a complex network formed by inducers and mediators of inflammation: the firsts start the inflammatory response; the second ones are molecules that acts on blood vessels, inflammatory cells or other cells to contribute to the inflammatory response.

Due to its absorptive function, the mucosal surface of the intestinal tract is thin and permeable and, as consequence, vulnerable to infection. Any alteration of intestinal barrier further increases the permeability of the epithelium. In this way, toxins, bacteria and feed-derived antigens can cross the barrier and lead to malabsorption, inflammation processes and, in animal, impaired growth and production. The gastrointestinal tract (GIT) is the most extensively exposed surface in the organism and it forms a protective barrier. In addition to be a vital organ for the digestion, absorption and metabolism of dietary nutrients, the GIT forms part of the mucosal immune system (Murphy and Weaver, 2017). The gut associated lymphoid tissue (GALT) contains about 70-80% of the total amount of immune cells (Genton and Kudsk, 2003) and is involved in the mediation of the innate and adaptive immune responses (Pitman et

al., 2000). The immune population of the GIT consists of macrophages, T and B cells, dendritic cells and immunoglobulins A. For this reason, GIT is so important for the maintenance of health in both, humans and animals. Kogut and Arsenault (2016) defined gut health as the "absence/prevention/avoidance of disease so that the animal is able to perform its physiological functions in order to withstand exogenous and endogenous stressors". The GIT equilibrium is guaranteed by the complex interactions existing between the environment, the microbiota and the mucosa (Niewold, 2015a). At the same time, these interactions are the responsible for the potential antigens overload of the organism (Wershil and Faruta 2008).

1.1.1 Nutrition: friend and foe

On one side, nutritional diseases are considered the first cause of oxidative stress condition, on the other, nutrition itself and the gastrointestinal tract play a pivotal role in the control of oxidative balance and inflammation.

Several dietary compounds have been shown to determine huge effects on oxidative stress and inflammation in animals such due to their deficiency or optimal supplementation in the diet. Among others, microelements, vitamins, polyunsaturated fatty acids, natural extracts and contaminants are the most important.

Selenium and Vitamin E deficiency is one of the main nutritional disease altering oxidative homeostasis. They are involved in the protection of membranes' phospholipids and their deficiency can lead to biological membrane damages, and, as consequence, to injury of the other cellular components, including DNA. After a membrane injury, cell is not able to maintain its electrophilic balance, phospholipases are activated, and calcium released. These events lead to mitochondrial apoptosis (Maxie, 2007).

Mycotoxicoses are diseases caused by exposure to mycotoxins that are secondary metabolites of fungi with adverse effect on humans and animal health (Zain, 2011). Mycotoxins feed contamination is a worldwide problem. Consumption of naturally contaminated multiple mycotoxins has been proved to be more toxic than consumption of pure mycotoxin, probably due to the synergism existing (Hou et al., 2013).

Aflatoxins (AF) are produced by *Aspergillus flavus* and *Aspergillus parasiticus*. Those toxins have a mutagenic, teratogenic and carcinogenic effect and are active mainly in the liver (Bennet and Klich, 2003). AF metabolism is linked to a huge production of ROS, which damage membrane's lipids, proteins and nucleic acids (Kodama et al., 1990).

Metabolic disease, strictly connected to nutritional factors, in periparturient dairy cows are also related to oxidative stress and inflammation. The onset of insulin resistance, for example, is related to an increased production of ROS (Abuelo et al., 2016) and increased expression of inflammatory mediators (Olefsky and Glass, 2010). Insulin resistance then predisposes animal to other diseases such as ketosis and lipomobilization syndrome (Ohtsuka et al., 2001; Youssef and El-Ashker, 2017), abomasal displacement (Pravettoni et al., 2004), subclinal mastitis (Turk et al., 2012) and reduced fertility (Baruselli et al., 2016).

Nutrition has been claimed to improve immune system function through feed components such as probiotic, prebiotic and antioxidant.

FAO defined probiotics as living microorganisms that provide beneficial effects to the health of the host if administrated in adequate amount. Probiotics help the host to counteract pathogens colonization of the gastro intestinal tract. This control activity is imputable to antimicrobial substances produced by probiotics (Shim et al., 2012) as well as the adhesion to intestinal epithelium that lead to competition with pathogens and activation of immune response.

Commonly used products in animal and human nutrition derived from yeasts, as *Saccharomyces cerevisiae*, *Saccharomyces boulardii*, or bacteria, as *Lactobacillus, Enterococcus* and *Pediococcus* spp. and *Bifidobacterium* (Chaucheyra-Durand and Durand, 2010; Gaggia et al., 2010).

Prebiotics are "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon" (Gibson and Roberfroid, 1995). In 2007, Roberfroid revised the definition of probiotics, expanding the action of prebiotics to the entire gastrointestinal microflora and not limited at the colon compartment. Most common prebiotics are carbohydrates and oligosaccharides normally present in human and animal diet (Gaggia et al., 2010), but the most promising are the non-digestible oligosaccharides such as fructooligosaccharides (FOS, oligofructose and inulin), galactooligosaccharides (GOS), transgalacto-oligosaccharides (TOS), and lactulose.

Antioxidants are defined as substances able to delay or inhibit oxidation even at low concentrations (Halliwell and Gutteridge, 1995).

Antioxidant defence system of the organism is structured on different stages and need to be considered as an antioxidant network in which different substances work in synergy in order to optimize the reaction to oxidative input (Jacobs et al., 2010).

Dietary antioxidants includes, among others, vitamin C, vitamin E, plant polyphenol and carotenoids (Shahidi and Zhong, 2010).

Vitamin C or ascorbic acid is a six-carbons compound. It is a water-soluble scavenger and, cooperating with GSH or other compounds able to donate reducing equivalents, it regenerates Vitamin E in cell membranes (Niki, 1991).

Vitamin E, also known as α-tocopherol, is a liposoluble vitamin. It is a chain-breaking antioxidant, that means it stops the lipid peroxidation process by acting as electrons' donor (Traber and Atkinson, 2007).

Carotenoids are a wide group of natural pigments (Landrum, 2010). Carotenoids and their metabolites protect the organism in a variety of ROS-mediated disorders (Fiedors and Burda, 2014), acting as scavengers. However, the antioxidant capacity of these compounds depends on the oxygen tension in the system (Palozza, 1998). At low partial pressure of oxygen, β-carotene was found to inhibit the oxidation, but at the high oxygen tension, initial antioxidant activity of β-carotene is followed by a pro-oxidant action.

Some plants contain phenolic compounds with antioxidant, antimicrobial and anti-inflammatory activity (Pereira et al., 2009). Natural polyphenols antioxidant activities have been widely studied, including reduction of hydroperoxide formation, inhibition of lipid peroxidation and scavenging of free radicals (Sato et al., 1996). Natural antioxidants, in the form of extracts, may be obtained from many different sources such as fruits (melon, pomegranate, grapes), vegetables (broccoli, potatoes and pumpkins) or herbs and spices (oregano, rosemary, tea and cinnamon) (Shah et al., 2014).

A consideration apart should be done for polyunsaturated fatty acids. In literature, there are different opinion about the role of fatty acids in human and animal diet and the debate between saturated and polyunsaturated fatty acids and their beneficial properties is still open. Dietary fatty acids both, in human and animals, have been claimed to modulate inflammation by the production of lipid mediators. It was observed in human that saturated fatty acids are related to cardiovascular disease, while n-3 PUFA plays a significant role in immune cells functions (Calder, 2011). In goat, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), the most abundant type of 3-PUFA presents in diet, improve the phagocytic activity of neutrophils, whereas DHA reduces the extracellular release of ROS (Pisani et al., 2009). The administration of n-3 PUFA improves also fertility by lowering the production of Prostaglandin F2α and enhancing embryos' quality (Cerri et al., 2004; Mattos et al., 2004).

However, dietary lipids such as supplemental fat, oil seeds, and distiller grains, if not stabilized, can be significant contributors to the load of free radicals in the animal (Andrews et al., 2006).

Indeed, it was proved that supplementation of polyunsaturated fatty acids in dairy cows diet decreases the antioxidant defense by reducing both Super Oxide Dismutase (SOD) activity and Total Antioxidant Capacity (TAOC) and increasing also plasmatic concentrations of Malondialdehyde (MDA). (Wang et al., 2010; Hashemzadeh-Cigari et al., 2015).

1.1.2 Nrf2, NF-κB, MAPK and JAK signalling pathways

Oxidative stress response as well as inflammatory response are regulated by intracellular signalling pathways.

Kelch-like ECH associating protein 1-Nuclear Factor E2-related factor 2 (Keap1-Nrf2) pathway plays a pivotal role in the antioxidant defence system. Nrf2 is a transcription factor involved in the regulation of antioxidant and phase II detoxification genes (Itoh et al., 1997). Nrf2 is a basic region-leucine zipper (bZip) transcription factor negatively regulated by Keap1 (McMahon et al., 2006). Under basal conditions, Nrf2 is rapidly degraded by the ubiquitin-proteasome pathway through the association with Keap1 (Kelch-like ECH associating protein 1) (Zhang and Hannik, 2003). Upon exposure to oxidative stress, Nrf2 is released from Keap-1 mediated degradation and translocated to the nucleus, where interacts with a member of the small Maf family proteins (Figure 2). This complex activates the transcription of a wide range of cytoprotective genes via a cis-acting DNA element, known as the antioxidant/electrophile responsive elements (ARE) (Rushmore et al., 1991). The ARE is commonly located in the regulatory region of genes dedicated to encode antioxidant enzymes and proteins.

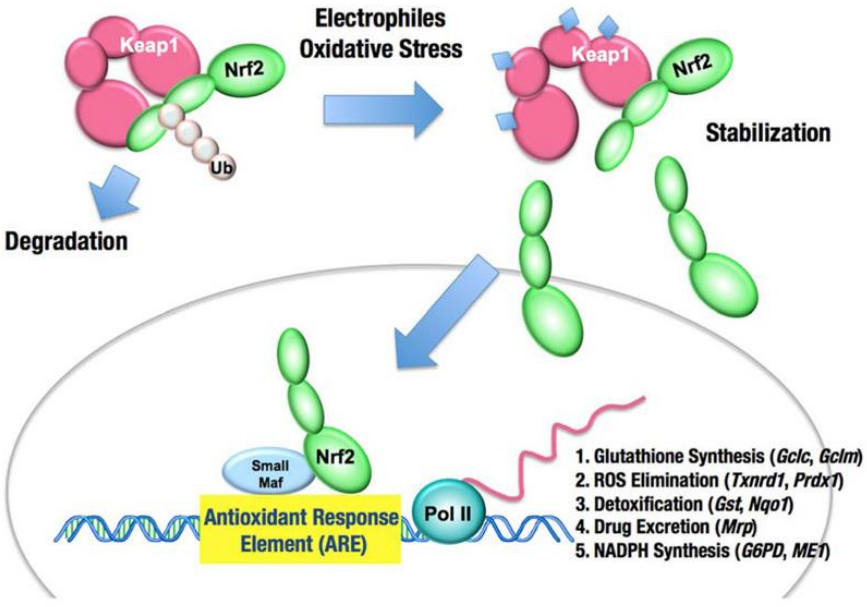

Figure 2: The Keap1-Nrf2 system (Mitsuishi et al., 2012)

Intracellular signalling pathways, activated by inflammatory stimuli, are responsible for the production of inflammatory mediators. The most common pathways are NF-κB, MAPK, and JAK-STAT pathways.

NF-κB induces the expression of different genes involved in the immune and inflammatory response, such as genes encoding cytokines and chemokines or involved in inflammasome regulation (Liu et al., 2017). Under physiological conditions, NF-kB dimers are retained in the cytosol by IkB proteins. After the degradation of IkB proteins by the IkB kinase (IKK) complex, NF- kB is translocated in the nucleus and induces the transcription of target genes (Oeckinghaus and Gosh, 2009).

The Mitogen-activated protein kinases (MAPK) proteins, especially p38 MAPK, are essential in macrophages-mediated inflammatory response (Yang et al., 2014). Cytokines and other stress/inflammatory stimuli upregulate the expression of p38, which is involved in the expression of inflammatory mediators such as Interleukins, Ciclooxygenase and Tumor Necrosis Factor α (Yang et al., 2012; Garcia et al., 1998).

JAK (Janus kinase protein) family is formed by four members: JAK 1 to 3 and tyrosine kinase 2 (TYK2) (Kiu and Nicholson, 2012). The signal transducers and activators of transcription (STAT) is a family of 7 members. The JAK/STAT pathway is responsible for the transduction of the signal from extracellular cytokine stimuli to the nuclear compartment in order to initiate the cellular response (O'Shea and Plenge, 2012).

1.2 Biomarkers

In the past, different definitions of biomarkers were released, of which two are particularly interesting.

In 2001 the Biomarkers Definitions Working Group stated that a biomarker is "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention".

The same year, the World Health Organizations declared that biomarkers are "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease".

In this view, biomarkers are objective and quantifiable characteristics of biological processes that can be used to predict disease, in our case oxidative stress and inflammation. The main characteristics of optimal biomarkers are stability, availability of assays for the detection and affordability (Niewold, 2015 b).

1.2.1 Oxidative stress biomarkers

Redox status of animals can be measured directly or indirectly by detecting free radical production (ROS) or by detecting antioxidant compounds and enzymes or, in addition, oxidative damage biomarkers, such as lipid and protein oxidation products formed from reaction between ROS and cellular macromolecules (Rossi et al., 2013; Tóthová et al., 2015).

Enzymatic antioxidants are the primary defence; their role is to catalyse chemical reaction to neutralize ROS. The quantification of antioxidant enzymes is useful to monitor the oxidative defence status of the animal. The most important enzymes involved in oxidative response are Superoxide dismutase (SOD), Catalase (CAT) and Glutathione Peroxidase (GPx).

Superoxide dismutase (SOD) catalyses the dismutation of superoxide radical into hydrogen peroxide and molecular oxygen:

$$\bullet O_2^- + \bullet O_2^- + 2H+ \rightarrow H_2O_2 + O_2$$

This reaction involves alternate reduction and reoxidation of a redox active transition metal, (Abreu and Cabelli, 2010).

Based on the catalytic metal and on the subcellular location, SODs can be classified into three isoforms:

- SOD1 is a homo-dimeric protein containing two ions, Zinc and Copper, and represents about 90% of total SOD activity (Liu et al., 2004). CuZnSOD is principally located in

the cytosol and in the nucleus; more recent studies, however, show that part of the inactive enzyme is stored into the intermembrane space of mitochondria (Okado-Matsumoto et al., 2001; Iñarrea, 2002). Acquisition of Cu ion is mediated by the copper chaperone for SOD1 (CCS) (Wong et al., 2000), whereas the acquisition method of Zn enzyme is still unknown.

- SOD2 is a tetramers enzyme with a single manganese atom per subunit (Abreu and Cabelli, 2010). It is synthetized in the ribosomes and then translocated in the mitochondrial matrix (Karnati et al., 2013). Because of its sequence and structure homology to FeSOD (a prokaryotic enzyme), SOD2 is able to bind iron instead of Mn in the active centre to the detriment of its enzymatic activity (Wintjens et a., 2004)
- SOD3 is glycosylated high molecular weight homotetramer. Even though SOD3 binds copper and zinc as SOD1, it is the only isoform that is expressed extracellularly (Weydert and Cullen 2010). SOD3 is one of the main defence system of blood vessel (Fukai and Ushio-Fukai, 2011).

Catalase (CAT) is a tetrameric protein with one heme moiety at the active site for each subunit. Its activity is heme-dependent and catalyse the conversion of hydrogen peroxide into water and oxygen:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Although CAT is an ubiquitary enzyme, most of it is localized in the peroxisomes, where high concentrations of hydrogen peroxide might be found (Kehrer et al., 2010).

Glutathione Peroxidases (GPx) are an enzymatic family able to reduce hydrogen peroxide producing water and oxidized glutathione (GSSG):

$$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$$

The GSSG produced by the reaction is then reduced again to GSH through the Glutathione reductase (Deponte, 2013), using NADPH as cofactor (Kehrer et al., 2010):

$$2NADPH + GSSG \rightarrow 2GSH + 2NADP^+$$

8 isoforms of GPx has been discovered: 5 (GPx1-4 and GPx-6) present a selenocysteine on the active site, whereas in GPx 5, 7 and 8 selenocysteine is replaced by cysteine. The non-selenocysteine-containing GPx has lower peroxidase activity compared to the others. GPx 7

and 8 do not have even GSH binding domain (Nguyen et al., 2011) and their function is still under investigation.

Several methodologies exist for the determination and quantification of the antioxidant enzymes, such as Western Blot, activity assays and immunohistochemistry (Weydert and Cullen, 2010)

Non-enzymatic antioxidants are the second line of defence against ROS. They are molecules able to rapidly inactivate radicals and oxidants (Mirończuk-Chodakowska et al., 2018)

Glutathione can be found in the reduced (GSH) or the oxidized form (glutathione disulphide- GSSG). It is the main element of the so called "glutathione system": GSH donates electrons to a pro-oxidant molecule, becoming GSSG. Reconversion to GSH is catalysed by the enzyme Glutathione Reductase through the co-enzymatic action of NADPH (Morris et al., 2014).

Vitamin E, as previously stated, plays a pivotal role in the protection of membranes' phospholipids and its serum or plasma levels reflects the oxidative status of the animals.

The ratio between copper and zinc is often used as biomarker of oxidative stress, due to the pro-oxidant activity of copper molecule and antioxidant activity of zinc. Under oxidative stress condition the Cu/Zn ratio increases.

Total Antioxidant Capacity (TAOC) is an index that evaluates the cumulative activity of various antioxidant systems. TAOC is a general term for different measurement methods able to detect the total antioxidant capacity in a biological fluid (Sies, 2007). There are different methodologies to determine TAOC that differ for substrate, quantification methods and reaction conditions (Huang et al., 2005): Oxygen Radical Absorbance Capacity (ORAC), Total Radical-trapping Antioxidant Parameter (TRAP) Trolox equivalent Antioxidant Capacity (TEAC) and Serum Antioxidant Capacity (SAC).

KRL assay is specific for the antioxidant capacity of blood samples and it test the time needed to haemolyse 50% of red blood cells when sample is under a free radical attack (Prost, 1992)

The evaluation of oxidative reaction products is a quantitative indirect method to assess the oxidative status of the organism.

8-isoprostane belongs to isoprostanes family that are eicosanoids of non-enzymatic origin produced by the random oxidation of tissue phospholipids by oxygen radicals.

Specifically, are non-COX derived prostanoids originated from arachidonic acid degradation (Roberts and Morrow, 2000).

Protein carbonyls are protein derivatives or peptide fragments produced by the interactions between proteins and ROS (Stadtman and Levine, 2003). The presence of these compounds in cells directly reflect the damage induced by ROS.

Thiobarbituric acid reactive substances (TBARS) are low-molecular-weight compounds, mainly malondialdehyde, (MDA), that originate from the decomposition of lipid peroxidation products.

1.2.2 Intestinal health biomarkers

Appropriate intestinal health biomarkers should originate from the GIT or be related to it. They can be obtained in a non-invasive or minimally invasive way from samples of blood (plasma or serum), faeces, saliva, urine, or other bodily fluids.

Thanks to the greater availability of research funding, human medicine is at the cutting edge in this research field and has already validated biomarkers as diagnostic tool for intestinal inflammation and dysfunction in humans and experimental animals.

However, due to differences between species, their results cannot be easily translated on production animals (Table 1). In the genome of livestock species, homologous sequences with humans have been found. Anyway, the same function cannot be guaranteed and, therefore, they cannot be assumed as reliable biomarkers.

Indeed, the greater the evolutionary distance between species, the more likely that differences in homology, gene function and immunological cross-reactivity will occur (Niewold, 2015 b).

509 **Table 1:** Intestinal health biomarkers, their specificity, and presence in species, sampling method, and
510 the availability of reagents[1] (Adapted from Niewold, 2015b)

Marker	Specificity	Species other than human	Sample	Test reagents/asssay
Intestinal fatty acid binding protein (I-FABP)	Small intestine Enterocyte damage	Porcine	Blood Urine Faeces[3]	Imm: porcine, chicken
Pancreatitis associated protein (PAP, RegIII)	Small intestinal Inflammation	Porcine	Urine Faeces	Imm: *porcine*
Myeloperoxidase (MPO)	Intestine Inflammation	Absent in chicken	Faeces	Imm: / Biochem: porcine
Acute phase proteins (APP)	Inflammation	Porcine	Blood Saliva	Imm: *porcine* Biochem: all

511 [1] In italics: claimed but not proven; [2] Imm(unoassay), biochem(ical assay), for species other
512 than human; [3] In pig faeces (T.A. Niewold, unpublished data).

513

514 Intestinal Fatty Acids Binding Protein (IFABP) is part of a group of proteins whose
515 function is to bind long chain fatty acids in a reversible and non-covalent manner in order
516 increase their aqueous solubility and therefore facilitate their transport both extra- and
517 intracellular (Glatz and van der Vusse, 1996). A distinction can be made between two main
518 types of FABPs, namely those present in association with the plasma membrane and the
519 intracellular or cytoplasmic FABPs. At least nine immunologically different types of
520 cytoplasmic FABPs exist: liver (L-) FABP, intestinal (I-) FABP, heart (H-) FABP, adipocyte
521 (A-) FABP, epidermal (E-) FABP, ileal (IL-) FABP, brain (B-) FABP, myelin (M-) FABP, and
522 testicular (T-) FABP, named after the first tissue in which they were isolated (Zimmerman and
523 Veerkamp, 2002). Intestinal enterocytes express both I-FABP and L-FABP (Glatz and van der
524 Vusse, 1996; Zimmerman and Veerkamp, 2002). The release of cytoplasmic FABPs from
525 damaged cells can be measured, making it a prominent aid to diagnose and monitor the
526 occurrence and extent of cell damage or tissue injury, both in experimental studies and in the

clinical setting. The duodenum, jejunum and ileum contain significantly higher I-FABP levels than the colon ($p < 0.001$) with the jejunum expressing the highest level. I-FABPs are detectable in blood, urine and faeces after damage of cells, for this reason are considered suitable biomarkers of enterocyte damage in various species. Its half-life of approximately 10 minutes makes I-FABP useful as acute enterocyte damage marker (Niewold, 2015 b).

Niewold et al., (2004) detected the presence of the I-FABP protein in pig jejunum using a commercially available human ELISA kit. In addition, based on the acute increase (within 30 minutes) of I-FABP in plasma after experimentally induced ischemia in pigs, it was concluded that plasma I-FABP concentration in pigs might be used as a sensitive marker of mild damage to the intestinal mucosa.

Chicken I-FABP gene show 71% to 72% similarity to human, mouse, and pig I-FABP genes (Wang et al., 2005). In addition, the chicken I-FABP gene was also expressed exclusively in the intestinal tissues (Wang et al., 2005), which suggests that I-FABP could be a useful biomarker in chicken as well; however, reagents are still unavailable (Niewold, 2015 b).

Expression and localization of FABPs protein in ruminants has not been deeply investigate. One of the first study in ruminants (Hayashi et al., 2013) showed that IFABP is expressed in duodenum, jejunum and ileum of both, calves and cows, but in calf mRNA expression was higher. No study has been conducted on the possibility to use IFABP as intestinal marker of inflammation in ruminants.

Myeloperoxidase (MPO) is an enzyme found in inflammatory cells, mainly in the granulocytes of neutrophils (Prokopowicz et al., 2012). During the degranulation process, MPO is released into the phagosome where it reacts with hydrogen peroxide and halide to produce hypochlorous acid or with tyrosine to produce tyrosyl radicals. These substances are extremely cytotoxic and are released to kill pathogenic microorganisms. However, they can also further contribute to inflammation due to their toxic nature (Klebanoff, 2005). Myeloperoxidase can be measured by performing an easy biochemical assay for total peroxidase activity (PO). In order to differentiate MPO from total PO activity, specific antibodies are used (Niewold, 2015b).

Since MPO is often overexpressed in various inflammatory diseases, faecal MPO is used as a biomarker to determine the level of intestinal inflammation in diseases such as inflammatory bowel disease (IBD) in humans (Saiki, 1998; Wagner et al., 2008; Kosek et al., 2012 and Hansberry et al., 2017) as well as animal models. Myeloperoxidase reflects the concentration of neutrophils present in faeces from individuals with intestinal inflammation

(Saiki, 1998). Myeloperoxidase can potentially be employed to monitor disease activity and treatment outcomes in patients with ulcerative colitis (Wagner et al., 2008).

The regenerating islet-derived (Reg) protein family are C-type lectins of which several members have been identified and grouped, according to homology, into four subfamilies: RegI, RegII, RegIII, and RegIV (Cash et al., 2006). C-type lectins mediate several functions including cellular adhesion, phagocytosis, and recognition of microbe-associated molecular patterns (Cash et al., 2006). RegIII comprises Pancreatitis Associated Protein (PAP) or RegIIIα and RegIIIγ in humans and RegIIIα, RegIIIβ, RegIIIγ and RegIIIδ in mice (Graf et al., 2006; Lieu et al., 2006; Parikh et al., 2012). Being PAP able to bind and aggregate bacteria in the intestines, it may act as an antibiotic agent (Iovanna et al., 1991). Moreover, it is suggested that PAP has anti-inflammatory activity (Folch-Puy et al., 2003) and regulates inflammatory response (Vasseur et al., 2004), reducing the secretion of pro-inflammatory cytokines in the intestinal mucosa (Gironella et al., 2005). In addition, PAP grants protection to cells against oxidative stress and apoptosis (Ortiz et al., 1998) induced by TNFα which leads to PAP up-regulation and, at the same time, apoptosis inhibition (Malka et al., 2000).

Acute Phase Proteins (APPs) are blood proteins and change their serum concentration by 25% during inflammation processes (Eckersall and Bell, 2010). In humans, APPs such as C-reactive protein (CRP) and haptoglobin (Hp) are employed as markers of intestinal inflammation in cases such as Inflammatory Bowel Disease (IBD). Haptoglobin is a haemoglobin (Hb) binding protein that regulates innate immunity reactions in white blood cells, has a bacteriostatic effect and a chaperone activity (Ceciliani et al., 2012).

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Chapter 2

Study objectives

2.1 Study objectives

The growing world population is expected to reach about 9 billion people in 2050 and, according to FAO, global food requirement will be double, compared to 2010. This dramatic increase will lead to a strong pressure on farmers and animals to increase productivity, but further improvements by breeding are unconvincing. Attention should now be paid to alternative approaches to yield higher productivity by reducing the loss in efficiency caused by disease and increasing feed efficiency.

Throughout their lifetime, production animals can undergo situations of physiological imbalance, like the transition period in dairy cows as well as the post-weaning period for young piglets. In these critical phases, regulatory mechanisms are altered, therefore, there is a major risk of metabolic and infectious diseases. Oxidative stress and intestinal inflammation are main problem in livestock animals and their monitoring is essential to avoid reduced productivity and huge economical losses for farmers.

The aim of this work was to evaluate the effect of antioxidant compounds on oxidative status and immune system and to study different markers of oxidative stress and inflammation in monogastric species and ruminants. The present thesis accounts for the oxidative stress and inflammation monitoring starting with a mouse model, moving to the development of a new ELISA test for the non-invasive detection of inflammation biomarkers in swine and concluding with the evaluation of a composite index for oxidative stress in transition dairy cows.

Chapter 3

Saturated fatty acids downregulate Nrf2 expression in an estrogen dependent way: an *in vivo* imaging nutritional study using ARE-luc2 reporter mice.

Adapted from:

E. Mariani, N. Rizzi, N. Cesari, A. Agazzi, D. Cattaneo, E. Brunialti, P. Ciana, G. Savoini and A. Maggi. Saturated fatty acids downregulate Nrf2 expression in an estrogen dependent way: an *in vivo* imaging nutritional study using ARE-luc2 reporter mice. – Manuscript submitted to Scientific Report

3.1 Abstract

In the literature, the debate about the effect of the degree of saturation of dietary fatty acids on the immune response, lipid metabolism and oxidative stress is still open, and outcomes are contradictory. Also, recent studies suggest an effect of estrogen (E_2) on oxidation processes through regulation of the Nuclear factor-E2 related factor 2 (Nrf2) pathway. The aim of the study was to evaluate the oxidative stress response in mice, as experimental model, fed with different fatty acid sources and in the presence or absence of E_2.

We measured Nrf2 expression using a novel ARE-*luc2* transgenic reporter mouse. In this study, ovariectomized female (OVIX), intact female (SHAM) and male ARE-*luc2* mice were fed for 10 consecutive weeks four isonitrogenous diets with two different levels of inclusion of lard (LL: 7.5% or HL: 20.0%), as saturated fatty acids (SFA) source, or fish oil (LF: 7.5%; HF: 20.0%), as polyunsaturated fatty acids (PUFA) source. At the end of the trial mice were submitted to acute oxidative stress by intraperitoneal injection with sodium arsenate (ASN). *In vivo* activation of Nrf2 was measured twice weekly by bioluminescent imaging.

Our results showed that dietary fatty acids content or composition alone was not sufficient to up or downregulate Nrf2 expression within each group (OVIX, SHAM and MALE). However, comparison between groups showed that SHAM and MALE had a higher expression of Nrf2 than OVIX. In addition, OVIX mice fed SFA (irrespective of amount) had a lower Nrf2 activation than SHAM and MALE.

In conclusion, these results suggest that E_2 deficient animals are more susceptible to oxidative stress, especially if fed saturated fatty acid. Beside the possible translation on farm animals species, the present results may have also bearing on dietary advice for the post menopause phase in women.

Keywords: Luciferase, Fatty acids, Nuclear factor-E2 related factor 2, Oxidative stress, Estrogen, Bioluminescent Optical Imaging, reporter transgenic mouse.

3.2 Introduction

The health effects of saturated (SFA) and polyunsaturated (PUFA) fatty acids in the diet are still discussed. Fatty acids (FAs) are important in the structure of the plasmatic/cellular membranes – being responsible for e.g., membrane fluidity, and in metabolic/signalling processes. They are important sources of energy and precursors of signalling molecules (including proinflammatory, anti-inflammatory, vasoactive, and many other mediators) (Nowak et al., 2013).

Several studies in human medicine describe nutritional benefits of consuming long-chain (LC) n-3 PUFA from fish, in particular, docosahexaenoic acid (DHA, 22:6 n-3) and eicosapentaenoic acid (EPA, 20:5 n-3) to protect against different pathologies (Fedor and Kelley, 2009; Carpentier et al., 2006), particularly cardiovascular diseases (Simopoulos, 2008). Therefore, nutritional recommendations in Western societies have been established for n-3 PUFA intake of 500 mg/day of EPA and DHA, in order to reduce the incidence of chronic diseases.

However, current studies reveal that the n-3 PUFA may not be devoid of risk. Possible harmful effects of high levels of n-3 PUFA on retinal membrane degeneration have been described by Tanito et al., (2009). Dietary LC n-3 PUFA are highly vulnerable to oxidation, which is one of the major problems in food chemistry and may decrease their nutritional value. Indeed, peroxidation causes loss of nutritional quality and further leads to the generation of genotoxic and cytotoxic compounds, such as the 4-hydroxy-2-alkenals (4-HHE) and 4-hydroxy-2-nonenal (4-HNE) that are major end-products derived from n-3 and n-6 PUFA peroxidation, respectively.

Oxidative stress plays a key role in several pathological conditions and can cause decreased health status including: mutagenesis, cell transformation, cancer, heart attacks, strokes, atherosclerosis, ischemia/reperfusion injury, chronic inflammatory diseases, acute inflammatory problems, neurological disorders, such as Parkinson's and Alzheimer's diseases and other age-related disorders, perhaps even including factors underlying the aging process itself (Davies, 1995). Commonly defined as imbalance between oxidants and antioxidants, high oxidative stress lead to a disruption of redox signalling and could cause excessive reactive oxygen species (ROS) formation, and subsequent damage on proteins and nucleic acids, DNA mutations, and a variety of degenerative processes and diseases (Rahal et al., 2014).

The most important endogenous sources of oxidants are the mitochondrial electron transport chain, nitric oxide synthase reaction, peroxisomal beta-oxidation and the respiratory burst of phagocytic cells (Poljsak and Milisav, 2013). Oxidative stress can be influenced by dietary

intake of exogenous antioxidants or in an increased consumption of pro-oxidants such as lipids, carbohydrates, high processed food/feeds and metal ions (Rahal et al., 2014).

A key role in the activation of the antioxidant response is played by the Kelch-like ECH associating protein 1-Nuclear Factor E2-related factor 2 (Keap1-Nrf2) pathway. Nrf2, a basic region-leucine zipper transcription factor, is rapidly degraded by the ubiquitin-proteasome pathway through the association with Keap1, a substrate adaptor protein of the Cul3-based ubiquitin E3 ligase complex (Zhang and Hannik, 2003). Upon exposure to oxidative or electrophilic stress, reactive cysteine residues in Keap1 are covalently modified, leading to the release of Nrf2 from Keap-1 mediated degradation. The stabilized Nrf2 is then translocated to the nucleus and interacts with a member of the small Maf family proteins. This complex activates the transcription of more than 200 cytoprotective genes via a cis-acting DNA element known as the antioxidant/electrophile responsive element (ARE) (Rushmore et al., 1991).

Recently, a strong relationship between Nrf2 pathway and estrogen (E_2) was established, suggesting that E_2 exhibits its antioxidant role by upregulating Nrf2. It was demonstrated that lacking estradiol in ovariectomized female mice attenuates the Nrf2-ARE pathway in the hippocampus CA1 region (Li et al., 2017), with significantly decreased Nrf2 mRNA expression (Yoo et al., 2016), while a long-term E_2 administration showed positive effects on lipid peroxidation in the liver (Sobočanec et al., 2015). In previous *in vitro* experiments, a direct relationship was demonstrated: E_2 treatment led to Nrf2 dissociation from Keap1, the main negative regulator of Nrf2 activity in the cytoplasm and increased the protein level of Nrf2 in the nucleus with a significant increase in HO-1 expression and SOD activity in Hcy-treated cells (Chen et al., 2013).

An increasing body of evidence now links estrogenic signalling also with the metabolic syndrome (Maggi and Della Torre, 2018). Ovariectomy, resulting in low estrogen levels, led to increased Body Weight (BW) and basal blood glucose with impaired glucose tolerance in a mouse model, which were reversible when estrogen level was restored (Matic et al., 2013). Estrogen is believed to protect, at least in part, against obesity. However, it is still unclear whether saturated or polyunsaturated fatty acids in the diet are the primary driver of this effect (Mamounis et al., 2017). For this reason, many studies were performed to investigate the relationship between fatty acids in the diet, but none about the possible relationship with estrogen.

In the present study, we aimed to clarify the relationship existing between dietary fatty acids, estrogen and oxidative stress response: we performed an *in vivo* experiment using mice fed either saturated or polyunsaturated fatty acids and in the presence of physiological or low

level of estrogen while measuring Nrf2 activity. We used a recent developed ARE-*luc2* reporter transgenic mouse fed with different dosage of lard or fish oil. The ARE-*luc2* model (Rizzi et al., 2017a; Rizzi et al., 2018) is a genetically modified mouse that leads to measure the activity of Nrf2 transcription factor *in vivo* by optical imaging. The employment of reporter mice mainly allows to monitor the oxidative stress response over time (temporal dimension) and provides a global view of the potential target organs in all spectrum of body action of whole mouse (spatial dimension) (Maggi and Ciana, 2005). The different estrogen status in the experimental animals was obtained by using a male group compared to intact and ovariectomized female groups; indeed, ovariectomized female have been reported to have lower level of serum estrogen compared to intact female (2.44 ± 0.77 µg/ml vs. 6.13 ± 0.62 µg/ml) (Mok et al., 2018).

3.3 Materials and methods

3.3.1 Experimental design, animals' management and dietary treatment

All animal rearing and handling procedures were carried out in accordance with the ARRIVE and European Guidelines for Animal Care. The experimental trial was validated by the Ethical Committee of the University of Milan and approved by the Italian Ministry of Research. Thirty-two females and 16 males (MALE) ARE-*luc2* (Rizzi et al., 2017a; Rizzi et al., 2018) transgenic reporter mice (8-10 weeks old) were selected on a body weight basis from several litters, and individually identified by ear tags. Two weeks before starting the trial, half of the total number of females underwent an intervention of ovariectomy (OVIX, n.16) or sham ovariectomy (SHAM, n.16). Female mice were anesthetized with a 50 µl subcutaneous injection of a ketamine (93.6 mg/kg, Ketavet 100; Intervet, Milan, Italy) and xylazine (7.2 mg/kg, Rompun; Bayer, Milan, Italy) solution. Surgical procedures where than performed as previously described by Brufani *et al*., (2017).

Each group of 16 mice (MALE, OVIX, and SHAM) was then further divided in four dietary groups of four animals each for a total period of 70 days, and fed: a) a low (10% ether extract) fat diet with 7.5% lard inclusion rate (LL); b) a low (10% ether extract) fat diet with 7.5% fish (tuna) oil inclusion rate (LF); c) a high (22.5% ether extract) fat diet with 20.0% lard inclusion rate (HL); d) a high (22.5% ether extract) fat diet with 20.0% fish oil (tuna) inclusion rate (HF). All diets were formulated to be isonitrogenous and meet the nutrient requirements recommended by National Research Council for laboratory animals (NRC, 1995). Diets LL and

LF were isocaloric within each other, as well as diets HL and HF (Table 2). The four experimental diets were provided by Sniff Spezialdiäten GmbH (Soest, Germany).

Table 2: Ingredients (% w/w) and chemical composition (g/100g dry matter) of the four experimental diets.

Ingredients	LL	LF	HL	HF
Casein, 80 Mesh	20.00	20.00	20.00	20.00
L-Cystine	0.30	0.30	0.30	0.30
Corn Starch	39.59	39.62	27.05	27.12
Maltodextrin	12.50	12.50	12.50	12.50
Sucrose	6.88	6.88	6.88	6.88
Cellulose, BW200	5.00	5.00	5.00	5.00
Soybean Oil	2.50	2.50	2.50	2.50
Lard	7.50	0.00	20.00	0.00
Fish (Tuna) Oil	0.00	7.50	0.00	20.00
tBHQ	0.0015	0.0000	0.004	0.0000
Mineral Mix	1.00	1.00	1.00	1.00
DiCalcium Phosphate	1.30	1.30	1.30	1.30
Calcium Carbonate	0.55	0.55	0.55	0.55
Potassium Citrate H_2O	1.65	1.65	1.65	1.65
Vitamin Mix[1]	1.00	1.00	1.00	1.00
Choline Bitartrate	0.20	0.20	0.20	0.20
Cholesterol	0.02	0.00	0.061	0.00
Chemical composition				
Crude protein	17.60	17.60	17.60	17.60
Ether extract	10.10	10.10	22.60	22.60
Crude Fiber	5.00	5.00	5.00	5.00
Ash	3.30	3.30	3.30	3.30
Kcal/Kg[2]	4,112.00	4,111.00	4,737.00	4,737.00

[1]The vitamin-mineral premix provided the following quantities of vitamins and micro minerals per kilogram of complete diet: vitamin A, 4 000 IU; vitamin D3, 1 000 IU; vitamin E, 50 mg; vitamin K3, 0.5 mg; Cu, 6 mg.

[2]Calculated values

The animals were housed in ventilated plastic cages and maintained at a temperature of 22°–25°C, with a relative humidity of 50%±10%. An automatic cycle of 12 hours light/dark (lights on at 0700 am) was adopted for all the trial period and *ad libitum* access to feed and water was granted.

Individual BW and feed intake (FI) were recorded weekly for all the trial period, while *in vivo* imaging sessions were performed twice weekly, and *ex-vivo* imaging was performed on day 70 from the beginning of the experiment.

3.3.2 In vivo and ex vivo imaging

To evaluate the activity of transcription factor Nrf2, in the present trial we used a recent developed ARE-*luc2* reporter mouse (Rizzi et al., 2017a) that permits to investigate the *spatio*-temporal activation of this signal in the context of a living organism. ARE-*luc2* reporter mice allow the measurement of Nrf2 activity in the whole body through *in vivo* imaging technologies. The specific features of the reporter system based on the presence of MAR sequence with insulating activity, together with the constitutively open state of the genomic locus where the transgene was inserted, granted a correct and ubiquitous activity of the biosensor.

In vivo bioluminescent imaging was performed by the semi-quantitative analysis of photon emission. Luciferin (80 mg/kg) (E1601, Beetle Luciferin Potassium Salt; Promega) was injected intraperitoneally 15 minutes prior the imaging session. Mice were then anaesthetized using Isofluorane (ISOFLU250, Isofluorane-Vet; Merial, Lyon, France) and kept under anesthesia for a five-minutes imaging session using a CCD-camera (IVIS Lumina II Quantitative Fluorescent and Bioluminescent Imaging; PerkinElmer, Waltham, MA, USA). Photon emission in chest, hepatic area, abdomen and mammary gland was measured using the Living Image Software (PerkinElmer, Waltham, MA, USA) (Figure 3a). Data were expressed as average radiance (p/s/cm^2/sr).

Ex vivo imaging was performed on a total number of 48 experimental mice. Twenty-four subjects in total, two for each surgical x dietary treatment group were intraperitoneally injected with 12.5 mg/kg of a well-known oxidant: Sodium (meta) arsenite (ASN, S7400 Sigma Aldrich, St. Louis, MO 63103, USA) (Oikawa et al., 2012) to cause high oxidative stress. Vehicle consisted of an intraperitoneal injection of an equivalent amount of saline vehicle in further two ARE-*luc2* mice for each surgical x dietary treatment group. Six hours after ASN or saline injection, mice were sacrificed through decapitation and the stomach, the liver and the adipose tissue were rapidly extracted. A five-minutes imaging session for each organ was then

performed immediately after each dissection. Photon emission was quantified with the Living Image Software (PerkinElmer, Waltham, MA, USA).

Since high dietary fat can be significant contributor to the load of free radicals in body, and since the fish oil is more prone to oxidation than lard for its elevated levels of n-3 PUFA (Awada et al., 2012), we also tested the stability of our LF and HF diets. With this purpose, we decided to maintain the same feed in the feeder until visible oxidation (11 days) before to change it with the fresh diet and we performed the *in vivo* bioluminescent imaging as previously described.

3.3.3 Statistical analysis

Body weight, BW gain and FI were analysed by a MIXED procedure for repeated measurements in SAS 9.4 (SAS Inst., Inc., NC, USA) accounting for the mouse as the experimental unit. Statistical model included the effect of the diet (lard or fish oil), diet inclusion rate of fat (7.5% or 20%), time, and their interactions.

In vivo and *ex vivo* photon emission quantification was analysed by ANOVA followed by Bonferroni's test for multiple comparisons by Graph Pad 6.1. (GraphPad Software Inc., La Jolla, CA).

3.4 Results

3.4.1 Performance Results

No difference in BW was detected between all experimental groups at the beginning of the trial. Average BW overall the trial period was higher in MALE (27.42g) than ovariectomized female (OVIX) (25.40g; $P<0.05$) and intact female (SHAM) (24.11g; $P<0.01$), while no differences were detected between SHAM and OVIX. Both high fat diets supplementation (HL=4.04g; HF= 3.75g) increased BW gain ($P<0.01$), when compared to low fat diets (LL=2.16g; LF=1.23g), but no differences in FI were observed between the four experimental dietary groups (HF=2.61g/d; HL=2.53g/d; LL=2.65g/; LF=2.76g/d; $P>0.05$).

3.4.2 In vivo, long-time evaluation of the effect of E2 and PUFA-diet on Nrf2 activity

In the present trial, using the ARE-*luc2* reporter mouse we observed patterns of expression of Nrf2 target genes *in vivo* similar to those observed by Rizzi et al. (2017a; 2018) who described that photon emission coincided with the location of Nrf2 mRNA expression, (Figure 3b).

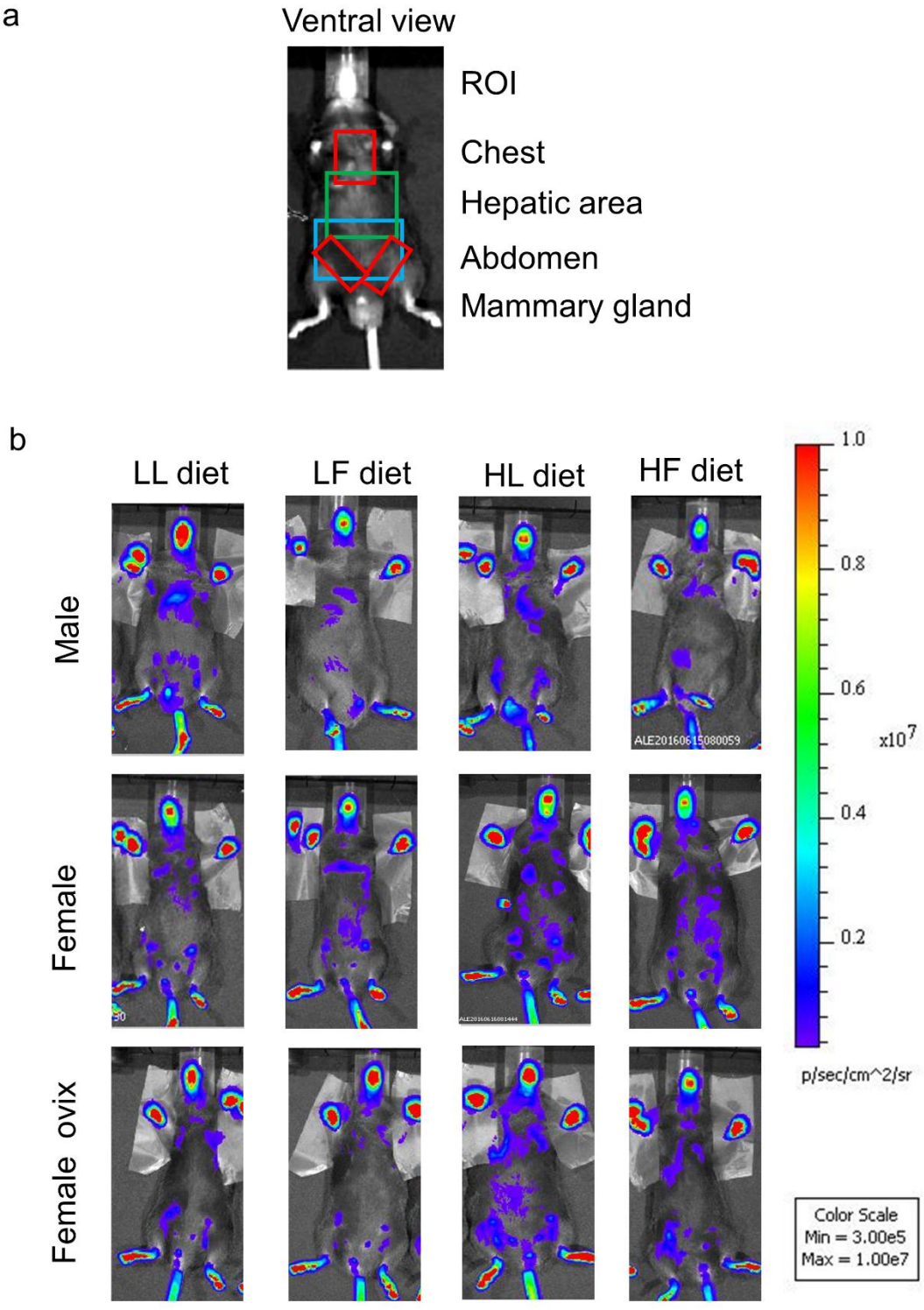

Figure 3: Representation of regions of interest (ROIs) considered for *in vivo* quantification of photon emission: chest, hepatic area, abdomen and mammary gland (a). *In vivo* bioluminescent imaging of a single representative mouse for each experimental group indicates the pattern of luciferase expression. Pseudocolor images of each individual mouse were obtained 15 min after injection of 80mg/kg luciferin with 5 min of exposition time (b).

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Concerning photon emission over 10 weeks (area under the curve), we detected significant differences in the abdominal area, in the mammary gland and in the chest and hepatic region. The results of our experiments showed that photon emission in the abdomen area (Figure 4) was increased in MALE and SHAM compared to OVIX in all four diets, suggesting a significant effect of E_2 in Nrf2 activity.

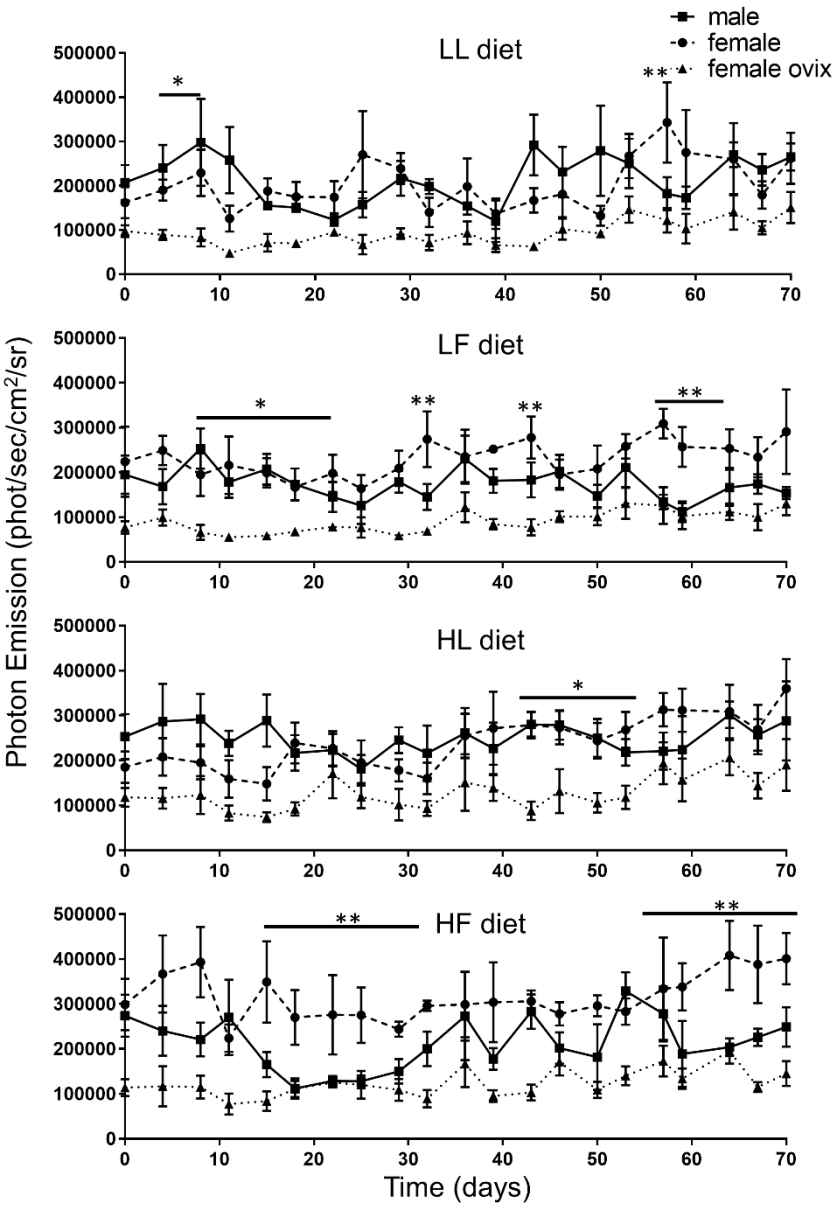

Figure 4: *In vivo* bioluminescent analysis of Nrf2 activity in abdomen of ARE-luc2 mice. Quantification of photon emission from abdomen of ARE-luc2 mice at the indicated time points fed with four different diets (LL= low lard diet; LF=Low fish oil diet; HL= high lard diet; HF= high fish oil diet) Each point corresponds to mean ± SEM (n = 4), expressed as photon emission (p/s/cm2/sr). * P < 0.05 male and SHAM vs OVIX; ** P < 0.05 SHAM vs OVIX calculated with ANOVA followed Bonferroni test.

In the abdominal region, photon emission over 10 weeks (area under curve) showed a low Nrf2 activity in OVIX female irrespective of diet. A comparable result was observed in the mammary gland area (Figure 5), OVIX being lower then SHAM, independent of diet. The photon emission from the hepatic area and chest (Figure 6) was lower ($p < 0.05$) in OVIX compared to MALE and SHAM in mice fed LL and HL diets only and not in LF and HF, showing the different influence of the type of dietary fat depending on E_2 status.

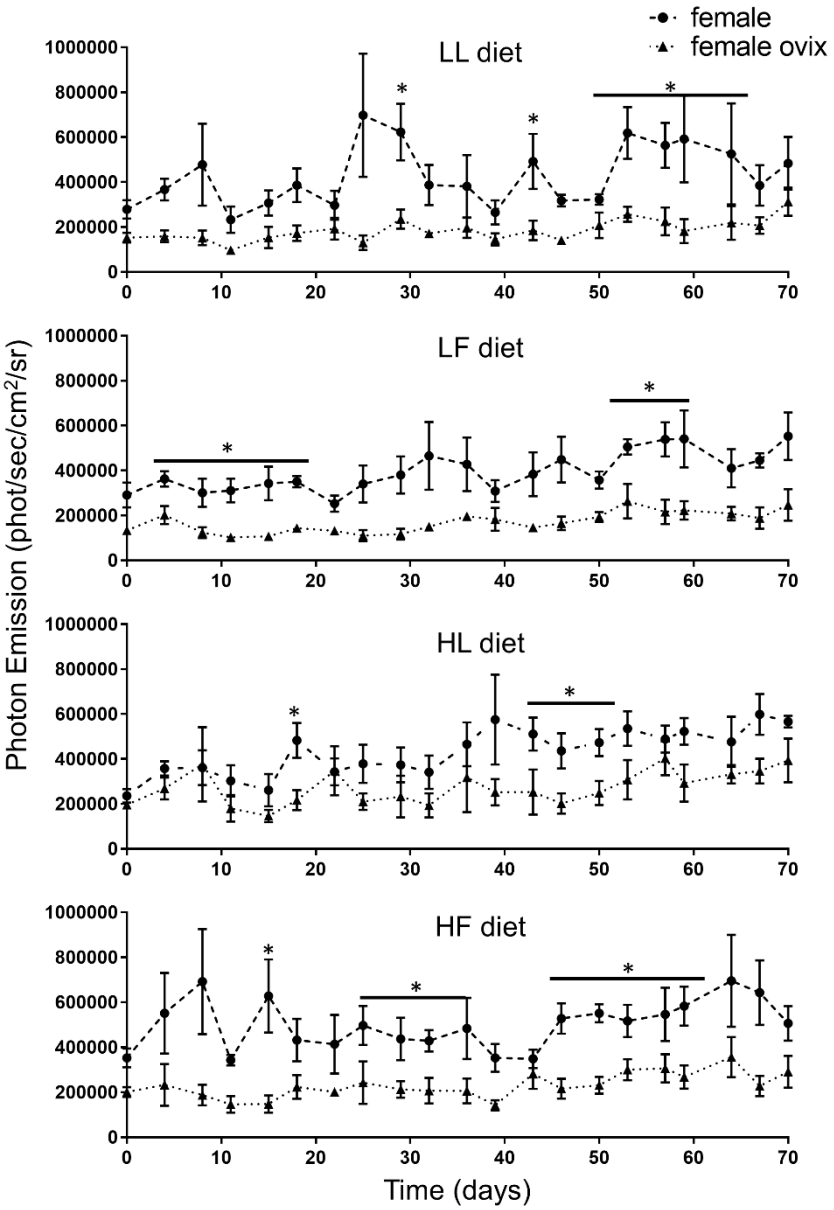

Figure 5: Quantification of photon emissions from mammary gland of ARE-luc2 mice at the indicated time points fed with four different diets (LL= low lard diet; LF=Low fish oil diet; HL= high lard diet; HF=high fish oil diet). Each point corresponds to mean ± SEM (n = 4), expressed as photon emission (p/s/cm^2/sr). * P < 0.05 SHAM vs OVIX calculated with ANOVA followed Bonferroni test.

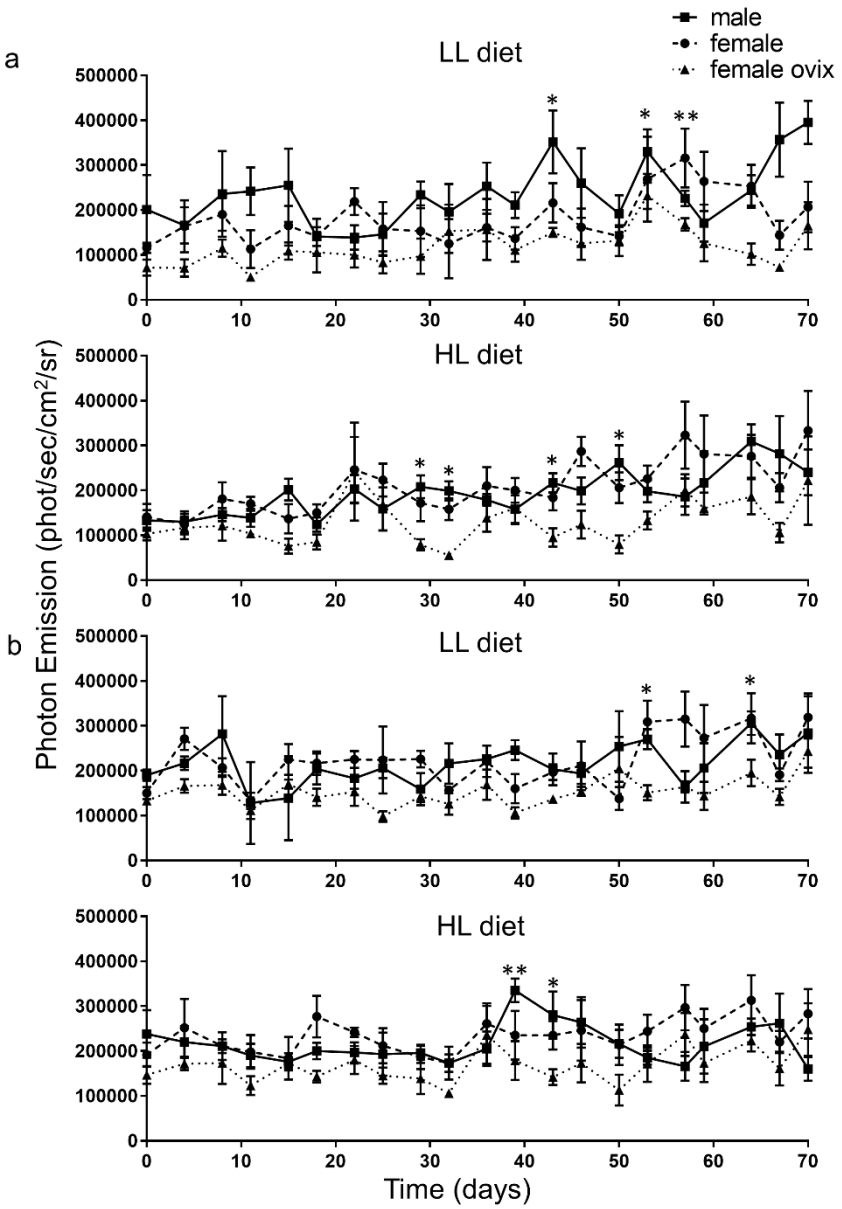

Figure 6: Quantification of photon emissions from hepatic area (a) and from chest (b) of ARE-luc2 mice at the indicated time points fed with two diets containing lard (LL= low lard diet; HL= high lard diet). Each point corresponds to mean ± SEM (n = 4), expressed as photon emission (p/s/cm2/sr). (a) LL diet * P < 0.05 male vs OVIX; ** P < 0.05 SHAM vs OVIX; HL diet ** P < 0.05 SHAM and male vs OVIX calculated with ANOVA followed Bonferroni test. (b) LL diet * P < 0.05 SHAM and male vs OVIX; HL diet * P < 0.05 SHAM and male vs OVIX, ** P < 0.05 male vs OVIX calculated with ANOVA followed Bonferroni test

Moreover, it was demonstrated that the different diets, with 7.5% inclusion of lard or fish oil and with 20% inclusion of lard of fish oil, had no effects on the Nrf2 activity alone.

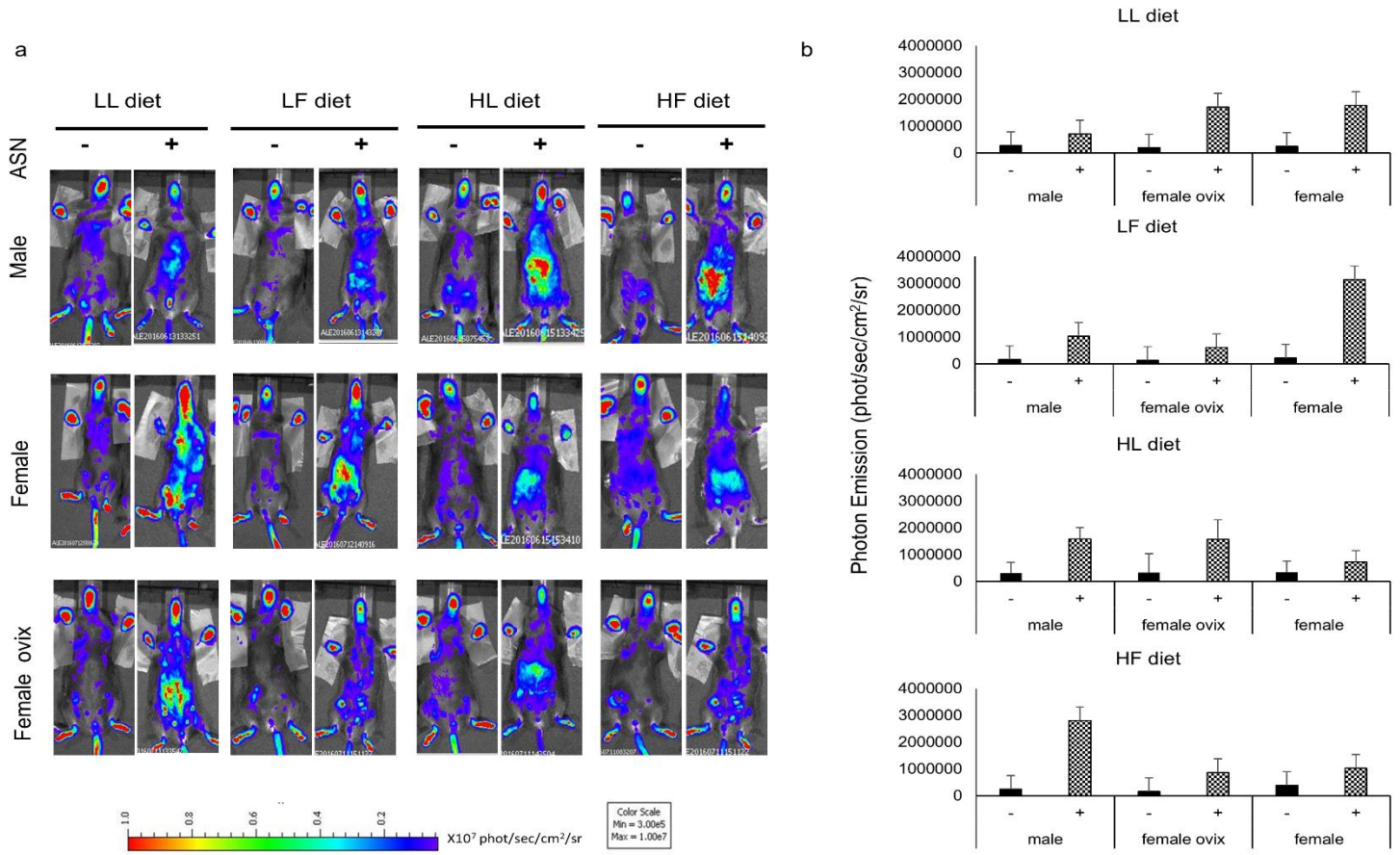

Figure 7: In vivo bioluminescent imaging of representative ARE-luc2 mice indicates the pattern of luciferase expression before (-) and 6 hours after (+) injection of 12.5mg/kg ASN. Pseudocolor images of each individual mouse were obtained 15 min after the intraperitoneal injection of 80 mg/kg of luciferin with 5 minutes exposition time (a). Quantification of photon emission from abdomen before (-) and 6h after (+) injection of 12.5mg/kg ASN in the four different dietary treatments (LL= low lard diet; LF=Low fish oil diet; HL= high lard diet; HF=high fish oil diet). Each bar corresponds to mean ± SEM expressed as photon emission (p/s/cm2/sr) (b).

3.4.3 In vivo and ex vivo evaluation of E2 and PUFA-diet effect on Nrf2 activity after a strong oxidative stress

The *in vivo* imaging results (Figure 7a) demonstrated that expression of the reporter was appropriately regulated in the different body areas and well corresponded with observations on the toxic effects of ASN, where major effects were observed in gastro-intestinal apparatus (Tchounwou et al., 2012). The quantification of photon emission from abdomen area (Figure 7b) showed that ASN increased the Nrf2 activity in all experimental groups, independently from E_2 content and diet. This result was confirmed by *ex vivo* analysis (Figure 8) where the organs showed a great increase of *Luc2* respect to vehicle.

The presence or absence of E_2 and the different content of PUFA or SFA seem to do not have any influence in the Nrf2 pathway modulation in the presence of a strong oxidative stress as acute 12.5 mg/kg ASN treatment.

3.4.4 In vivo evaluation of E2 and PUFA-diet effect on Nrf2 activity after a mild oxidative stress

Bioluminescent imaging performed after the consumption of oxidized feed revealed an increase of Nrf2 activity that was reversible by removing the old diet and suppling the fresh LF and HF diet. The quantification of photon emission from abdomen area demonstrated that lipid peroxidation derived products triggered the anti-oxidative answer, potentially with consequent damage for the animals. The main increase of Nrf2 signalling was recorded in the male and SHAM female animals, while OVIX female showed a very low anti-oxidative response (Figure 9).

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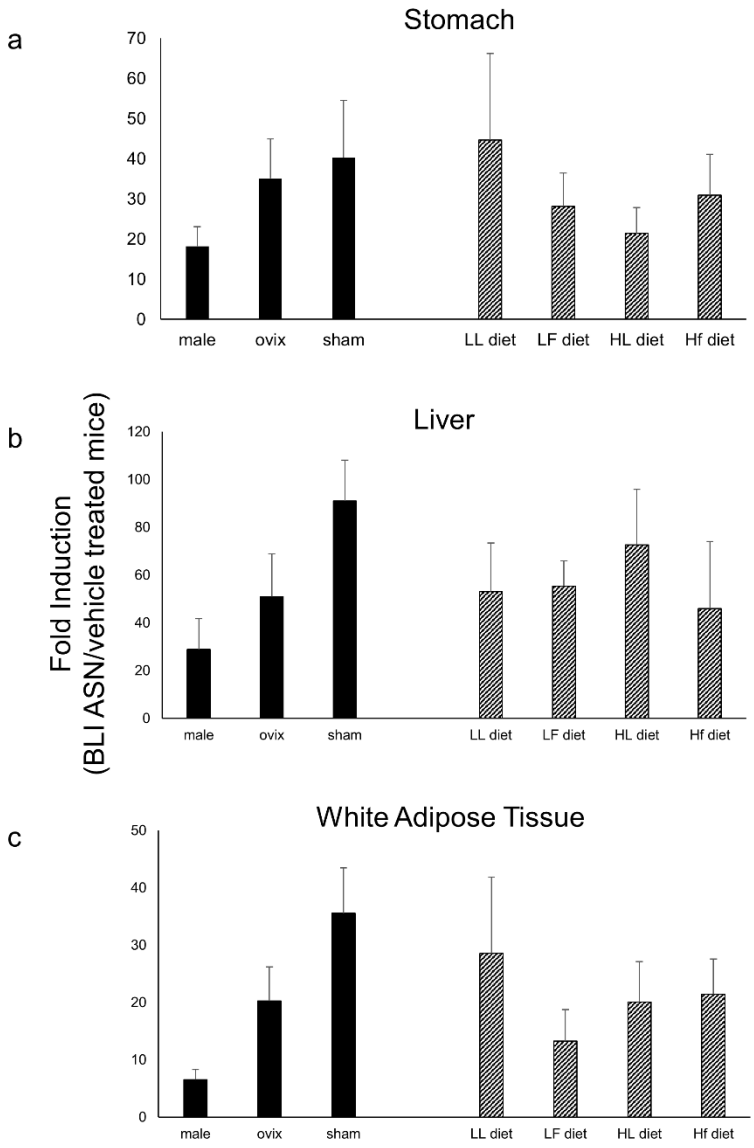
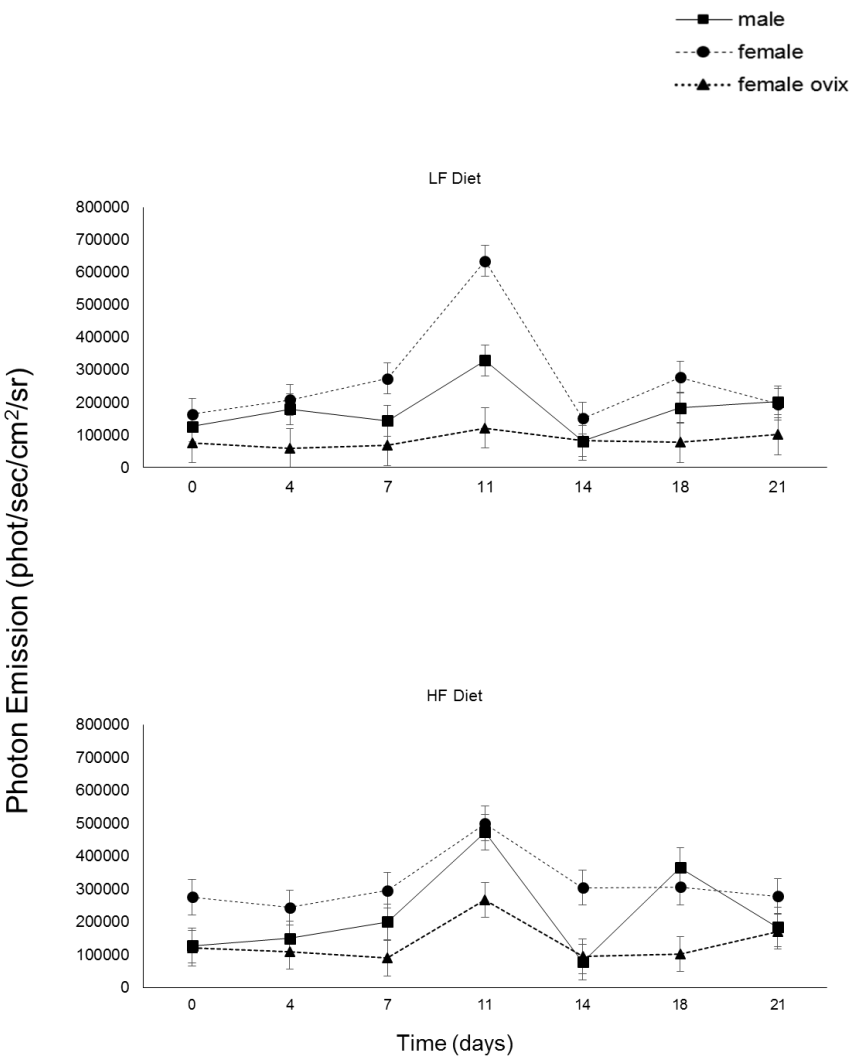


Figure 8: Quantification of photon emission measured ex vivo in stomach (a), liver (b) and white adipose tissue (c) after ASN injection (12mg/kg). Each bar corresponds to ratio between BLI measured from ASN treated mice and BLI measured from vehicle treated mice.



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1228 **Figure 9:** Quantification of photon emissions in abdomen at the indicated time points in the two
1229 dietary treatments containing oxidized fish oil (LL= low fish oil diet; HF= high fish oil diet). Each
1230 point corresponds to mean ± SEM, expressed as photon emission (p/s/cm^2/sr).

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3.5 Discussions and Conclusions

The aim of the present study was to determine the influence of the degree of saturation of dietary fatty acid on oxidative stress and the effect of estrogen on that process. We evaluated the oxidative stress response in mice with different E_2 status fed with different fatty acid sources by measuring the regulation of the Nuclear factor-E2 related factor 2 (Nrf2) pathway. Since Nrf2 plays an important role in oxidative stress response, the upregulation of Nrf2 pathways is considered a positive factor.

Our results showed that the type of dietary fatty acids alone did not influence Nrf2 expression within each group (OVIX, SHAM and MALE). Sodium arsenate (ASN) treatment strongly increased the Nrf2 activity independently of group and therefore estrogen and the supplied diet. Concerning the role of E_2, according to Yu et al. (2012), the results obtained in this in vivo longitudinal study confirmed the role of E2 increasing Nrf2 pathway in physiological condition. Comparison between groups showed that SHAM and MALE had a higher expression of Nrf2 than OVIX in all body regions. In addition, OVIX has a further decreased in Nrf2 activation in the hepatic and chest areas, compared with SHAM and MALE, but only if fed SFA (irrespective of the amount). This phenomenon is observed especially in the hepatic area probably because the liver is the main organ involved in lipid metabolism, which includes synthesis, desaturation, elongation and oxidation of fatty acids (Wang et al., 2006).

We conclude that the presence of high content of n-3 PUFA (HF diet) was not sufficient to trigger a protective effect against oxidative stress. This was unexpected because of the purported beneficial properties of PUFA (Fedor and Kelley, 2009; Carpentier et al., 2006). The beneficial effect of E_2 on immune system is well documented in the literature and this is supported by our findings. We confirmed these data *in vivo*, in a longitudinal study and in physiological condition by using the recent developed bioluminescent reporter mouse ARE-*luc2* that was used, for the first time, for a nutritional study. Finally, with our results on the hepatic and chest regions, we found that the type of dietary fatty acids can influence, at least in part, the oxidative stress response in mice with low E_2 level, further reducing the Nrf2 activation.

These outcomes show the importance to increase the knowledge on the relationship between the dietary fatty acid profile, the total amount of fat provided with the diet and their effect on oestrus cycle, characterized by E_2 presence. The possibility to follow in time, in entire body, *in vivo* and with a significant reduction of animal stress, make the technology of reporter

mice very useful for nutritional studies, maybe also for other biological events that are object of nutraceutical investigation as inflammation (Rizzi et al., 2017b).

Finally, another secondary result originating from our study was the demonstration of major sensitivity to oxidation of fish oil diet compared to lard diet. We measured the anti-oxidative stress answer, in term of Nrf2 activity, in ARE-*luc2* mice fed with evidently oxidized feed, and this effect was reversible by removing the old fish diet. We did not observe any Nrf2 activity in the mice fed with old lard diet, we conclude that the fish oil diet is more sensitive to lipid peroxidation due the presence of n-3 PUFA. Moreover, the main increase of Nrf2 signalling was recorded in the male and SHAM female animals, while OVIX female showed a very low anti-oxidative answer confirming the protective anti-oxidative role of E_2.

The observation of no effects of the degree of unsaturation of diets on oxidative status, can be useful also for livestock species, considering that diets fed to these animals usually contain a lower level of added lipids.

It is well known in women, that during menopause the level of endogenous estrogen decreases and the endogenous androgens increase. This may put women at risk for dietary fats intake during this critical phase. The present results may have bearing on dietary advice for the post menopause phase in women.

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Chapter 4

Melon pulp concentrate in combination with yeast did not improve oxidative and anti-inflammatory response in post-weaning piglets after an LPS challenge

4.1 Abstract

During the weaning period, piglets are subjected to remarkable stress that contributes to the onset of pro-inflammatory and pro-oxidative status. As consequence, during the post weaning phase transient anorexia, impaired growth, and up regulation of genes encoding inflammatory cytokines and proteins involved in oxidative stress can occur. The aim of the study was to test the effect of a feed additive containing melon pulp and yeast on antioxidant status, immune and inflammatory response in challenged weanling piglets. Twenty-four female piglets weaned at 24 days of age were divided in two homogeneous experimental groups of twelve animals each in a randomised block design and fed a basal diet (C, n.12) or a basal diet plus experimental premix (T, n.12). The trial considered a pre-challenge phase (from 1 to 19 days trial), and challenge phase (from 20 to 27 days on trial). The challenge was performed with repeated increasing intramuscular injections of LPS to mimic chronic inflammation, every two days starting from the 20th day of the experiment. Individual growth performance, including live body weight and average daily gain were recorded weekly until the end of the experiment. Feed intake was recorded daily per pen and feed conversion rate calculated. Antioxidant status was evaluated by TAOC, KRL test on blood and red blood cell, protein carbonyls levels, Cu/Zn ratio, 8-isoprostane. In addition, inflammatory response was evaluated by haptoglobin. Our results showed that dietary treatment did not affect growth performance. Moreover, the antioxidant supplementation did not affect immune response, but our results pointed out that the markers react differently to inflammatory stimuli. Cu/Zn ratio is useful as immediate markers, while 8-isoprostanes and protein carbonyls are more tardive.

4.2 Introduction

Pigs can suffer of chronic, subclinical or mild clinical diseases that imply a chronic impact of immune system stimulation (Bontempo et al., 2014; Rakhshandeh and De Lange, 2012; Di Giancamillo et al., 2010; Savoini et al., 2002). During the weaning period, piglets are subjected to remarkable stress that contribute to the onset of pro-inflammatory and pro-oxidative status (Sauerwein et al., 2005; Degroote et al., 2015). As consequence, during the post weaning period transient anorexia, impaired growth, and up regulation of genes encoding inflammatory cytokines (Piè et al., 2004) and proteins involved in oxidative stress (Wang et al., 2008) can occur.

European Union has banned the indiscriminate use of antibiotics in livestock diets (European Community Regulation no. 1831/2003) and has encouraged the investigation of alternative methods to antibiotics.

The primary function of Selenium has always been associated with Vitamin E in the role of antioxidant. Vitamin E and Selenium are involved in the protection of the phospholipidic layer of the membrane (Patel and Edwards, 1988; Flohè et al., 1973) and their deficiency is linked to structural damage of the membrane itself and also of the other cellular components, including DNA (Rao et al., 2001)

Selenium binds glutathion forming the glutathion peroxidase (GSH-PX) enzyme. This enzyme inactivates hydrogen peroxide trough a redox reaction (Brzezinska-Slebodzinska et al., 1994):

$$ROOH + 2GSH \rightarrow ROH + H_2O + GSSG$$

The bioavailability of selenium is determined by its forms and sources (Jlali et al., 2014) and some studies established that selenized yeast is one of the most suitable source for animal nutrition purpose (Wang and Xu, 2008; Calamari et al., 2010).

The supplementation of the diets with *S. cervisiae* spp in pig challenged with LPS has been proven useful to decrease the inflammatory response and reduce mortality (Collier et al., 2011); in addition, a beneficial effect on porcine epithelial cell lines through the reduction of inflammatory genes expression after ETEC (Enterotoxigenic *Escherichia coli)* infection has been described (Trckova et al., 2014).

Melon pulp concentrate (MPC) is a feed supplement that contains a primary antioxidant, superoxide dismutase (SOD), naturally present in melon (Vouldoukis et al., 2004). SOD is one of the main antioxidant enzymes of living cells and organisms, largely studied and used as a

treatment for different diseases in humans and rats (Corvo et al., 2002). High levels of dietary SOD demonstrated good antioxidant and anti-inflammatory properties in humans by inducing macrophages to produce IL-10 (Vouldoukis et al., 2004). In this view SOD is expected to lower the incidence of inflammatory disorders (Yasui and Baba, 2006) strengthening the health status and immunity of animals and, thus, contributing to improve the overall performance of pigs. Efficacy of melon concentrate is also related to the improvement of the barrier preventing the passage of potentially harmful microorganisms or toxic substances to the portal circulation (Petrof et al., 2004).

The aim of this study was to test a feed additive containing selenized yeast, melon pulp concentrate and inactive yeast from the species *Saccaromices cerevisiae*.

Selenized yeast is an additive already authorized by EU, melon pulp concentrate (MPC) and inactive yeast from the species *S. cerevisiae* do not require EU registration as they are listed in the catalogue of feed materials (EU 68/2013).

4.3 Materials and Methods

4.3.1 Experimental animals, diets and management

The experimental trial was validated by the Ethical Committee of the University of Milan and approved by the Italian Ministry of Research. The experiment was performed at the Animal Production Research and Teaching Centre of the Faculty of Veterinary Medicine of the University of Milan in Lodi.

A total number of twenty-four female piglets (Topigs 40 x Topdelta; Topigsdelta = Topigstalent x Topigstempo) weaned at 24 days of age coming from the same herd and vaccinated at two days of age for *Mycoplasma hyopneumoniae* were used for the trial.

The selected animals did not show any signs of disease and had an average initial body weight of 7.01 kg ± 0.36 kg. Piglets were divided in two homogeneous experimental groups of twelve animals each in a randomised block design and were allocated in the post-weaning room, in individual cage (0.47m^2) on slatted floor.

Each pen was equipped with one standard nursery pig bite-style nipple drinker and a self-feeder to allow *ad libitum* access to water and feed. Room temperature and ventilation were electronically controlled over a 24-h period. Starting room temperature was 28°C with a

ventilation of 10 m^3 /h per piglet and was decreased by 1°C/week until 25°C at the end of the trial.

In order to counteract potential infections derived from the previous herd, a prophylaxis procedure with amoxicillin and clavulanic acid (7+1.75 mg/kg/die) was provided from the first day to 5 days of trial.

Each experimental group was composed by 12 pens housing one animal per pen, thus 12 replicates per group were used.

Animals were fed two different meal-form diets (Table 3):

- Control group (C): basal diet + challenge with LPS
- Treatment group (T): basal diet + antioxidant mix containing selenized yeast melon pulp concentrate and inactive yeast from the species *Saccharomyces cerevisiae* + challenge with LPS

All diets were iso-nutritive and calculated to meet or exceed the nutrient requirements recommended by the National Research Council in 2012 (NRC, 2012).

Treatments lasted 27d starting on day 24 of age until day 51 of age of piglets. The trial considered a pre-challenge phase (from 1 to 19 days trial), and challenge phase (from 20 to 27 days on trial).

The challenge was performed with repeated increasing intramuscular injections of LPS from Escherichia coli (serotype 055:B5; cat. no. L2880; Sigma-Aldrich Canada Ltd, Oakville, ON, Canada) to mimic chronic inflammation, every two days starting from the 20th day of the experiment (20, 22, 24, 26 days). Initial LPS dosage of 60 μg/kg of body weight was increased by 12% at each subsequent injection to reduce endotoxin tolerance (Rakhshandeh and de Lange, 2012). At days 20, 22, 24 and 26 body heat temperature was recorded twice, the first time in correspondence to feed administration (basal temperature) and the second one 2 hours after LPS injection.

Table 3: Composition and expected chemical analysis of experimental diets

	Groups	
Composition (kg/100 kg as fed)	**C**	**T**
Barley meal	24.93	24.93
Flaked Barley	16.83	16.83
Wheat meal	14.96	14.96
Flaked Corn	11.78	11.78
Soycomil	8.97	8.97
Sweet Whey	7.48	7.48
Soybean meal (48%)	3.99	3.99
Dextrose	3.49	3.49
Soybean Oil	2.49	2.49
Coconut Oil	1.99	1.99
Calcium diphosphate	0.70	0.70
L-Lysine	0.60	0.60
Calcium carbonate	0.50	0.50
Dl-methionine	0.30	0.30
L-treonine	0.25	0.25
Vitamin premix	0.25	0.25
NaCl	0.20	0.20
Experimental premix C	0.30	---
Experimental premix T	---	0.30
Total	100.00	100.00
chemical analysis (% DM basis)		
Dry Matter	91.40	91.42
Crude Protein	18.95	18.34
Ether Extract	5.91	5.64
Crude Fibre	7.96	7.49
Ashes	5.09	4.89
ME (kcal/Kg)	3,546.00	3,579.00
Met	0.58	0.605
Lys	1.40	1.34
Treo	1.02	1.01
Trp (%)	0.23	0.22

4.3.2 Data and samples collection

Individual growth performance, including live body weight (BW) and average daily gain (ADG), were recorded weekly until the end of the experiment. Feed intake (FI) was recorded daily per pen and feed conversion rate (FCR) calculated.

BW was recorded at days 0, 7, 14, 20, 22, 26, 27 with an electronic scale (Ohaus ES100L) and the values were used to calculate the average daily gain.

Feed Conversion Rate (FCR) and *gain:feed ratio* (G:F) were calculated taking in account two different trial periods (pre and post-challenge).

Morbidity, medications and mortality were recorded.

Blood samples were collected at days 0, 20, 22, 24, 26, 27. Each blood sample under the challenge period was collected prior the LPS injection. For sampling procedures, two different 10 ml vacutainer tubes were used to yield serum from the cranial vena cava from each piglet:

- K_2EDTA tubes (366643, BD Vacutainer®, Italia) for KRL test
- Tubes containing clot activator (367820, BD Vacutainer®, Italia) for Total Antioxidant Capacity (TAOC), protein carbonyls levels, Cu/Zn ratio, 8-isoprostane and haptoglobin.

Aliquots from K_2EDTA tubes used for the KRL test were immediately stored at 4°C, processed within 3 h from sampling, and analysed in the next 24 h after collection.

The leftovers were centrifuged 5 minutes at 1400 rpm and plasma collected and stored at -80°C for further analyses.

Blood samples with the clot activator were kept 45 minutes at room temperature and then centrifuged 5 minutes at 1400 rpm. The collected serum was then stored at -80°C for further analyses.

4.3.3 Chemical analyses

Total Antioxidant Capacity, 8-isoprostanes and protein carbonyls levels were measured using commercial kits according to the recommendations of the manufacturer (Cat. No. 709001; Cat. No. 516351 and Cat. No. 10005020, respectively; Cayman Chemical).

KRL test was performed as previously described by Ahasan et al., (2018) only on samples collected at days 20, 26 and 27. Results were expressed as the minimum time required to reach 50% of maximal haemolysis. Half-haemolysis time for total blood cells (HT_{50}WB) and

for red blood cells (HT_{50}RBC) refers to the whole blood and the red cell resistance to free-radical attack, respectively.

The serological levels of copper and zinc were determined through spectrophotometry and the ratio of the values analysed

The serum concentrations of haptoglobin were determined by colorimetric assay (Tridelta Phaserange serum haptoglobin assay, Cat. No. TP-801) and expressed on the basis of a standard curve (Cooke and Arthington, 2013).

4.3.4 Statistical analysis

Growth performance and blood parameters were analysed using a MIXED procedure for repeated measurements with the piglet as the experimental unit. Dietary treatment, time and their interaction were set as fixed effects.

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.

4.4 Results

4.4.1 Performance

Performance were not affected by the dietary treatment, neither the interaction between time and treatment affected the values of BW (P=0.50), FI (P=0.22), ADG (P=0.44), FCR (P=0.40), G:F (P=0.62) among two experimental groups (Table 4).

Even if not significant, a huge decrease in the ADG (-45% C group; -31.3% T group) and FI (-36.3% C group; -57% T group) values occurred after the first LPS injection.

4.4.2 Oxidative status

The dietary treatment with the antioxidant compound did not affect the oxidative stress markers (Table 5).

Between days 20 and 22, the serum level of Zn decreased (P<0.01) and copper showed a tendency to increase (P=0.07), irrespective of dietary treatment. As consequence, in response to the first injection of LPS, the Cu/Zn ratio increased (P<0.0001).

Serum levels of protein carbonyl started to increase only after the second injection of LPS. Indeed, whereas no difference was recorded between days 20 and 22 (P=0.17), the carbonyl levels at day 22 differs from days 24 (P=0.04), 26 (P<0.001) and 27 (P=0.013).

Similarly, plasmatic levels of 8-isoprostane changed late during the challenge, indeed the variations were recorded after the second LPS injection (P=0.003). In contrast to protein carbonyl, 8-isoprostans had not a linear trend and a great variation was registered during the challenge phase.

1554 **Table 4:** Growth performance of post-weaning piglets during the trial period

Item	Day				P-value		
		C	T	SEM	treatment	time	Treatment xTime
BW (g)	0	6,969	7,041	516.08	0.65	<.0001	0.50
	7	7,915	7,875				
	14	10,491	10,168				
	20	13,181	12,657				
	22	13,314	12,655				
	24	13,957	13,535				
	26	14,461	14,091				
	27	14,906	14,652				
ADG (g/d)	0-7	135	119	53.72	0.74	<.0001	0.44
	7-14	368	328				
	14-20	448	415				
	20-22	67	-1				
	22-24	321	440				
	24-26	252	278				
	26-27	356	449				
FI (g/d)	0-7	216	212	40.37	0.78	<.0001	0.22
	7-14	507	445				
	14-20	722	668				
	20-22	460	400				
	22-24	441	530				
	24-26	556	558				
	26-27	641	661				
FCR	0-7	2.08	1.77	0.63	0.30	0.43	0.40
	7-14	1.38	1.38				
	14-20	1.63	1.63				
	20-22	1.12	2.96				
	22-24	1.04	1.37				
	24-26	1.66	3.07				
	26-27	2.39	1.97				
G:F	0-7	0.60	0.37	0.15	0.52	<.0001	0.62
	7-14	0.72	0.77				
	14-20	0.62	0.62				
	20-22	-0.04	-0.10				
	22-24	0.64	0.84				
	24-26	0.43	0.50				
	26-27	0.28	0.61				

Table 5: Oxidative stress biomarkers at the beginning of the trial and during the LPS challenge phase

Item	Treatment	Days							P-value		
		0	20	22	24	26	27	SEM	Treatment	Time	Treatment X Time
Cu (µmol/L)											
	C	33.77	23.46	27.78	26.51	26.10	26.01	1.59	0.50	<.0001	0.47
	T	32.15	23.98	24.06	27.17	24.97	24.72				
Zn (µmol/L)											
	C	13.08	12.03	8.77	10.99	9.88	9.73	0.78	0.61	<.0001	0.32
	T	11.55	11.93	8.23	9.99	10.66	9.40				
Cu/Zn ratio											
	C	2.68	2.02	3.27	2.51	2.72	2.88	0.23	0.88	<.0001	0.55
	T	2.94	2.15	3.11	2.79	2.55	2.77				
Protein Carbonyl (nmol/ml)											
	C	15.04	16.89	14.26	18.82	17.81	19.58	1.94	0.89	0.0003	0.14
	T	10.37	16.90	14.34	17.55	23.79	18.48				
8-isoprostane (pg/ml)											
	C	136.13	124.82	93.1273	145.4	84.0644	201.31	31.26	0.49	0.0009	0.77
	T	175.05	111.71	71.9121	200.62	106.29	193.5				

The dietary treatment did not affect TAOC level (P=0.86) as well as the time (P=0.37) (Figure 10).

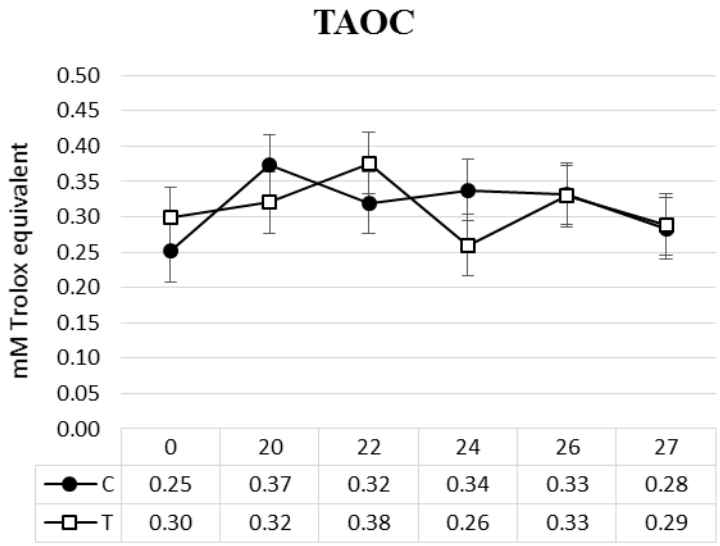

Figure 10: Average TAOC values (expressed as mM Trolox equivalent) in the two dietary treatment groups during the trial period

As previously stated, the antioxidant compound did not influence the oxidative stress markers and that is true even for the KRL test results.

KRL values on both, red blood cells and whole blood, markedly decreased the last day of the trial (Figures 11 and 12).

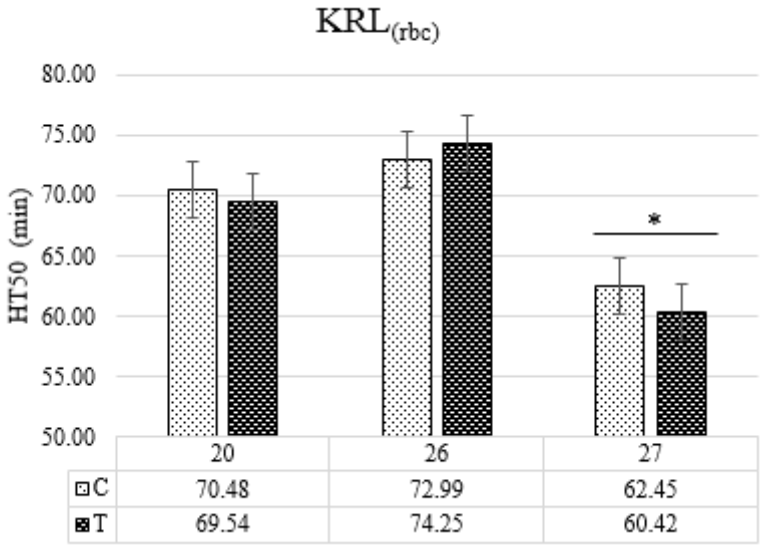

Figure 11: Average values of HT_{50} on red blood cells in the two experimental groups

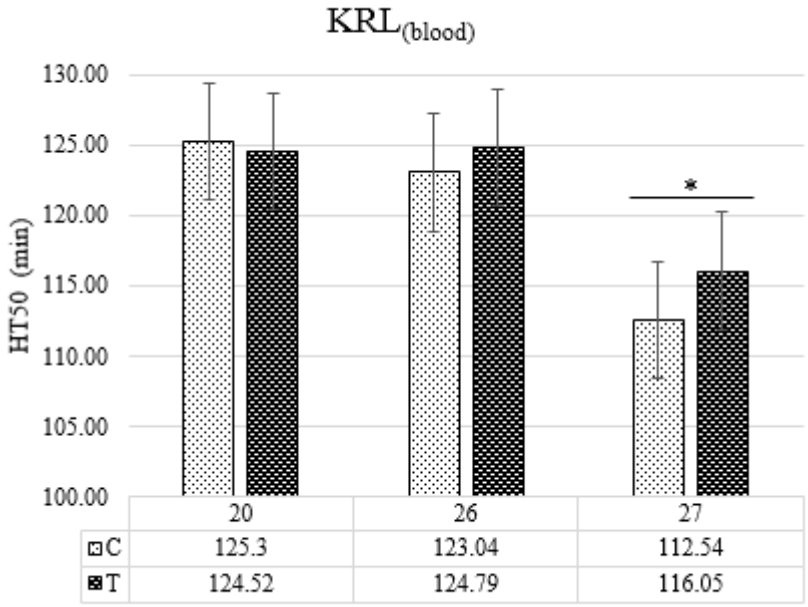

Figure 12: Average values of HT_{50} on whole blood in the two experimental groups

4.4.3 Inflammatory status

Considering the inflammatory status, day 20 was considered the control point, because of the stress the animals were subjected to at day 0 (weaning and transport to the faculty's facilities). The challenge effect of LPS was confirmed by their pyrogenic effect. Body temperature of the animals increased during the whole challenge period ($P<0.05$), irrespective of the dietary treatment (Figure 13).

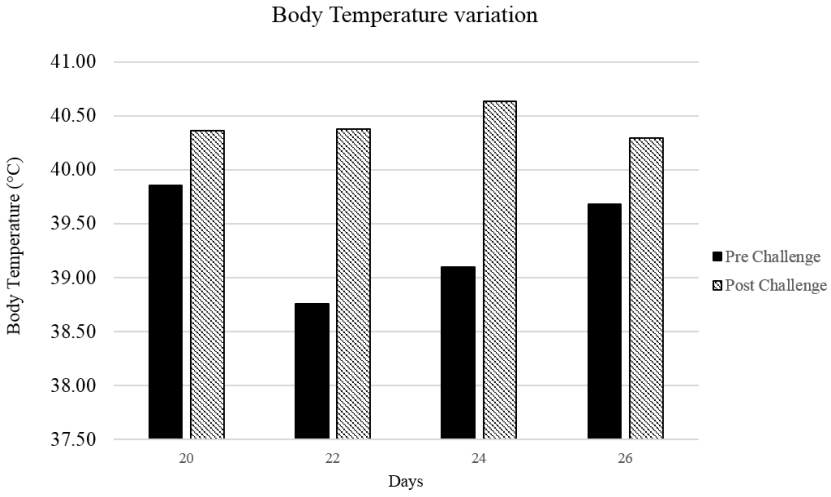

Figure 13: Body temperature variations during the pre and post challenge phase

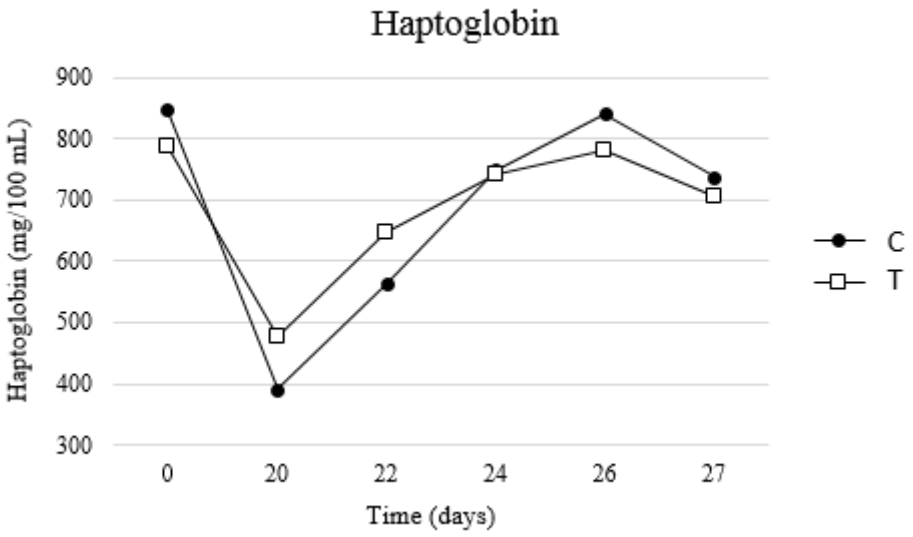

Figure 14: Comparison between Haptoglobin's trends during the whole trial period in the two experimental groups

Compared to day 20, inflammatory stimulus of LPS challenge gradually increased haptoglobin concentration in the serum of all experimental subject ($P<0.01$), irrespective of dietary treatment.

4.5 Discussions and Conclusions

A correct management of the weaning period is of main importance in pig production. This stressing phase is characterized by decreased feed intake and nutrient absorption, as well as a worsening in the immune response (Niekamp et al., 2007). As consequence, if not well managed, could compromise the long-term productivity of the animals. Inclusion of antioxidant compounds in the diets of piglets aims to prevent the oxidative and inflammatory events typical of the weaning phase (Zhu et al., 2012).

With this study, we wanted to test the effect of a feed additive containing selenized yeast, melon pulp and inactivated yeast on antioxidant status, immune and inflammatory response in challenged weanling piglets.

In contrast to Ahasan et al. (2018), but according with Làlles et al. (2011) dietary treatment of weaning piglets with an antioxidant mix containing among others, melon pulp concentrate did not improve the performances of the animals, either during the challenge period.

Furthermore, and still in contrast to Ahasan et al. (2018), the supplementation with melon pulp and yeast did not influence the antioxidant and inflammatory response at least for the tested parameters. The reason could be linked to a different dosage of the additives used in the diets or to a different mechanism of action of the melon pulp concentrate administrated in combination with the yeast and further investigation are needed.

The experimental protocol implemented in this study used an LPS challenge in order to induce oxidative stress status (Rakhshandeh and De Lange, 2012) in addition to the physiological stress induced by weaning.

Even if not successful for the feed additives administration, this study allowed us to better investigate the mechanism of action of the considered biomarkers and to improve the protocol for the next studies. The strong effect of LPS challenge on performances confirms the goodness of the experimental design. Growth curve is characterized by a decline after the first LPS injection. Minor influence of next inoculations could be due to an increased tolerance of piglets against the endotoxins (Deitch, 1998). The effect of challenge on body weight is sustained by the decrease of FI and ADG and by FCR increase. These outcomes are due to the anorexia induced by LPS, which compromises the general health status of the animal (Toepfer-Berg et al., 2004; Gòmez-Laguna et al., 2010; Che et al., 2011).

All the biomarkers considered for the oxidative stress evaluation were affected by the LPS challenge, but the velocity of the response was different.

Levels of Zinc, Copper and Cu/Zn ratio registered a variation after the first inoculation of LPS, whereas protein carbonyls and 8-isoprostans changed later during the infection period.
Our hypothesis is that precursors of protein carbonyls and 8-isoprostans need more exposition time to oxidative damage in order to start structural modification and for that reason, they may be considered good biomarkers for a chronical oxidative stress situation.
In contrast with what we observed about protein carbonyls and 8-isoprostans, the only relevant difference on KRL results was between days 26 and 27, i.e. the only measure obtained 24 hours after the LPS injection instead of 48h. This different variation in time of KRL test let us think about the possibility that the effect of LPS on this parameter shows up and finishes in a shorter lapse of time. As consequence, it might be a better test if applied on samples collected within 24h from LPS injection. Concerning the inflammatory status, temperatures measured after the challenge extensively exceed the reference value for weaning piglets (39.3±0.3 °C) (Dewey and Straw, 2006), confirming again the success of LPS to induce inflammatory response.

In conclusion, the antioxidant product added to the diet was not able to improve growth performances or to protect the experimental subjects from the damages induced by LPS. However, our results can be useful to enhance the quality of the experimental design and to bring new insight on the mechanism of action of some of the most used biomarkers in pigs.

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Chapter 5

Non-Invasive Intestinal Health Biomarkers: a new ELISA test for Pancreatitis Associated Protein Detection in Pig

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Study presented at EAAP 2018, Dubrovnik

5.1 Abstract

Nowadays, the effectiveness of new feed additives as alternatives to in feed antibiotics are under investigation and their effect on intestinal health need to be demonstrated. In this research it is essential to have intestinal health biomarkers, non -or minimally invasive, for the early detection of intestinal inflammation in pigs. Candidates are Myeloperoxidase (MPO), an enzyme that permits to quantify the number of inflammatory cells present in tissues and faeces, and Pancreatitis Associated Protein (PAP) a protein mainly produced in the small intestine with anti-inflammatory and bactericidal activity. Currently, no commercial ELISA kit for porcine PAP detection are available, so the main aim of this study was to develop and validate a new sandwich ELISA test for the quantification of PAP in pig faecal samples. The development phase consisted in the optimization of the protocol on the standard curve. We used polyclonal antibodies from serum from a rabbit previously immunized with a pure peptide containing the N-terminus of pig PAP. The validation of the test was then performed using faecal extraction samples derived from animals with known high or low MPO activity values, and samples with different faecal consistency. Moreover, we tested the temperature stability of PAP in faeces and we used Western Blot analyses to confirm our findings. Our results show a good relationship between PAP faecal concentrations and both, faecal consistency and MPO activity levels. PAP immunoreactivity remained stable after 24h of incubation at 37 °C, 4°C or room temperature. This study suggest that PAP is a very promising candidate as a non-invasive (faecal) biomarker for intestinal health. Furthermore, considering its apparent immune-stability, it is exceptionally suitable for field tests.

Keywords: Non-invasive biomarkers, Pancreatitis Associated Protein, Myeloperoxidase, ELISA, Intestinal Health, Immunoreactivity.

5.2 Introduction

Intestinal diseases markedly affect productivity and profitability of pig production all over the world; moreover, legal restrictions on antibiotics usage aggravate their incidence. Early detection of intestinal diseases in intensive pig farming is essential to avoid large economic losses; however, obvious clinical signs in pigs affected by intestinal disease can vary greatly and are dependent on the location, type, severity and duration of the disease process (McOrist and Corona-Barrera, 2015). *In vivo* determination of intestinal health in pig still represents a challenge. The available techniques, such as endoscopy, mucosal biopsies or stomata do not cover a large part of intestine and, most of all, are invasive and suitable exclusively under experimental conditions (Niewold, 2015). To be able to keep track of intestinal condition over time, different animals must be sacrificed, which further raises costs, while test results may not be representative (Niewold, 2015 b). In addition, the sacrifice of the animals make these techniques clearly not appropriate for large scale routine screening on farms.

The Gastrointestinal tract (GIT) is the largest site of interaction between pig and its environment and, for this reason, it is of main importance for growth and health.

GIT has a barrier function, comprised of epithelial cells, immune system and enteric nervous system (Moeser et al., 2017). Intestinal diseases lead to an increase in epithelium permeability and as consequence to inflammation processes that can result in morbidity, reduced productivity, mortality, and consequently lead to economic losses to pig farmers (Niewold, 2015; McOrist and Corona-Barrera, 2015).

In this view, there is a need for new biomarkers that allow early, *in vivo* and non-invasive determination of intestinal inflammation.

The Pancreatitis associated protein (PAP), also known as regenerating islet derived III alpha, is a C-type lectin secreted protein. It was thought that its production was mainly in the exocrine pancreas, but now it is clear that is produced also by the small intestine, most likely in Paneth cells .(Cash et al., 2006; Murphy and Weaver, 2017). In 2015, Soler et al., demonstrated that the protein known as Reg3α/PAP expressed in the intestinal mucosal of pigs is a different isoform, i.e. Reg3γ. They also demonstrated that it is expressed along the whole intestinal tract, but in particular seems to be highly expressed in pig jejunum during ETEC infections. Reg3γ is reported to be stored in secretory granules and released into the lumen of the small intestine. It has anti-inflammatory and anti-bacterial activity (Cash et al., 2006). Reg3γ protein is a 176 AA protein, with a signal- and a pro- peptide.

The porcine Reg3γ signal peptide consists of amino acids 1 to 27; it is removed before the secretion from the Paneth cells to produce the mature, but inactive form of the protein (Cash, 2006). In order to be activated and acquire its bactericidal functions, the mature protein undergoes to a further proteolytic cleavage in the intestinal lumen (Mukherjee et al., 2009; Murphy and Weaver 2017). Trypsin acts at level of residues A^{37} -R^{38}, removing the so called pro-peptide. In this way the carbohydrate binding domain is revealed, and the protein achieved its active form (Mukherjee et al., 2009; Mukherjee et al., 2014; Murphy and Weaver 2017).
Once active, the protein is able to bind to the bacterial membrane forming a hexameric pore, which directly kills bacteria. The bactericidal activity of PAP is reported to be specific against Gram-Positive species, as the lipopolysaccharide (LPS) layer of Gram-negative's membrane inhibits the pore-forming ability of PAP (Mukherjee et al., 2014; Murphy and Weaver, 2017).

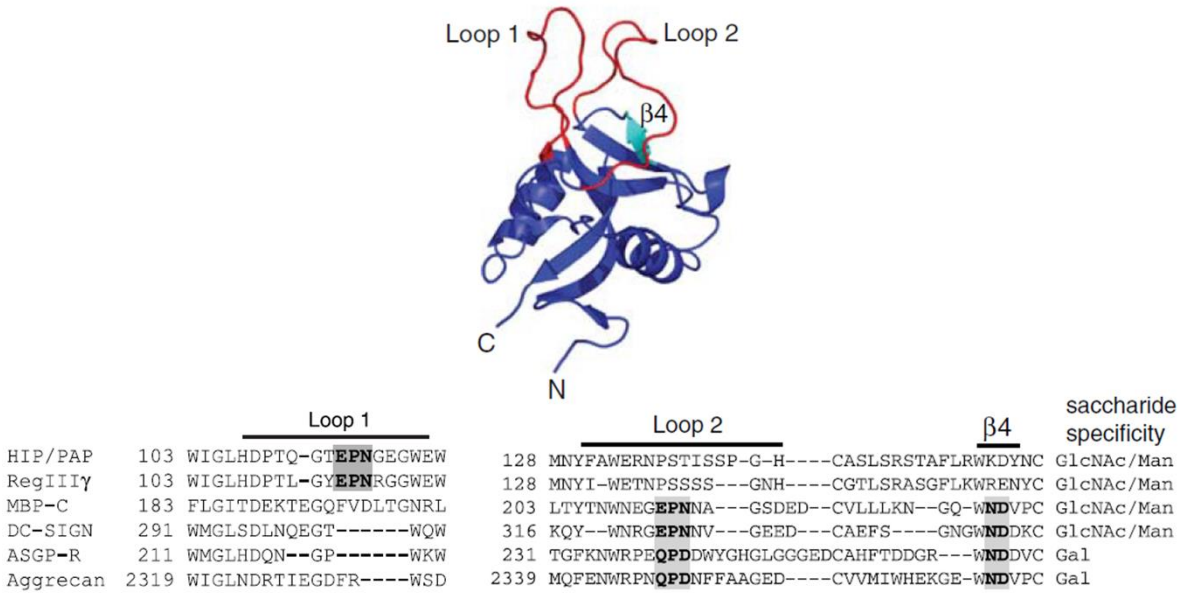


Figure 15: PAP structure and Loops sequences (modified by Lehotzky et al., 2010)

In contrast to most C-type lectins, Reg3 proteins bind the bacterial membranes through carbohydrates, but in a calcium-independent way. Lehotzky et al. in 2010 showed that HIP/PAP and Reg3γ has the EPN motif in Loop1 instead of Loop2 and that the ND motif in β4 strand is missing (Figure 15). These two motifs together are responsible for the Ca^{2+} dependent carbohydrate binding. According to their results, the shift to Loop 1 of EPN and the lack of ND is probably why Reg3 proteins do not need calcium to bind carbohydrates.
Due to its characteristics, Reg3γ seems to be an appropriate intestinal health biomarker: it originates from the gastrointestinal tract and could be obtained in a non-invasive way

investigating faecal sample. The N-terminus of porcine Reg3γ showed some characteristics that apparently impeded detection with anti-mouse or human antibodies (Soler et al., 2015) and that is why no commercial kit are available yet. The aim of this study was to develop and validate a new sandwich ELISA test for the detection of Reg3γ in porcine faecal samples.

5.3 Materials and Methods

5.3.1 Enzyme-linked immunosorbent assay (ELISA): development and validation

The development phase consisted in the optimization of the assay on the standard curve. We used polyclonal antibodies previously immunized from rabbit serum with a pure peptide containing the N-terminus of pig PAP.

We followed a standard ELISA protocol with different combinations and concentrations of catching and detection antibodies.

To build the standard curve, the synthetized peptide (sequence DSPADTPSARISCPKGSMAYASY) was used (Soler et al., 2015). The peptide is the N-terminus region of the whole pro-protein and contains the trypsin cleavage site (A^{37} -R^{38}) that is responsible for protein activation. The aforementioned peptide was previously identified as the most immunogenic part of the protein and was used to produce the rabbit polyclonal antibody (Proteogenix, Schiltigheim, France) (Soler et al., 2015). Part of the obtained antibodies was then biotinylated using a biotinylation kit, following the manufacturer's instructions (Cat. #21326 Thermo Fisher Scientific, Walthman, MA, USA).

For the determination of the optimal antibodies concentrations, a checker board experiment was performed. Catching antibody concentrations tested were 10 µg/mL, 5 µg/mL, 3.125 µg/mL and 0 µg/mL. Biotinylated antibody concentrations tested were 5 µg/mL, 2.5 µg/mL and 0 µg/mL. For each combination, serial dilutions of the pure peptide were analysed.

As detection system, streptavidin-HRP conjugated and ABTS were used. Absorbance measurements were performed using VICTOR3™ multi-label plate readers, after 90 minutes in dark incubation.

After the optimization of the protocol with the pure peptide, we analysed pig faecal samples with unknown RegIIIγ content. Two faecal samples, with high (350.09 million units per ml) or low (<78.85 million units per ml) level of MPO activity, were tested, as well as pooled samples with different faecal consistency, ranked using the following scale: 0 = solid; 1 = semi-solid; 2

= semi-liquid; and 3 = liquid. A sample is considered diarrhoeic with a score of 2 or 3 (Liu et al., 2010).

We assumed that high level of Myeloperoxidase and low faecal consistency corresponded to higher Reg3γ concentrations.

Samples with known MPO values were prepared as follow: 1g faecal sample was diluted with 1 mL of PBS and centrifuged 10 minutes at 3500 rpm; before supernatant's collection, a second centrifugation at 21000 g for 15 minutes was performed.

After testing other extraction methods (data not shown), samples characterized by different faecal consistency were extracted using HTAB buffer instead of PBS.

5.3.2 Temperature stability test

The extraction protocol used for the stability test was the same adopted for the samples with known values of MPO activity. The temperature stability test was performed keeping the faecal extracts at different temperatures (+4°C, room temperature and +37°C) for different times (4, 8, 24 hours) with or without addition of a protease inhibitor cocktail (PIC). As control we used faecal extracts (+/- PIC) immediately frozen at -20°C after the extraction procedure. The faecal extracts were then analysed following the ELISA protocol optimized during the development phase.

5.3.3 Statistical analysis

Statistical analysis and figures were generated using GraphPad Prism® v.7.04 software (GraphPad Software Inc., San Diego, CA, USA). For standard curve analysis, the linear regression and R-squared (R^2) values were calculated to evaluate the goodness of fit. With the same software, data from faecal samples were interpolated using a four-parameter logistic model. Results from stability test were analysed using SAS 9.4 (SAS Inst., Inc., NC, USA). A GLM procedure was used to analyse the effect of temperature and protease inhibitor cocktail, as well as their interaction. A MIXED procedure for repeated measurements was used to analyse the effect of incubation time and its interactions with all the other variables. Final Reg3γ concentration is expressed as equivalents peptide in μg/mL.

5.4 Results

5.4.1 Enzyme-linked immunosorbent assay (ELISA): development and validation

For the development phase of the sandwich ELISA test, we followed a standard ELISA sandwich protocol and we tested different concentrations of primary and secondary antibodies (10- 5- 3.125- 0 μg/mL and 5- 2.5- 0 μg/mL, respectively).

Considering the two different concentrations of biotinylated antibody (5 and 2.5 μg/mL), the optimal standard curve, yielding the highest absorbance values, was obtained by using a capture antibody concentration of 10 μg/ml (Figure 16a and 16b). Absorbance values were approximately zero when either no capture or no detection antibodies were loaded in the wells (Figure 16c).

The curves have a Goodness of Fit (R^2) respectively of 0.946 and 0.905, that means the optimal signal and response were reached when the assay is performed with a 10 μg/ml of primary antibody and 5 μg/ml of biotinylated antibody.

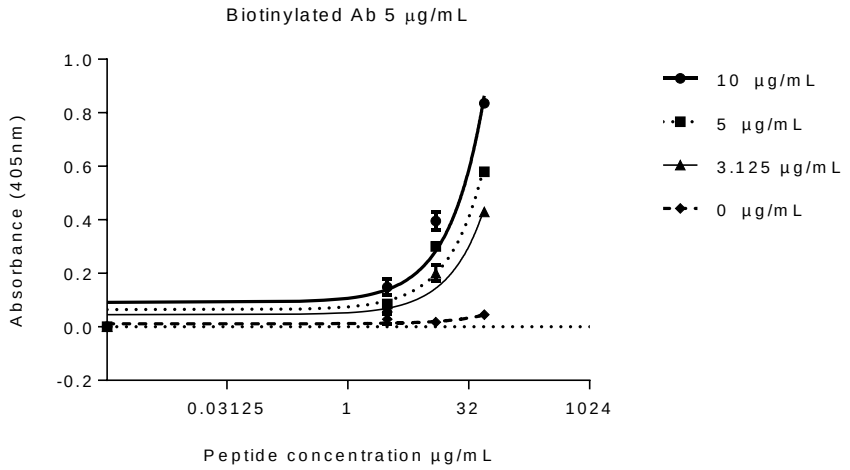
For the validation of the test, two faecal samples, with high (350.09 millU/ ml) and low (<78.85 millU/ ml) level of MPO activity, were tested, as well as pooled samples with different faecal consistency.

We assumed that high level of Myeloperoxidase and low faecal consistency corresponded to higher Reg3γ concentrations.

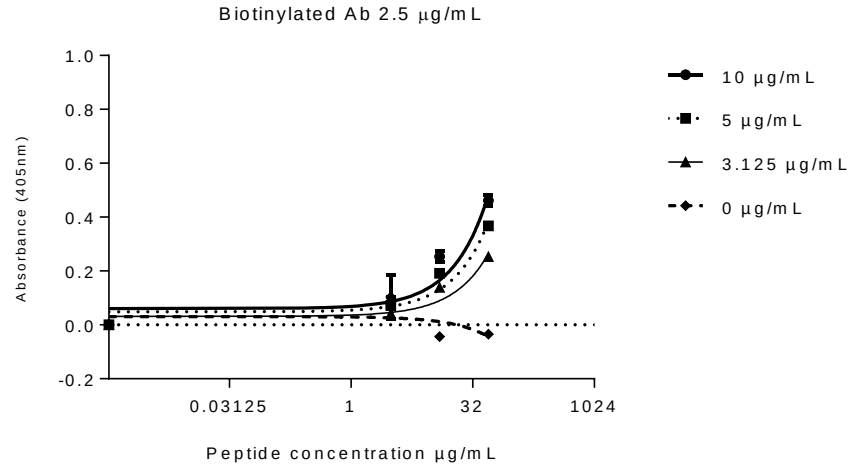
As shown in Table 6, a high value of MPO activity corresponded to a higher content of Reg3γ. The concentration of Reg3γ in sample 2 is almost five times higher than in sample 1, paralleling the values of the MPO activity.

In Table 7 the concentrations of Reg3 γ in faeces with different faecal scores are reported. As hypothesized, Reg3γ concentration is related to faecal consistency.

a



b



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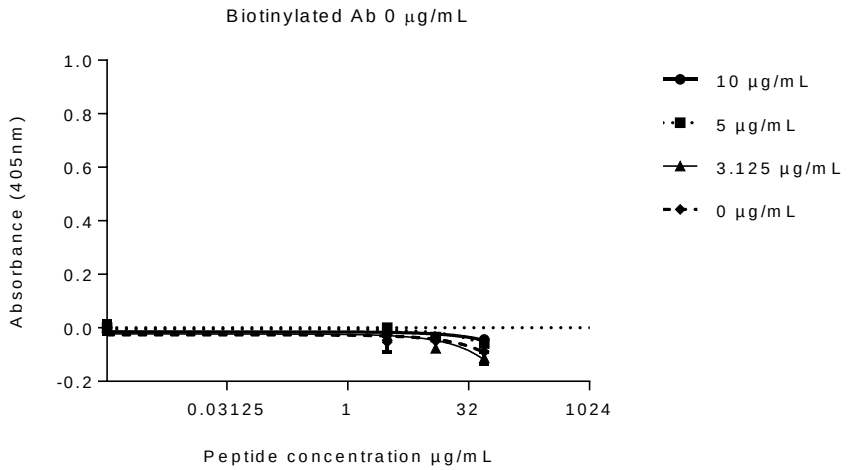


Figure 16: Standard curves with absorbance at 405 nm versus log 2 RegIIIγ peptide concentration (µg/ml). Capture antibody concentrations used were 10 µg/ml ( ); 5 µg/ml ( ); 3.125 µg/ml ( ) and 0 µg/ml ( ). Detection antibody concentration used was 5 µg/ml (a), 2.5 µg/ml (b) or 0 µg/ml (c).

Table 6: Absorbance values and Reg3γ concentrations of samples with known MPO values. Each sample was test in three different dilutions

Sample ID	MPO Activity (millU/mL)	Baseline corrected average absorbance value (405nm)	Diluted Reg3γ concentration (µg/mL)	Dilution factor	Final Reg3γ concentration (µg/mL)
1	78.85	0.169	12.955	10	129.552
		0.083	5.938	50	296.907
		0.028	1.775	100	177.474
2	350.09	0.505	50.703	10	507.032
		0.157	11.981	50	599.053
		0.079	5.626	100	562.570

Table 7: Absorbance values and Reg3γ concentrations of samples with different faecal score (0 = solid; 1 = semi-solid; 2 = semi-liquid; and 3 = liquid)

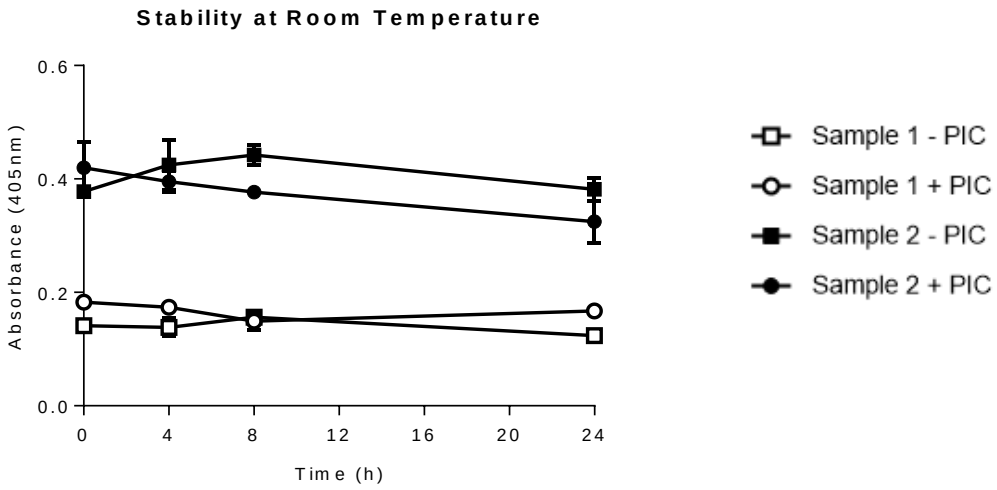
Sample ID	Faecal score	Baseline corrected average absorbance value (405nm)	Diluted Reg3γ concentration (µg/mL)	Dilution factor	Final Reg3γ concentration (µg/mL)
3	2	0.4055	20.2094	10	202.09
4	1	0.1895	5.897176	10	58.97
5	0	0.1055	1.026278	10	10.26

5.4.2 Stability test results

Figure 17 reports the results obtained during the temperature stability test. To perform the test we extracted new supernatant from the same samples used during the validation phase (high and low MPO activity).

Time point 0 represents the control value, i.e. the supernatant immediately frozen at -20°C after the extraction procedure. As expected, also in this assay sample 1 (low MPO) had lower absorbance value than sample 2 (High MPO). Our results showed that the addition of the protease inhibitor cocktail (P=0.70), the temperature (P=0.99) and the time of incubation (P=0.97) did not affect the results of the test.

a



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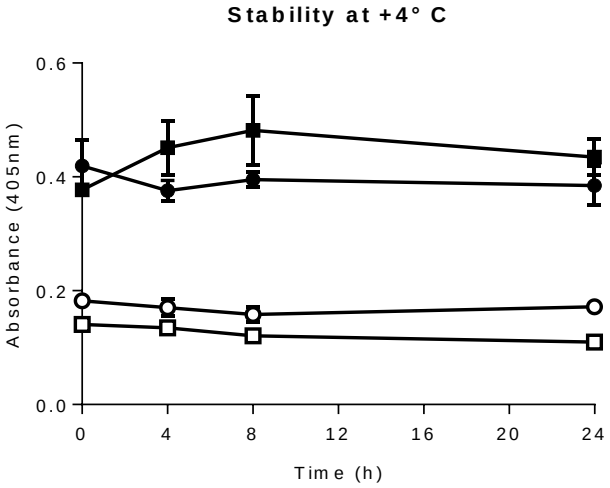

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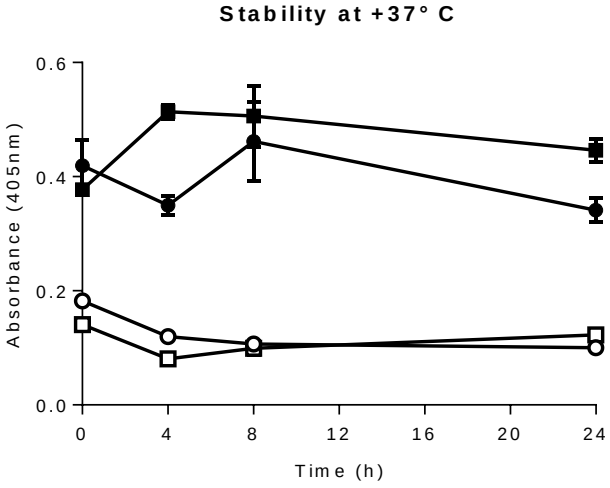

Figure 17: Absorbance value derived from faecal extracts with or without Protease Inhibitor cocktail (PIC) kept 4, 8 or 24 hours at different temperature conditions: room temperature (a), +4°C (b) and +37°C (c).

5.5 Discussions and Conclusions

Reg3α/PAP is expressed in the intestinal mucosal of pig as a different isoform (i.e. Reg3γ) and, as consequence, anti-mouse and anti-human antibodies have no cross reaction with porcine PAP and no commercial ELISA kit is available yet. Therefore, the main aim of this study was to develop and validate a new sandwich ELISA test for the quantification of PAP in pig faecal samples.

For the development phase of the sandwich ELISA test, we followed a standard ELISA sandwich protocol and we tested different concentrations of primary (10- 5- 3.125- 0 μg/mL) and secondary antibodies (5- 2.5- 0 μg/mL). The obtained results allowed us to establish a standard protocol for Reg3γ quantification. The antibodies are effectively able to detect the synthetic pure peptide and have a high specificity for it. The high specificity is corroborated by the absence of signal obtained when one or both antibodies were not loaded in the well. The combination assuring to reach the highest absorbance signal is 10 μg/mL of catching antibody and 5 μg/mL of secondary antibodies.

For the validation of the test, two faecal samples, with high (350.09 millU/ ml) and low (<78.85 millU/ ml) level of MPO activity, were tested, as well as pooled samples with different faecal consistency. Results from those samples are promising. MPO activity and diarrhoea were used as signs of intestinal disease and Reg3γ seems to be positively correlated. Concentrations of Reg3γ in sample 2 is almost five times higher than in sample 1, paralleling the values of the MPO, and a worsening in faecal score resulted in an increased concentration of Reg3γ.

Stability is one of the most important characteristic of a biomarker, especially if it derives from excretions such as faeces (Niewold, 2015b).

Our results revealed that the PAP immunoreactivity in a complex matrix such as faeces is extraordinarily and surprisingly stable. The addition of the protease inhibitor cocktail, the temperature and the time of incubation did not affect the results of the test and after 24h at 37°C the immunoreactivity is still detectable like it was immediately frozen. This makes the current ELISA exceptionally suitable for field tests, because it implies that samples do not need to be really fresh.

This study is only preliminary, but contributes to increase the knowledge about non-invasive biomarkers to predict intestinal health disease in production animal. In addition, our results are a starting point for the further development of a commercial ELISA kit for establishing pig intestinal health by using faecal samples.

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Chapter 6

Associations between oxidative status and negative energy balance markers in the periparturient period in dairy cows: An observational study

Adapted from:

Guido Invernizzi, Panagiota Koutsouli, Giovanni Savoini, Elena Mariani, Raffaella Rebucci, Antonella Baldi, Ioannis Politis. Short communication: Associations between oxidative status and negative energy balance indexes in the periparturient period in dairy cows: An observational study. Manuscript submitted to Journal of Dairy Research.

6.1 Abstract

The objective of this Research Communication was to determine changes in reactive oxygen species (ROS), serum antioxidant capacity (SAC) and oxidative stress index (OSi; ROS/SAC) during the periparturient period in dairy cows and thus, test the hypothesis that OSi predicts better the oxidative status than ROS or SAC alone. Furthermore, the relationship between all three indexes of oxidative status (ROS, SAC, OSi) with markers of energy balance (blood free fatty acids, FA and β-hydroxybutyrate, BHB) and α-tocopherol (α-T) was determined. Blood samples were collected from 131 dairy cows belonging to 4 commercial farms, located in Italy and Greece. Blood samples from all animals were collected at dry-off, calving and 30 d postpartum. Results indicated that ROS and OSi were low at dry-off and 30 d postpartum and high at calving. The serum antioxidant capacity followed exactly the opposite trend. There was a strong negative correlation of ROS and OSi with α-T at all three sampling points, whereas no correlation was found between SAC and α-T. Reactive oxygen species were positively correlated with BHB at all three sampling points and with FA levels only at dry-off. A negative correlation of SAC with FA was found at dry-off and 30 d postpartum. The oxidative stress index was positively correlated with FA and BHB at dry-off, with FA at calving and with BHB at 30 d postpartum period. Thus, associations between parameters related to oxidative status and those related to energy balance were found, mainly at dry-off and postpartum. The oxidative stress index does not appear to describe this relationship better than ROS or SAC alone.

Key words: oxidative stress index, free fatty acids, β-hydroxybutyrate, periparturient period

6.2 Introduction

The majority of the evidence available suggests that dairy cows experience oxidative stress during the transition period (Sordillo and Aitken, 2009; Abuelo et al. 2013). During this critical period, transition cows experience also negative energy balance, a problem that becomes more prominent in high-yielding animals. Severe negative energy balance impairs, among others, milk production, DMI intake, energy efficiency and fertility in dairy cows. Fatty acids (FA) and β-hydroxybutyrate (BHB) are the two markers used to assess the severity of the negative energy balance. In a previous study including four commercial farms located in Italy and Greece (Pilotto et al. 2016), our group showed as these two markers are not well correlated during the transition period. In fact, the concentration of fatty acids in blood became maximal during calving and declined postpartum, while BHB continued to increase in the postpartum period. Furthermore, significant negative correlations between BHB and α-tocopherol (α-T) were found.

Even though it is widely accepted that increased oxidative stress participates to the improved incidence of impaired health status and diseases in the transition period, the negative energy balance (NEB) seems to account as the major contributing factor. Abuelo et al. (2013) reported that the oxidative stress index (OSi), which is the ratio between reactive oxygen species (ROS) and serum antioxidant capacity (SAC), predicts more accurately the oxidative status in transition cow.

The fact that we have already determined FA and BHB values in blood samples from 4 commercial farms (Pilotto et al. 2016) gave us the incentive to extend our previous investigation and determine ROS, SAC and OSi and, thus, test the concept proposed by Abuelo et al. (2013) in a larger scale. The objectives of the present study were: (1) to determine changes in ROS, SAC and OSi levels during the periparturient period to test whether OSi predicts more accurately the oxidative status in transition cows, (2) to examine the relationship between the three markers (ROS, SAC, OSi) of oxidative status and the two markers of negative energy balance (FA, BHB).

6.3 Materials and Methods

6.3.1 Experimental design, animals and dietary treatment

A total of 131 Holstein cows belonging to four commercial farms were sampled in an observational field study. Two of the farms were located in North Italy and the other two in North Greece. A total of 59 cows belonged to the Italian farms (30 and 29 from each farm) and 72 belonged to the Greek farms (36 from each farm). According to Pilotto et al., (2016), milk yield was approximately 20% lower in the Greek farms. Table 8 reports the diets on all farms (adapted from Pilotto et al. 2016)

Table 8: Composition of the diet on Dry Matter basis in the farms located in Italy and Greece for the dry period and after calving

Ingredient (% on DM basis)	Italian farms		Greek farms	
	Dry period	After calving	Dry period	After calving
Corn silage	43.3	23.8	42.0	48.5
Soybean meal	12.8	17.6	9.6	16.5
Molasses		2.0	8.4	3.7
Alfalfa hay		5.8		14.0
Extruded flaxseed		1.9		
Meadow silage		13.5		
Meadow hay		7.6		
Corn		22.0		12.0
Straw hay	35.0		40.0	
Corn meal	5.9			
Rumen protected fat		1.5		1.7
Mineral and vitamin premix	3.0	3.5		3.5

6.3.2 Data collection and analyses

Blood samples were collected from all cows at dry-off, at calving, and at 30 d postpartum. Serum was obtained following centrifugation of the blood samples, and it was frozen at −80°C until analysis. Reactive oxygen species were determined using the method of Trotti et al. (2001) by the spectrophotometric d-ROM test (Diacron International, Grosseto, Italy). The method determines the amounts of hydro-peroxides using the chromogenic substrate N,N-diethyl-

paraphenylenediamine. Results are expressed in arbitrary 'Carratelli Units' (CarrU), where 1 CarrU is equivalent to the oxidizing power of 0.08 mg H_2O_2/dl.

Serum antioxidant capacity was determined with the OXY-Adsorbent Test (Trotti et al. 2001). This test exploits the capacity of HClO to oxidize the complete pool of antioxidants present in serum (albumin, bilirubin, uric acid, thiol groups, vitamins, glutathione, glutathione peroxidase, superoxide dismutase, catalase, etc.). Thus, SAC's estimation is based on the cumulative action of all the antioxidants present in serum. Results are expressed as mmol HClO/ml. Oxidative stress index was calculated as the ratio of ROS/SAC.

The amounts of FA and BHB were determined, and the results were described in detail in a previous paper published by our group (Pilotto et al. 2016).

Briefly, for determination of blood fatty acids an enzymatic colorimetric assay based on acyl-CoA synthetase–acyl-CoA oxidase method was performed (Wako Chemicals, Richmond, VA); to determine plasma concentrations of BHB we used a test based on the oxidation of D-3-hydroxybutyrate to acetoacetate by the enzyme 3-hydroxybutyrate dehydrogenase (Cayman Chemical, Ann Arbor, MI).

6.3.3 Statistical analysis

The statistical analysis was performed using a linear mixed model with two fixed factors (farm and sampling point) and three repeated measures for each cow. Cow was considered as a random factor nested within farm. We used heterogeneous first order autoregressive covariance structure, resulting in the smallest Akaike information criterion. The model used was

$$Yijk = \mu + Fi + Tj + Fi\,X\,Tj + Ck(i) + eijk,$$

where Yijk is the individual value for each dependent variable (ROS, SAC, OSi); μ is the overall mean; Fi is the fixed effect of farm (1 and 2 = Italian farms and 3 and 4 = Greek farms); Tj is the fixed effect of 3 repeated measures factor "sampling point" for each cow (1 = dry-off, 2 = calving, 3 = +30 d post-partum); Ck(i) is the random cow effect, nested within farm; and eijk is the random error assumed to be normally and independently distributed with zero expectation and common variance σ^2. Values in the tables are presented as least squares means (± SEM). The Bonferroni test for P-values was used when performing multiple comparisons and assigned significance at an α level of 0.05 unless otherwise noted. All analyses were performed by PROC

MIXED in SAS, version 9.0 (SAS Institute, 2004). Spearman's rho bivariate correlations of variables at sampling points were estimated by PROC CORR statement in SAS.

6.4 Results

Table 9 shows that ROS and OSi were maximal at calving and lower values were found at dry-off and 30 d postpartum (P<0.05), conversely SAC was lowest at calving while higher values were found at dry-off and 30 d postpartum (P<0.05).

Table 9: Changes in levels of reactive oxidative substances (ROS, CarrUnits), serum antioxidant capacity (SAC, µmol HClO/ml) and oxidative stress index (OSi, ROS/SAC) during the periparturient period in dairy cows[1]

Time of sampling	ROS	SAC	Osi
Dry-off	60.924[a] ± 1.141	459.085[a] ± 6.630	0.134[a] ± 0.003
Calving	73.871[b] ± 1.223	380.412[b] ± 6.847	0.200[b] ± 0.004
30 d postpartum	64.015[a] ± 1.275	461,863 [a] ± 6.555	0.143[a] ± 0.004

[a-b]Means within the same column followed by different letters differ at $P < 0.05$

[1]Dairy cows from 4 herds, 2 of them in Italy and 2 in Greece. All values are LSM ± SEM.

Considering the correlation between α-tocopherol (α-T) and ROS, SAC and OSi, our results show that ROS and OSi were negatively correlated with α-T at all three sampling, whereas SAC was not correlated with blood α-tocopherol in any of the three sampling points (Table 10).

Table 10: Spearman's rho correlations between ROS, SAC, OSi and blood α-tocopherol (α-T) during the periparturient period in dairy cows[1]

		α-T		
Item		Dry-off	Calving	30 d postpartum
ROS	Rho	-0.500	-0.282	-0.671
	P-value	***	**	***
SAC	Rho	0.123	0.100	0.178
	P-value	NS	NS	NS
OSi	Rho	-0.552	-0.267	-0.634
	P-value	***	**	***

[1]Dairy cows from 4 herds, 2 of them in Italy and 2 in Greece

Correlation is significant at $P < 0.01$;* $P < 0.001$ (2-tailed). NS: non-significant differences.

Table 11 presents the Spearman' s rho correlation coefficients between the three markers of oxidative status (ROS, SAC, OSi) and the two markers of negative energy balance. Reactive oxygen species were positively correlated with BHB at all three sampling points, but only at dry-off with FA. Oxidative stress index was positively correlated with BHB at dry-off and 30 d postpartum, and with FA at dry-off and calving. Serum antioxidant capacity was negatively correlated with FA at dry-off and 30 d postpartum but was not correlated with BHB at any of the three sampling points.

Table 11: Spearman's rho correlations between the levels of blood free fatty acids (FA), β-hydroxybutyrate (BHB), ROS, SAC and OSi during the periparturient period in dairy cows[1]

Time of sampling	item		FA	BHB	ROS	SAC	OSi
Dry-off	FA	rho	1	-0.108	0.233	-0.197	0.327
		P-value	-	NS	**	*	***
	BHB	rho		1	0.274	-0.004	0.261
		P-value		-	**	NS	**
	ROS	rho			1	0.104	0.841
		P-value			-	NS	***
	SAC	rho				1	-0.314
		P-value				-	***
	OSi	rho					1
		P-value					-
Calving	FA	rho	1	0.118	0.121	-0.138	0.215
		P-value	-	NS	NS	NS	*
	BHB	rho		1	0.184	0.169	0.040
		P-value		-	*	NS	NS
	ROS	rho			1	0.225	0.517
		P-value			-	**	***
	SAC	rho				1	-0.610
		P-value				-	***
	OSi	rho					1
		P-value					-
30 d postpartum	FA	rho	1	-0.030	0.037	-0.400	0.162
		P-value	-	NS	NS	***	NS
	BHB	rho		1	0.272	-0.023	0.254
		P-value		-	**	NS	**
	ROS	rho			1	0.052	0.852
		P-value			-	NS	***
	SAC	rho				1	-0.396
		P-value				-	***
	OSi	rho					1
		P-value					-

[1]Dairy cows from 4 herds, 2 of them in Italy and 2 in Greece

* Correlation is significant at $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (2-tailed). NS: non-significant differences.

6.5 Discussions and Conclusions

In the present study, our findings suggest that OSi does not describe the oxidative status of cows better than ROS or SAC alone. This is in contrast with Abuelo et al. (2013) who reported as OSi was more accurate in the detection of oxidative stress when compared to ROS and SAC. Specifically, during the periparturient period Abuelo et al. (2013) found significant changes only in OSi, whereas changes in ROS only showed trends (P=0.07) and SAC variation was not significant. This discrepancy could be attributed to the larger number of farms and cows involved in our trial.

α-tocopherol (α-T) is the main antioxidant vitamin in dairy cows (Weiss et al. 1997; Baldi et al. 2000; Politis et al. 2012a). Concerning the negative correlation of α-T with ROS and OSi, our findings show similarities with those reported by Politis et al. (2012b), who found that α-T was inversely related with two measures of oxidative stress, namely ROS and thiol groups, during the periparturient period.

In our study SAC decreased approximately by 18% in the dry off period and increased by 21% in the first month of lactation, whereas Pilotto et al. (2016) on the same cows showed a reduction of α-T by 50% during dry period and increased level of 100% at 30 d postpartum. Surprisingly, SAC was not correlated with blood α-tocopherol in any of the three sampling points. The lack of expected positive correlation between SAC and α-T can be related to the inability of OXY adsorbent test to correlate with the single antioxidant measure (Costantini, 2011); the test indeed accounts simultaneously for the contribution of different antioxidants families that could affect the total antioxidant capacity.

In conclusion, calving was associated with the highest values of two measures of oxidative stress (ROS and OSi) and the lowest value of serum antioxidant capacity. Our findings suggest that a positive relationship does exist mainly at dry-off and 30 d postpartum between markers of oxidative stress (OSi and ROS) and those of negative energy balance (FA, BHB), while OSi does not appear to describe the oxidative status of cows more accurately than ROS or SAC alone.

6.6 References

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Chapter 7

General Conclusions

7.1 General conclusions

The main objectives of this thesis were to evaluate the effects of dietary antioxidant compounds, namely polyunsaturated fatty acids and melon pulp concentrate combined with yeast, on oxidative stress and immune system and to study the possible application of non-invasive biomarkers for oxidative stress and inflammation.

It is not often possible to divide the incidence of diseases and pathologies from economical losses for the farmers. Indeed, the total losses of piglets born in EU was estimated by approximately 17% and the greatest cause was related to infection of the mucosal surface of the intestine (Lallès, 2007). In dairy cows, oxidative stress is recognized to negatively affect mammary gland health and can cause reproductive disorders (Turk at al., 2011 and 2012), decreasing milk production, with an average loss of 300€/mastitis, and lowering reproduction (Lykkesfeldt and Svendsen, 2007).

Nutrition is one of the main factors affecting animal health, reproduction and performance. This thesis focused its attention on the possibility to improve health, reproduction and performance, throughout nutritional additives and monitoring and developing oxidative stress and inflammation biomarkers.

Our results on the supplementation of polyunsaturated fatty acids to mice as model or melon pulp concentrate and yeast in post-weaning challenged piglets did not outline significant advantages in terms of animal benefits, at least for the tested parameters. In both cases, the animals did not benefit for the growth performance from the respectively supplementation, in addition, both dietary treatments were unexpectedly not able to improve oxidative status and immune response.

Specifically, in the first trial, we found that the combination of saturated fatty acids and low estrogen levels seems to worsen the oxidative defence of hepatic and chest areas, highlighting the necessity to increase the knowledge about the relation existing between dietary fatty acids and oestrus cycle. In this view, it would be interesting to consider, in addition, changes in circulating levels of estrogens and ovarian/uterine weight, but also to add a group of mice that are exogenously treated with 17β estradiol to understand what happens if circulating estrogens are very high.

The experiment performed on challenged piglets, did not lead to the same results as previously found by our group when piglets were fed melon pulp concentrate alone, rather than in combination with yeast (Ahasan et al., 2018). In particular our previous trial stated as dietary melon pulp concentrate alone ameliorated the total antioxidant capacity and the half-haemolysis

time in red blood cell of post-weaning piglets, with positive results on growing performance. The lack of effectiveness found administering melon pulp concentrate together with yeast could be probably be attributable to a possible interference between the yeast and the antioxidant compound, or to the different dosage of the additives used in the diets.

A positive implication of this study concerns the investigated biomarkers.

With this study, we highlighted different pattern and time of reaction of the markers and that should be took into consideration during the experimental trial design. With KRL test, for example, we obtained significant results only with the samples collected 24h after the challenge and no results were obtained when the test was performed on samples collected 48 h after LPS injection. Thus, the haemolysis time of red blood cells could be considered a good biomarkers only if assessed in the first 24h after the inflammatory stimulus; otherwise the researcher might lose the data.

Beside the effect of the nutritional additives, the used markers in the post weaning piglets can be considered as invasive due to the need of blood samples collection for the specific assays. The need of reliable non-invasive markers of oxidative stress and inflammation is raising during these years, also due to the recent regulation on animal welfare in Europe that poses serious limitations on the use of invasive methodologies to monitor animal health status.

For this reason, the third experiment was aimed to create a new sandwich ELISA test for Reg3γ detection and quantification in pig faecal samples.

Our preliminary results are promising, assessing the suitability of Reg3γ as intestinal non-invasive biomarkers of inflammation in pig. Indeed, PAP immunoreactivity was found to be extremely stable, even in an organic matrix such as faeces; this makes PAP a useful tool to detect inflammation, not only under experimental conditions, but also for the detection of intestinal disease in farm.

The Reg3γ sandwich ELISA test we developed may be helpful in the elaboration of an affordable on-field test where farmers could be able to quickly measure the presence and the degree of intestinal inflammation in their animals, and adjust management and nutritional strategies accordingly.

Due to the fact that non-invasive biomarkers are requested at all animals levels, the developed marker in the present thesis could be further tested and validated also in other livestock such as in ruminant and poultry.

The main objective of the last study was to determine changes in Reactive oxygen substances (ROS), Serum antioxidant capacity (SAC) and Oxidative Stress Index (OSi) during

the periparturient period of dairy cows and test whether OSi predicts more accurately the oxidative status in transition cows.

The oxidative stress index is a derivate index that combine ROS and SAC and in 2013, Abuelo and colleagues found that it seemed to be a more accurate tool than the single markers but unexpectedly we were not able to obtain the same results on a large scale. It is possible, however, that on small population it is more sensitive than the evaluation of reactive oxygen species and serum antioxidant capacity separately.

Celi in 2011 pointed out that differences between models and methodologies of oxidative stress assessment make difficult to do meaningful comparisons with practical conclusions but we do not believe that OSi could add any improvement in this sense or any further information.

In conclusions, the lack of effectiveness of the tested product on both, oxidative and inflammatory markers, can be due to the dosages, the interaction with the other feed components, the management condition and the markers considered.

The development of new non-invasive biomarkers for intestinal inflammation can improve not only the available techniques for feed additives testing, but also broaden the knowledge on the mechanism of action of different beneficial molecules/compounds such as those used in the present thesis.

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