**Background.**

*My research activity was focused on dietary supplementation with several plant extracts and their effect on global antioxidant status of piglets.*

*Natural extracts are products used in animal nutrition for purposes to getting better feed quality and to improve the animals' performance and health. Moreover, the banning of the use of in-feed antibiotics in the European Union has increased interest towards plant additives (Hashemi and Davoodi, 2011). Interesting for animals' production, result to be the bioactive compounds that have antioxidant activities from plant origin ingredients. In particular, polyphenols are of special interest due to the several positive biological activities, including antiallergic, anti-inflammatory, antimicrobic and antioxidant activities (Manach et al., 2004). Although reports have demonstrated antioxidative, antimicrobial and immune stimulation efficacy in vitro, respective experimental in vivo evidence is still quite limited. It is also well recognized the relationship between the onset of several inflammatory and infectious diseases and the reduction of the animals' antioxidant status. Oxidative stress involves in several pathological conditions negatively affecting animal health, welfare and productive parameters (Lykkesfeldt and Svendsen, 2007). In fact, according with Brambilla et al., (2002) the response to oxidative stress could be considered as welfare parameter in swine.*

*Post-weaning piglets are particularly susceptible to diseases and to oxidative stress related to several environmental and physiological factors. Antioxidant status declines after weaning, increasing piglet's morbidity and mortality. One study reported that dietary plant polyphenols in pig enhance oxidative stress responses (Rossi et al., 2013). However, dietary polyphenols during the absorption are conjugated in the small intestine and later in the liver. Conjugation mechanisms are highly efficient, and aglycones are generally either absent or present in low concentrations in blood (Manach et al., 2004). It is still very difficult to evaluate the accumulation of the polyphenols in tissues, and the in vivo studies are still very scarce even though the number of them has rapidly increased over the last few years. Consequently, the determination of antioxidant reserve could be an important tool to better understanding the pigs' antioxidant status and to identify the plant with a high in vivo antioxidant activity. On this basis, further investigations are needed, using a specific adaptation of the biological KRL® test. In fact, the determination of antioxidant reserve (RESEDA test [1], Prost 2003) allows to identify the amount of antioxidant molecules stored in the organism, that could be released to counteract oxidative stress. Therefore, the influence of dietary natural antioxidant from plant*

1 *RESEDA test, RESErves de Défenses Antiradicalaires test*

*extracts on well-being of weaned piglets and their in vivo effects is worth further investigation.*

**Aims**

*In light of this, the aim of the two months stage conducted at Laboratoires Spiral in Couternon, France, was the acquisition of the technical know-how regarding:*

- *assessment of antioxidant capacity of plant extracts by KRL® test and RESEDA of plant extracts.*
- *assessment of the global antioxidant activity and the release of antioxidant reserves of blood of the piglets by KRL® test in order to highlight the effect of the dietary natural antioxidant supplementation.*

**Experience in foreign country: France**

*I spent the 2 months of my fellowship in the private research Laboratory (Laboratoires Spiral) in Couternon, France. I had the opportunity to work in deep in the topic as well as the well-being of pigs and natural extracts. During this period, I improved the knowledge about the biological determination of antioxidant activity of plant extracts and antioxidant status of animals, which received a dietary supplementation with natural extracts. My research project was focus on study in deep the effects of dietary natural extracts supplementation in weaned piglets on piglet's well-being and antioxidant status.*

*In the first week, I had several meeting with my supervisor Prof. M. Prost in order to organize my project. During the first period, I performed a training in order to adapt the use of KRL® test for 3 different methods: assessment of antioxidant activity of plant extracts, assessment of global antioxidant activity of blood samples, assessment of antioxidant reserves (RESEDA) of blood samples. I followed a Lab technical who received a samples of plant extracts or blood from private farm in order to establish the antioxidant activity by biological test KRL® test.*

*In the second period, I performed KRL® test on samples of plant extracts used in the in vivo experimental trial started at the University of Milan in order to obtain the data of antioxidant activity of extracts. Moreover, piglet's blood samples of the same trial have been also analyzed. Details of in vitro and in vivo experimental trial are described below.*

**Material and method.**

*Animals, housing and sampling in Italy.* *One hundred-sixty piglets (Topics x Tempo) at 28 days of age were used in the experimental trial in Italy by research group of Prof. Carlo Corino (University of Milan - Dept. VESPA). Piglets were housed in 4 post-weaning room that contained 8 pens per 5 piglets. The experiment was a completely randomized block design. Piglets were allocated randomly on the basis of litter, sex and live weight to one of four treatment groups as follows: C group fed with commercial diet; BOS group diet supplemented with Boswellia (500 mg /kg feed); UT group diet supplemented with Uncaria e Tanaceto (200 e 50 mg /kg feed); AOX group diet supplemented with mix of natural extracts (Verbenaceae, Liliaceae, Labiatae; 225 mg/kg feed). The health status of the animals and clinical signs were recorded. The body weight of each pens was recorded at 0, 18 and 28 days of the experimental trial. Feed intake was measured and feed utilization was calculated for each pen. At the end of the trial (28 days) the blood sampling (5 mL) from 2 piglets/pen were collected by puncture from anterior vena cava in in 10-mL vacutainer glass tubes containing EDTA (Venoject, Terumo Europe N.V., Leuven, Belgium) and stored at 4°C until the analysis in Laboratoires Spiral laboratory (France).*

*Analysis of plant extract in Laboratoires Spiral Lab.*

*KRL® test of the antioxidant activity of plant extracts.* *The effects of the plant extracts on the sensitivity to free radical aggression were tested by the capacity of control blood to withstand free radical-induced haemolysis (Prost, 1989; Prost, 1992). Plant extracts and standard Trolox were diluted in a serial dilution in a macroplate. Samples were placed in a microplate and control blood suspension (50 µl) were assayed in a 96-well microplate coated with a free radical generator 2,2'azobis (2-aminido-propane)dihydrochloride (AAPH;Spiral,Dijon,France) (Figure 1.). Kinetic of control blood resistance to haemolysis will be determined at 37°C by continuous monitoring of changes in absorbance at 450 nm. Half haemolysis time ($HT_{50}$) will be retained for group comparisons. Results were expressed in mg of Trolox equivalents per 100 g of product. The products are tested in triplicated.*

*KRL® test of plant extracts for RESEDA. In order to determine the possible changing of the molecules exposed to the specific enzymes the RESEDA of plant extracts in vitro were tested following the method described by Prost (2003). At the 3 extracts, Bosw- U+T and AOX, in concentration of 100 mg/L, are added the enzymes:*

- *R1 (hydrolysis with glucosidase),*
- *R2 (hydrolysis by means of sulfatase)*
- *R3 (hydrolysis with glucuronidase).*

*Finally, samples were placed in a microplate and control blood suspension (50 µl) were assayed in a 96-well microplate coated with a free radical generator 2,2'azobis (2-aminido-propane)dihydrochloride (AAPH;Spiral,Dijon,France). Kinetic of control blood resistance to haemolysis will be determined at 37°C by continuous monitoring of changes in absorbance at 450 nm. Results were expressed in mg of Trolox equivalents per 100 g of product.*

*Blood Analyses in Laboratoires Spiral Lab.*

*KRL® test of the global antioxidant activity. The Kit Radical Libres (KRL®) test is a biological test that evaluates the antioxidant status of an organism by testing the antioxidant defence systems (Prost, 1989; Prost, 1992). The KRL® test allows the ex vivo dynamic evaluation of the overall antioxidant defense potential of an individual. The antiradical potential of piglets' blood were evaluated using a KRL® test, which tests blood resistance based on free radical induced haemolysis. Piglets' blood and red blood cell (RBC), were diluted to 1/50 in isotonic saline solution, and were submitted to organic free radicals (AAPH) produced at 37°C. The extracellular and intracellular antioxidant defences contribute in maintaining blood cell membrane integrity and function until cell lysis. Haemolysis were recorded using a 96 well microplate reader by measuring the optical density decay at 450 nm. Results were expressed as the time that is required to reach 50% of maximal haemolysis. $HT_{50}$ for total blood and RBC will be expressed in minutes, refers to the whole blood and RBC resistance to free radical attack. The measurement of $HT_{50}$ has been shown to be representative of the overall defence against free radicals (KRL® value).*

*KRL® test of RESEDA. The release of antioxidant reserve in blood were evaluated by RESEDA test (Prost, 2003). Piglets' blood, were diluted to 1/50 in isotonic saline solution, and were submitted to organic free radicals (AAPH) produced at 37°C. In order to evaluate in depth the effect of the dietary natural extracts supplementation on the release of antioxidant reserves, several enzyme are added: Reserves R1 (hydrolysis with*

*glucosidase), R2 (hydrolysis by means of sulfatase) and R3 (hydrolysis with glucuronidase). Haemolysis were recorded using a 96 well microplate reader by measuring the optical density decay at 450 nm. Results were expressed in Eq mg trolox/g.*

*Statistical analysis. Statistical analyses of the data were performed using SPSS (SPSS/PC Statistics 23.0 SPSS Inc., Chicago, IL). The data on performance, KRL® value and RESEDA were analyzed by one–way Analysis of Variance (ANOVA) to evidence the effect of treatment. Data are presented as means ± SEM, and a value of P<0.05 was used to indicate statistical significance.*

**Results and discussion.**

*In vitro analysis of antioxidant activity of plant extracts. Low antioxidant activity was exerted by Boswellia and mix of Uncaria and Tanaceto (not more than 50 eq Trolox/100 g of products). While, AOX mix of natural antioxidant exhibited a high antioxidant activity more than 1000 eq Trolox/100 g of products. The study demonstrated that KRL® assay could be a promising approach for screening the plant extracts to determine the global antioxidant activity in a biological system. Moreover, the extracts at the concentration of 100 mg/L are exposed to specific enzyme hydrolysis: R1 (hydrolysis with glucosidase), R2 (hydrolysis by means of sulfatase) and R3 (hydrolysis with glucuronidase). The response to kinetic of control blood resistance to haemolysis has been changed in relation to the molecules that the enzyme release. An increase of control blood resistance to haemolysis was observed in Bow and UT (more than 200 eq Trolox/100 g of products), considering the previous KRL values without hydrolysis. While, AOX that exhibited a high antioxidant activity did not increase their activity after a specific enzyme hydrolysis. In thighs, suggested that Bow and UT could have an indirectly antioxidant activity and the compounds have a reserve form that can have an effect on the organisms.*

*In vivo performance parameters and ex vivo antioxidant activity of blood. No difference (P>0.05) of piglets growth performance in relation to dietary supplementation with several natural extracts tested by KRL® test was observed. These finding are in agreement with Fiesel et al., (2014) and Stelter et al., (2013), which reported that the integration of polyphenol in piglets had no influence on the parameters of growth performance. However, we observed a numerically higher feed efficiency in UT group than the other one.*

*The total antiradical activities of whole blood showed the total antioxidant defences of the organism at the time of sampling. Thus, whole blood parameter gives an indication of physiological status instantaneous. On the other hand, KRL® value of RBCs is important for the interpretation of the balance between attack and defence of organism in a medium/long period, considering that the RBC mean life in pig is 60-85 days. No significant differences (P> 0.05) in KRL® values for total blood and RBC, in relation to feed treatment, was observed. Previous studies showed that KRL® value of swine was improved through dietary supplementation for a long period whit antioxidant mixture containing plant extracts (Cannata et al., 2010; Rossi et al., 2013; Maghin et al., 2015). Thus, the no significant difference of whole blood KRL® value could be explained with a short treated diet period. The antioxidant defence of RBC was not influenced by dietary supplementation (P = 0.578). This because the supplementation of 28 days has been short to detect a chronic effect on RBSs and highlight a difference between treated and control. Moreover, it is important highlight that the piglets were healthy and not been subjected to any stress during all the experimental trial.*

*To establish conclusive evidence for the effect of dietary polyphenols, content in plant extracts, it is useful to better define the bioavailability of the polyphenols. Prior to passage into the blood stream, the polyphenols, that are simple aglycones, undergo to other structural modifications due to the conjugation process that takes place in the small intestine and liver. The conjugation, that includes methylation, sulfation, and glucuronidation, produces active metabolites from dietary polyphenols. Therefore, dietary polyphenol generates several metabolites in vivo, which exert a biological function and are accumulated in the tissue. In light of this, it is important to study in deep the antioxidant reserve in the organism. The data of blood antioxidant reserves showed that the piglets which received Uncaria and Tanaceto had a higher (P<0.05) RESEDA than the other experimental groups. No significant difference (P>0.05) among piglets fed with a dietary supplementation with Boswellia, mix of antioxidant and control was observed, considering all the specific enzyme (glucosidase, sulfatase, glucuronidase) hydrolysis. Although the mixture of Uncaria and Tanaceto did not show a higher antioxidant scavenger activity in vitro, this mixture is able to increase the antioxidant reserves, so it has an indirectly antioxidant activity which will be mobilized by the piglets in case of oxidative stress. Uncaria and Tanaceto mix had no a direct antioxidant activity, but increase indirectly the antioxidant defence of the organisms.*

*The parameters proposed could be useful especially for the evaluation of welfare in pigs system for studying the influence of nutrition. Moreover, the reserves give more information about the antioxidant status of the piglets and reflect the physiological status of the organism.*

**Conclusion.**

*The data presented has provided interesting results about the dietary supplementation with several natural extracts in pig. The biological KRL® test has proven an extremely sensitive tool to evaluate both the antioxidant capacity of the products and antioxidant status of piglets. The data relating to RESEDA is the first study regarding the action of the different dietary supplements with natural extracts on the physiological state of the subject. Moreover, the determination of antioxidant reserve is an important tool to better understanding the real pigs' antioxidant status. Further study are needed to study in deep how the dietary natural extracts supplementation can modulate the antioxidant status. Data will be available for an article.*

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