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# Livestock Precision Farming and the Kit Radicaux Libres Test: Two Simple Approaches to Detect Variation in Piglets' Behaviour and Welfare

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**Abstract**

In commercial practice, the mixing of pigs from different litters is very frequently done in order to minimise the within-pen variation in weight. Aggressive behavior can be easily observed when unknown pigs are mixed into a group. Post-mixing fighting occurs between pigs that are unfamiliar with each other and some times leads to serious injury. For the establishment of social hierarchy, aggression may occur. The aim of the study was to evaluate the response of piglets fed and not with dietary plant extract (PE) when exposed to mixing-induced **s**tress. Behavioural and physiological indicators combined with Precision Livestock Farming (PLF) methods were used. A total of 84 female piglets (Dalland), (average live weight 5.53 ± 0.1 kg, corresponding to day 0 of experimental trial) divided into 6 pens of 14 piglets each were randomly assigned to two dietary treatments: a control diet (CON) and a diet supplemented with 5 mg/kg feed of plant extract (PE). At 37 d of the experimental trial, half the animals of two treated pens (7+7) and half the animals of two control pens (7+7) were mixed. The experimental trial lasted 59 days. Blood from piglets of each treatment was collected by anterior vena cava puncture before mixing, on day 37, and after mixing, on day 39, for assessing total blood antioxidant activity, by Kit Radicaux Libres (KRL) test and haptoglobin (HPT) and cortisol concentration as biological indicators of stress. Behaviour measurements were performed using an ethological rating scale and Precision Livestock Farming methods as indicator of qualitative behavioural assessment. Dietary treatment and mixing affected total antioxidant activity ($P < 0.05$) of whole blood that was higher in the PE group and after mixing. A trend effect of mixing was found for cortisol and haptoglobin which were significantly ($P < 0.05$) lower in the PE group. The rating scale and PLF showed significant variation in the abnormal animal behaviours due to mixing; continuous monitoring of pigs by PLF showed more ($P < 0.05$) animal activity in the PE group than in CON. In this context, the behaviour measurements were demonstrated to be simple and reliable tools for evaluating pig behaviour and KRL test was an effective method for assessing antioxidant activity.

**Keywords:** piglets, mixing, precision livestock farming, KRL test

## 1. Introduction

Stress is a complex state and management systems and control of the environment and nutrition should minimise its effects in pig farms. In the newly weaned pig, stress is induced by the process of being separated from the sow, relocation and mixing with unknown piglets, introduction of a novel environment (temperature changes, different flooring types, air quality for example) and a radical change in diet. The digestive tract could be compromised on the basis of loss of nutrient intake and the capacity for digestion and absorption of nutrients may be reduced (Campbell et al., 2013). Reduced feed intake and growth immediately post-weaning has an impact throughout life. Providing a diet that is suitable for piglets as well as careful management, are therefore key to reducing the effects of stress in pigs. Natural plant can be considered as useful nutritional approaches througly applied and studied in the practical management of pig farms. Many plants compounds, such as polyphenols, carotenoids and anthocyanidins, display interesting antioxidant activities (Korkina, 2007). In addition, they are able to integrate

endogenous antioxidant defenses, which are depressed after weaning (Bouayed & Bohn, 2010). Weaning leads to intense pressure on the adaptive processes at the behavioural level as well as in terms of neuroendrocine and other physiological levels. Phenolic compounds include a large range of molecules and are often referred as "polyphenols" divided in different classes relating to the molecular structure (Bravo, 1998). The growing interest in the bioactivity of phenolic compounds is due to their efficacy *vs* the oxidative stress (Rangel Huerta et al., 2015). Lippia citriodora (Ort.) HBK (Verbenaceae) is a herbal species mainly used as a spice and medicinal plant. The leaves of this species are reported to possess digestive, antispasmodic, antipyretic, sedative, and stomachic properties (Newall et al., 1996) as well as antioxidant properties (Valentão et al., 2002). When an animal is stressed the natural process of cell turnover and replication is increased. This has many damaging influences in the body including the 'release' of free-radicals that are the products of the breakdown of cell fragments within body tissues. The balance between the processes and the prevention of oxidation is therefore critical in controlling cell integrity and health. If the balance shifts to a high level of oxidation with little inhibition then damaged immunity and poor growth will be the ultimate result for the growing pig and for piglets. The Kit Radicaux Libres is a biological test that evaluates the antioxidant status of an organism by testing the antioxidant defence system of both plasma and red blood cells (RBC) (Prost, 1992). The KRL test is a reliable one for monitoring oxidative stress in humans (Girodon et al., 1997; Lesgards et al., 2002), in rats Taleb-Senouci et al., 2009), birds (Bertrand et al., 2006), rabbits (Brzeziska-Slebodziska, 2001), pigs (Rossi et al., 2013) and piglets (Di Giancamillo et al., 2015). Concerning management, mixing unfamiliar pigs together is a common practice in pig production, particularly at the time of weaning, in order to minimise the within-pen variation in weight and to give all pigs an equal ability to access the feeder. After regrouping weaners, aggressive interactions usually take place in order to establish a new dominance relationship (Blackshaw et al., 1987). These transient fights during the first 48 or 72 hours can lead to skin lesions, especially in the front third of the body (Stukenborg et al., 2011). A crucial point is to quantify the behavioural and physiological response of pigs resulting from stress situations. Precision Livestock Farming is a new approach and uses the principles and technology of process engineering without imposing additional stress on the animals through on line continuous automatic monitoring of the animal (Wathes, 2010; Guarino et al., 2008). Indeed its application allows the farmer or stock manager to make optimal use of knowledge and information in the monitoring and control of farm (Fontana et al., 2015). The aim of the present paper is to evaluate the piglets' response, fed and not wih dietary plant extracts, to mixing practice by means of behavioural and blood physiological indicators combined with Precision Livestock Farming method.

## 2.Material and Methods

### *2.1 Housing and Animals*

Procedures involving animals were carried out in accordance with the European Communities Council Directive (86/609/EEC, 1986) and approved by the Italian Ministry of Health (Law No. 116/92). The animals were reared in an environmentally-controlled room, with 6 pens (1.9 m wide × 2.6 m long), each equipped with a fully slatted floor. The pens were delimited by pen profiles consisting of individual profile boards with a thickness of 35 mm. At the beginning of the experimental trial the internal temperature was 28 °C, relative humidity 60% and ventilation rate 859 $m^3$/h. During the entire test period these parameters varied according to the needs of the piglets; at the end of the experimental period the temperature was 22 °C, relative humidity 80% and ventilation rate equal to 3000 $m^3$/h. A total of 84 piglets (Dalland), weaned at 21 d of age (average live weight 5.53±0.1 kg, corresponding to day 0 of experimental trial) divided into 6 pens of 14 piglets each were randomly assigned to two dietary treatments: a control diet (CON) and a diet supplemented with 1 kg/t of plant extract (PE) to obtain 5 mg verbascoside/kg feed. The dose of plant extract in the feed was chosen on the basis of a previous study in piglets (Pastorelli et al., 2012). The antioxidant supplement contains a water-soluble extract of Lippia citriodora leaves (Verbenaceae), prepared on an industrial scale by a standardized procedure that includes ultrasonic extraction with 60% aqueous ethyl alcohol followed by spray-drying with maltodextrins as an excipient. The bioactive components of the feed supplement, according to a certificate of analysis provided by the manufacturer were verbascoside (4.47±0.08), methyl gallate (1.91±0.09), gallic acid (1.75±0.07), 3.4-dihydroxybenzoic acid (0.45±0.04) and isoverbascoside (0.43±0.04 g $kg^{-1}$). The quantitative analysis of the phenolic compounds was performed by HPLC-UV-DAD (high-performance liquid chromatography, UV diode array detector) according to Piccinelli et al. (2004). To avoid oxidation in the complete feed, the supplement is micro-encapsulated within a protective matrix of hydrogenated vegetable lipids using spraycooling technology (Sintal Zootecnica, Isola Vicentina, Vicenza, Italy). Piglets were given ad libitum access to water and a pelleted diet formulated to meet or exceed the nutritional requirements of a weaned piglet (NRC, 2012). All animals were individually identified by eartags. At 37 d of the experimental trial, half of the animals in two treated pens (7+7) were mixed and same mixing procedure was done for two control pens (7+7). Therefore the resulting total mixed pens were 4. Pen pig

weights were recorded at days 0, 37, 39 and 59 to determine growth performance of all animals involved.

*2.2 Collection of Blood Samples*

Blood samples were obtained from each piglet of mixed control pen (N = 14) and mixed treated pen (N = 14) by puncture of the anterior vena cava at day 37 (before being moved) and at day 39 (after two days from mixing) of the trial. Three different vacutainer glass tubes were used: one tube containing a gel and clot activator (Becton and Dickinson, Milano, Italy), for assaying haptoglobin (HPT) and cortisol concentration; two tubes containing EDTA (Venoject, Terumo Europe N.V., Leuven, Belgium), used for evaluation of total antioxidant capacity by means of the KRL test on whole blood and red blood cells. Blood samples, excepting those analysed for the KRL test, were centrifuged (1000 g, 4 °C, 15 min) within 1 hour from collection, and the serum was stored in the dark at -35 °C until assayed for haptoglobin and cortisol concentration. Blood for the KRL test was stored at 4°C and the assay was performed within 24 h.

*2.3 Blood Physiological Indicators*

Serum concentration of haptoglobin and cortisol were determined using a commercial kit (PhaseTM Range Tridelta Ltd., Kildare, Ireland and LKC01; Medical System, Genova, Italia) respectively. The total antiradical activity of whole blood and RBC for each pig was evaluated using KRL test (Laboratoires Spiral, France). The principle of the KRL test is to submit whole blood to thermocontrolled free radical aggression in order to mobilize all families of any free radical scavengers present in the blood to neutralize the oxidation processes. All the chemical and enzymatic antioxidant systems of the sample were triggered to protect cells integrity until lysis. Whole blood and RBC samples were submitted in an isotonic saline solution to organic free radicals produced at 37 °C from the thermal decomposition of a solution of 2.20–azobis (2-amidinopropane) dihydrochloride (AAPH) (Kirial International, Dijon, France). Haemolysis was recorded using a 96-well microplate reader by measuring the optical density decay at 450 nm. For each well, absorbance measurements were performed 75 times, once every 150 s. Results were expressed as the time required to reach 50% of maximal haemolysis (half-haemolysis time–$HT_{50}$–in minutes), which refers to the whole blood resistance to free-radical attack. Intra and inter-assay coefficients of variation of the KRL test were 2.5% and 4%, respectively (Laboratoires Spiral).

*2.4 Behaviour Indicators*

2.4.1 Rating Scale

Behaviour measurements were performed on CON and PE pen, filling the ethological Rating scale form before and after piglet mixing. The form contains 10 items regarding pig behaviours. In detail, data were recorded, on the $30^{th}$, $32^{th}$, $34^{th}$, $36^{th}$ day of the trial before mixing (indicated as T0) and on the $39^{th}$, $41^{th}$, $43^{th}$, $45^{th}$ day of trial after mixing (indicate as T1). The form was always filled in during the morning, during 1 hour of observation, with the same procedure:

- Waiting for the time necessary for the animals to calm down (value not recorded);
- Noting the presence (observed) or absence (not observed) of behaviour traits.

To the observed or not observed items are then attributed the score reported in Table 1 as reported by Candotti et al. (2007). The sum of individual scores is defined as the Global Score and varied from 14.745 to 0 that were taken to mean maximum or minimum welfare.

Table 1. Behaviour assessments form and score for weaning pigs in pen

| | | Score Observed | Score Not observed |
|---|---|---|---|
| 1 | Are there groups of huddling piglets? | 0 | 1.49 |
| 2 | Are there lesions referred to etherolesionism? | 0 | 1.679 |
| 3 | Are there lesions on anterior part of the body? | 0 | 1.435 |
| 4 | Are there lesions on back of the body? | 0 | 2.011 |
| 5 | Is there "bell noising"? | 0 | 0.922 |
| 6 | Are there competitive behaviours? | 0 | 1.353 |
| 7 | Are there ear lesions? | 0 | 1.714 |
| 8 | Are there tail lesions? | 0 | 1.394 |
| 9 | Is there ear biting behaviour? | 0 | 1.243 |
| 10 | Is there tail biting? | 0 | 1.504 |

2.4.2 Precision Livestock Farming

Precision Livestock data were collected by means of a camera and computer system that generates continuous information providing an objective measure of behavior. The Eyenamic system, using an infrared sensitive CCD camera, was mounted 2.5 m above the floor to get a top view of both pens (CON and PE) in order to record behavior of individual animal (Kashiha et al., 2014) and worked continuously for the entire period of the trial. A total of 1440 hours, corresponding to 60 days, were recorded and stored. Images were captured with a resolution of 768 × 586 pixels at a sample rate of 1 Hz, 1 frame per second. Later on, the frames related to the same time schedule of rating scale, (on $30^{th}$, $32^{th}$, $34^{th}$, $36^{th}$ day before mixing and on $39^{th}$, $41^{th}$, $43^{th}$, $45^{th}$ day after mixing) were labelled (57600 frames) in order to identify individual behaviours (nuzzling, ear biting). Before the starting of the registration, the area of each pen in the camera image was further subdivided into two equally sized areas called zones, one on the left size and one on the right side corresponding to the two observed pens. The recorded videos were labelled by one observer using the software "Labelling Tool". The image files were visually checked and manually labelled by observing frame by frame (one frame per second) to score the animal' behaviour according to Ismayilova et al. (2013) method.

Animal activity (fraction of moving pixels with respect to the total number of pixels within the monitored pen, ranging from 0 to 1) and occupation index (fraction of pixels corresponding to a region of the image occupied by pigs with respect to the total number of pixels within the same monitored pen ranging from 0 to 1) were calculated in real time from day 1 to day 59.

The activity index is a measurement that quantifies the activity of animals in practical field conditions and the occupational index is a measurement that calculates the fraction of the area occupied by the animals (Leroy et al., 2006).

*2.5 Statistical Analysis*

The following analyses were performed with SPSS software (SPSS/PC Statistics 18.0, SPSS Inc., Chicago, IL, USA) (SPSS Inc., Chicago, USA). Performance data were analysed using the ANOVA procedure with dietary treatment and mixing as main effect. For the growth data, pen was considered as the experimental unit. Blood parameter data were evaluated using dietary treatment and time (corresponding to mixing) as effects. Each pig was considered as the experimental unit. Dietary treatment, time, treatment and time interactions were included in the model. No effect of treatment and time interaction was observed for any blood variables; these data are therefore not presented. Treatment effects were deemed significant at $P < 0.05$ and a trend was noted when $P < 0.10$. Global score was calculated as sum of the single score of each items of rating scale and analysed using ANOVA with dietary treatment and mixing as effects; subsequently each item was calculated as frequency of observed item and a contingency table with the corresponding chi-squared statistics was used. Unpaired t-test was used to compare difference in activity and occupation indices between animals of CON and PE pen before and after mixing.

## 3. Results

*3.1 Growth Performance and Blood Welfare Indices*

At mixing, corresponding to day 37, mean body weight was 15.23 kg (CON) and 15.80 kg (PE) in the mixed pens; average daily gain in the period from d 37 to d 59 was 0.593 kg and 0.637 kg in CON and PE group

respectively. At the end of the trial mean average weight of all animals involved was 29.85 kg and 30.1 kg in PE and CON group respectively (P > 0.05). No effect of mixing was detected. Results on blood welfare indices are reported in Table 2. A higher blood antiradical capacity due to mixing ($P < 0.012$) and to dietary treatment ($P < 0.037$) was found. Dietary treatment ($P = 0.035$) affected serum haptoglobin, while no effect of mixing was found. Serum cortisol was not affected by dietary treatment and mixing showed a trend ($P = 0.091$) effect, with lower values in the PE group than the Control one.

Table 2. Welfare blood indicators of piglets fed control diet (CON) and diet supplemented with plant extract (PE) before and after mixing

| Blood Parameters | Diet (D) | | Mixing (M) | | s.e. | *P*-value | |
|---|---|---|---|---|---|---|---|
| | CON (N=28) | PE (N=28) | No (37d) (N=28) | Yes (39d) (N=28) | | D | M |
| Total antioxidant activity | | | | | | | |
| Blood, $HT_{50}$ min | 78.24 | 82.57 | 77.86 | 83.12 | 1.95 | * | * |
| RBC, $HT_{50}$ min | 63.98 | 62.02 | 64.22 | 61.79 | 2.48 | ns | ns |
| Cortisol, g/dL | 6.23 | 7.08 | 5.87 | 7.45 | 0.88 | ns | 0.09 |
| Haptoglobin, mg/mL | 2.54 | 1.94 | 2.28 | 2.20 | 0.27 | * | ns |

*Note.* CON = Control; PE = Plant extract; $HT_{50}$ = half-haemolysis time; s.e: Pooled standard error; *Level of significance of 0.05; ns: not significant.

### *3.2 Behaviour Indicators*

#### 3.2.1 Rating Scale

The global score resulting from the sum of the answers to the 10 items, before mixing showed a higher ($P < 0.001$) value than after mixing (9.92 vs. 5.41). No differences related to dietary treatment were found. Table 3 shows the frequency of abnormal behaviour observed (presence) at T0 and T1. Mixing of piglets resulted in a significant increase of lesions attributed to hetero lesions (from 0% to 50%). We also observed an increase of the lesions on the anterior part (from 25% to 75%) and posterior part of the body (from 12.5% to 100%) as well as an increase of the ear lesions (from 50% to 100%). There were no significant differences for other issue of rating scale examined.

Table 3. Frequency of abnormal behaviours observed (presence) of piglets in pen before (T0) and after (T1) mixing

| | | Frequency% | | *P*-value |
|---|---|---|---|---|
| | | T0 | T1 | |
| 1 | Are there groups of huddling piglets? | 0 | 0 | ns |
| 2 | Are there lesions caused by other piglets? | 0 | 50 | * |
| 3 | Are there lesions on anterior part of the body? | 25 | 75 | * |
| 4 | Are there lesions on back of the body? | 12.5 | 100 | ** |
| 5 | Is there “bell noising”? | 62.5 | 25 | ns |
| 6 | Are there competitive behaviours? | 12.5 | 0 | ns |
| 7 | Are there ear lesions? | 50 | 100 | * |
| 8 | Are there tail lesions? | 0 | 25 | ns |
| 9 | Is there ear biting behaviour? | 62 | 75 | ns |
| 10 | Is there tail biting? | 0 | 0 | ns |

*Note*. *, ** Levels of significance of 0.05 and 0.01, respectively; ns: not significant; T0: (30-32-34-36 d) not mixed; T1 (39-41-43-45 d) after mixing.

#### 3.2.2 Precision Livestock Farming

Observations of the recorded images relative to activity and occupation indices during the day are shown in Figure 1.

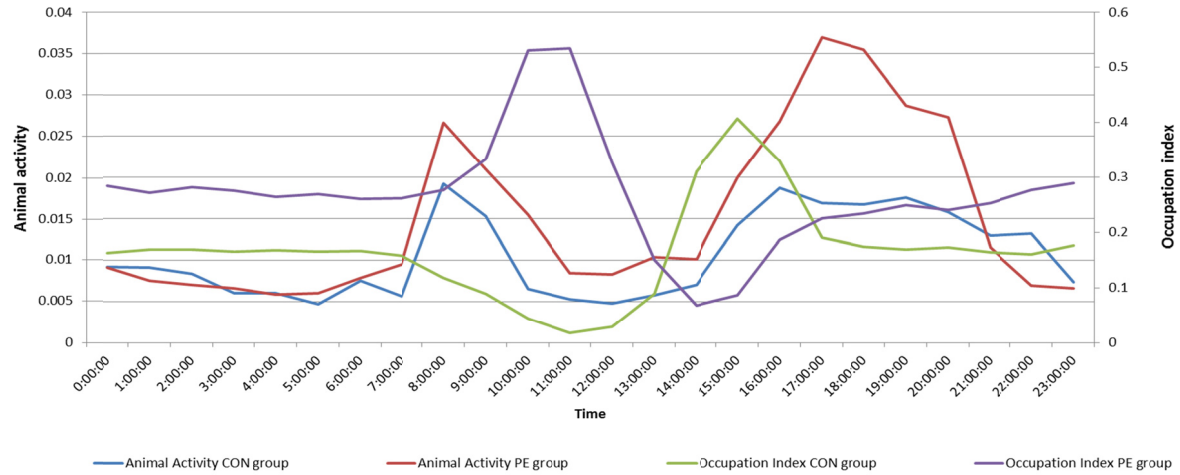


Figure 1. Daily piglet activity and occupation index in control (CON) and plant extract (PE) group

*Note*. CON group: piglets fed control diet; PE group: piglets fed diet supplemented with plant extract; Time: hours.

An increase in animal activity and occupation indices with the peaks related to diurnal rhythm and human interaction for management routine inspection is observed. The analysis of the images recorded from 12:00 am to 7:00 am, showed a mean activity index in a small range from 0.0070 to 0.0074 units.

Considering the time period T0-T1, corresponding to time before and after mixing respectively. animal activity was significantly influenced by dietary treatment (0.010 unit vs 0,015 in CON and PE group) but no effect of mixing was detected (0.013 vs. 0.012 units) (Figure 2).

Instead occupation index significantly increased after mixing of animals in both dietary groups (0.189 vs. 0.232 units) (Figure 3).

Moreover with labelling procedures it was observed that after mixing, behaviours were significantly increased ($P < 0.001$) showing (1286/57600 vs. 3549/57600) frame for nuzzling and (87/57600 vs. 526/57600) frame for ear biting respectively.

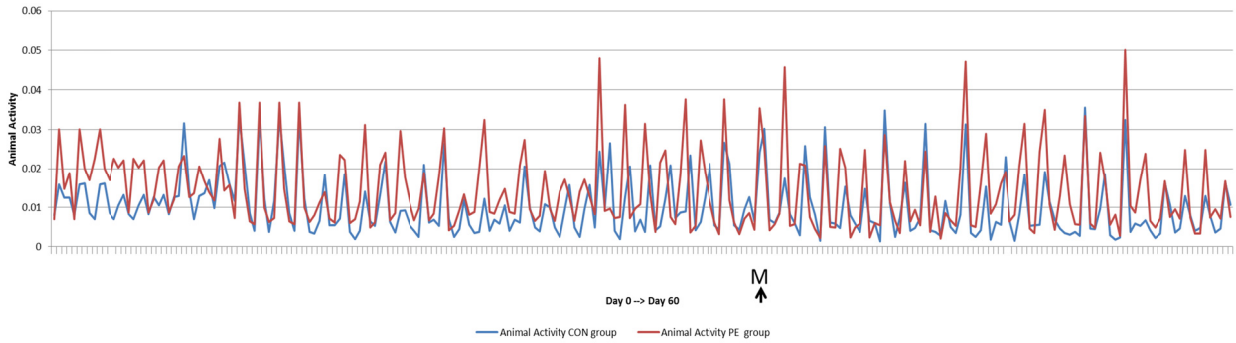


Figure 2. Piglet activity in control (CON) and Plant extract (PE) group during the experimental trial

*Note*. CON group: piglets fed control diet; PE: piglets fed diet supplemented with plant extract; M: mixing.

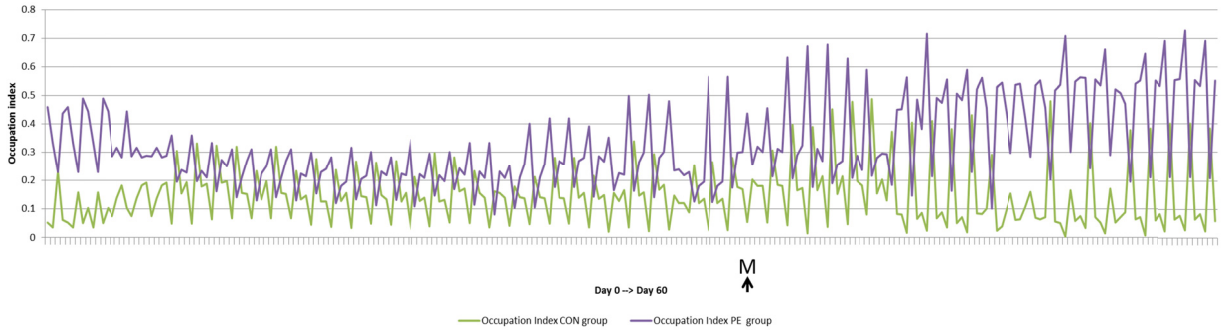


Figure 3. Piglet occupation index in the control (CON) and Plant extract (PE) group during the experimental trial

*Note*. CON group: piglets fed control diet; PE group: piglets fed diet supplemented with plant extract; M: mixing.

## 4.Discussion

### *4.1 Growth Performance*

Animals may use a variety of behaviours when they try to deal with a particular stressful situation; for this reason animal behaviour is an integral response to all thermal, nutritional and health factors (Broom, 2008). In modern production systems, a variety of stressors can modify normal behaviour and growth with resultant poor performance and negative effects on health status. The usual practice of mixing piglets to reduce weight heterogeneity within the pens from different litters post-weaning, may induce psychosocial stress.

After regrouping weaners or fatteners, aggressive interactions usually take place in order to establish new dominance relationships. The mixing of finishing pigs may also significantly depress their productivity. Stress and increased physical activity can imply greater energy expenditure and may affect the weight gain (Fels & Hoy, 2010). In the present study no effect of mixing was found, in agreement with the study of Blackshaw et al. (1987) in which performance parameters were not or slightly affected by regrouping from 1 to 8 litters and were similar between groups at the end of the post-weaning period.

### *4.2 Blood Physiological Indicators*

Stress is also generally thought to suppress the immune system and may lead to an increase in the occurrence of disease in the presence of a pathogen.Natural antioxidants have a role to play in helping the pigs to cope with stress and the linked suppression of the immune system. The blood and red blood cell antiradical activity values found are in line with the reference values for female piglets (Pastorelli et al., 2013).

In the present study the KRL test, that evaluates the antioxidant status of an organism by testing the antioxidant defence, showed a higher ($P < 0.05$) blood total antiradical activity in the PE group than in the CON group.

As recently observed, KRL test may be effective for assessing antioxidant activity of liposoluble and water soluble antioxidants fed to pigs (Rossi et al., 2013). This suggests that due to the content of natural antioxidant fed, endogenous antioxidants were less involved in the reduction of reactive oxygen species**,** thus improving the blood antioxidant status of pigs. This was also observed in a previous study on post-weaned piglets in which the same dietary supplementation improved the oxidative stability measured using the direct production of metabolite reactive oxygen (Pastorelli et al., 2012).

Mixing also showed a significant effect on blood antioxidant activity, probably due to the mobilization of antioxidant reserves in response to oxidative stress (Prost, 2004). One possible mechanism suggests that in humans an event causing oxidative stress such as exposure to oxidant air pollutants, or exercise can initiate chain reactions, cause lipid peroxidation and protein oxidation-generating bioactive molecules, and deplete the antioxidants at the target sites (Elsayed & Bendich 2001). These events would activate signal transduction pathways (Azevedo et al., 2000) that in turn might cause antioxidant mobilization if the body's antioxidant stores are high. In this case, tolerance, repair, and recovery may occur. However, if antioxidants are deficient and the antioxidant capacity is limited, injury will progress and direct or indirect damage and organ dysfunction will occur. Similar results were observed in rabbits and horses exposed to intense physical exercise (Alipour et al., 2006; Wiesław, 2010). At the end of the induced physical stress values returned to baseline.

Haptoglobin is considered to be a reliable indicator of inflammation (Le Floc'h et al., 2009), stress (Piñeiro et al., 2007) and health status as reviewed by Petersen et al. (2004). The concentrations of haptoglobin in serum

increase in animals chronically stressed and/or exposed to inflammatory activities. No effect was detected in serum of pigs after acute stress (Hicks et al., 1998). In the present trial haptoglobin was used as a marker of the health status of pigs. No effect was found after the induced stress of mixing, in accord with the results of Quinonero et al. (2012); we speculated that mixing in this case did not act as a chronic stress. The concentrations of Hp in the present research were lower ($P < 0.05$) in the PE group.

Haptoglobin increases as a result of inflammation caused by tissue damage or infection (Petersen et al., 2004) typical of the postweaning period and an integration of natural antioxidant may help. Our findings are in agreement with previous studies, in which PE has been shown to display anti-inflammatory effects (Kantas et al., 2015). Moreover the significant decrease in haptoglobin found in the treated group was in agreement with results of the KRL test showing that the water soluble antioxidants are integrated in a biological pathway, contributing to the protection of the cells' integrity (Lesgards et al., 2005).

Most data from the literature concern the acute response of cortisol after a single social challenge in an unfamiliar environment have demonstrated its increase in plasma (Otten et al., 1997). The pigs have to cope with novelty in their life environment and with social challenges, which may cause injuries and physiological reactions of acute stress. Fighting between newly mixed pigs over the first 24 h is part of the process necessary to establish a dominance order (Meese & Ewbank, 1972). As for other domestic mammals, once established, this social hierarchy regulates aggressive interactions, improves the predictability of social relationships between animals and thus reduces stressful social encounters in stable groups (Tennessen, 1989).

The increases in cortisol levels are in agreement with data of Merlot et al. (2004) and Coutellier et al. (2007) who found a significant effect after 4-5 hours after regrouping. In the present study, we found just a trend towards an increase in cortisol levels, probably due to the time of analysis that was done after two days from mixing. It is likely that there is an increase in serum concentrations during the acute phase, inducing neoglucogenesis and higher glucose utilization (Fagundes et al., 2008).

During fighting due to mixing, injuries can occur through physical contact (Turner et al., 2006), and the production of stress hormones such as adrenaline and cortisol can arise from unresolved aggression (Arey & Edwards, 1998). Supplementing natural extract in the diet did not affect the level of cortisol in the blood according to Bonnette et al. (1990) who found no effect of dietary antioxidant (vitamin E) on cortisol of piglets.

*4.3 Behaviour Indicators*

Both techniques, PLF and rating scale, showed a significant variation in the abnormal animal behaviours, as a result of the mixing of the animals. As a result of animal mixing, the rating scale showed a decrease of welfare, represented by a lower global score, according to labelling procedures (increase of ear biting and nuzzling behaviour). This could be explained by the fact that in nature piglets rarely allow new subjects to be a part of their social group. Continuous monitoring of pigs by PLF showed how animal activity was greater in the PE group than the CON that exhibited quieter behaviour. This result is not easy to explain; Helliòn-Ibarrola et al. (2006) in mice treated with 10.0 mg/kg of extract put orally of the *Aloysia polystachya* extract, (Verbenaceae), found a significant increase in the exploratory head-dipping behaviour observed after the treatment, reinforcing the anxiolytic-like activity. The results found herein could be considered as an indicator of greater well-being of the animals because welfare is improved when animals can express their normal behaviour (Hemsworth et al., 2015). Signs of depression, frustration, chronic apathy and boredom can be considered inactive behaviours as reported by Meagher and Mason (2012). Normally occupation indices change when the animals modify their behaviour in consequence of something that happened. The occupation index could be a sensitive indicator of the physiological status of the animals that is determined by a wide number of variables, including the microclimate condition of the building. In the present trial the microclimatic conditions followed the normal piggeries environment and no anomalies were recorded.

## 5. Conclusion

A main element of an indoor intensive system is the continuous monitoring of the animal itself as the most crucial element in the biological production process. In the present study, image analysis of animal activity proved to be a promising tool for monitoring of pigs' behaviour; moreover it does not involve high costs and does not interfere with or manipulate the animals. Rating scale and PLF revealed a modification of behaviour represented by an increase of ear biting and nuzzling after the induced stress of mixing. The KRL test was an effective method for assessing antioxidant activity following dietary treatments and mixing, whereas cortisol and haptoglobin tests were not able to detect any significant differences. Dietary natural antioxidant did not affect any other results. Further research on the response of animals to other induced stresses could take into account the measurements of behaviour and welfare with the new tools used in this study.

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