Effects of antioxidant mixtures in the diet of finishing pigs on the oxidative status and shelf life of longissimus dorsi muscle packaged under modified atmosphere^{1,2}

R. Rossi,³ S. Stella, S. Ratti, F. Maghin, E. Tirloni, and C. Corino

Università degli Studi di Milano, Department of Health, Animal Science and Food Safety, Via Celoria 10, 20133 Milan, Italy

ABSTRACT: The effect of pig dietary supplementation with an antioxidant mixture (AOX), containing vitamin E and verbascoside, on animal oxidative status, meat quality parameters, and shelf life of the longissimus dorsi (LD) muscle was examined. Seventy pigs with an average live weight of 95.2 ± 1.2 kg were selected and assigned to 2 dietary treatments. The control (CTR) group was fed a commercial diet, and the AOX group was fed the same diet supplemented with the AOX, containing vitamin E and verbascoside from Verbenaceae extract, for 45 d before slaughter. At the beginning and at the end of the trial, blood samples were collected to determine oxidative status, using the Kit Radicaux Libres test. At slaughter, carcass weight was recorded and LD muscles from 10 pigs per treatment were sampled. Physical, chemical, microbiological, and sensory parameters and oxidative

stability of LD muscle were assessed for up to 21 d of storage at 4°C under modified atmosphere packaging. Dietary AOX positively affected ($P < 0.05$) oxidative status and carcass dressing percentage. The oxidative and color stability of the LD muscle were improved ($P < 0.05$) in the AOX group compared with the control. The sensory shelf life revealed that at 15 d of storage, meat from the AOX group was comparable ($P < 0.05$) to the fresh meat in appearance and aroma. A lower ($P < 0.05$) *Pseudomonas* spp. load was observed in the AOX samples than in the control samples. No other microbiological parameters were affected by dietary treatment. Overall, the present data showed that dietary AOX supplementation in pigs improved in vivo antioxidant status and exerted antioxidant and antimicrobial effects, thus enhancing the shelf life of raw pork under commercial conditions.

Key words: antioxidant, meat quality, microbiological parameters, shelf life, verbascoside

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INTRODUCTION

Improving meat quality parameters and prolonging the shelf life are important for both producers and consumers. Attention has focused on lipid oxidation and undesired microbial replication, which represent the main spoilage phenomena affecting meat quality (Fung, 2010; Dave and Ghaly, 2011). In fact, both lipid oxidation and microbiological replication reduce meat shelf life, negatively affecting nutritional

value, color, and sensory parameters and, therefore, consumer acceptance (Zhao et al., 1994).

One promising strategy to reduce meat deterioration and improve shelf life is the use of antioxidant molecules (Engel et al., 2015). In previous works, functional properties of plant extracts containing verbascoside was investigated for their potential use as dietary antioxidants in pigs in enhancing oxidative status and meat quality (Pastorelli et al., 2012; Rossi et al., 2013a; Di Giancamillo et al., 2015). Verbascode has been considered an effective scavenger of free radicals and an inhibitor of lipid peroxidation (Korkina, 2007).

In addition, antibacterial properties of verbascoside have been reported (Soler-Rivas et al., 2000; Rajendran and Basha, 2010). Consequently, verbascoside could be used as a natural antioxidant and preservative in meat stored in commercial conditions to enhance the shelf life. Packaging is also fundamental to prolong product shelf life in commercial conditions.

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³Corresponding author: raffaella.rossi@unimi.it

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The storage of meat in modified atmosphere packaging (**MAP**) has been proved to enhance the shelf life of meat for fresh consumption (Hayes et al., 2010).

To the best of our knowledge, the effects of dietary antioxidant mixture supplementation in pigs on pork quality and shelf life of fresh pork stored under MAP are still unknown. Therefore, this study evaluated the effects of pigs' dietary supplementation with an antioxidant mixture on pig oxidative status and meat quality parameters and the shelf life of pork longissimus dorsi (**LD**) stored under MAP.

MATERIAL AND METHODS

Animal and Dietary Treatment

Procedures involving animals were performed in accordance with European Commission (2010) and approved by the Italian Ministry of Health.

The trial was performed on a commercial farm, located in the north of Italy, producing medium-heavy pigs slaughtered at about 130 kg BW. A total of 70 pigs (PIC [Pig Improvement Company, PIC Italia Spa, Perugia, Italy] × Maxgrow [Hermitage Italia srl, Reggio Emilia, Italy]), half barrows and half females, with a mean of 95.2 kg (SE 1.2) BW were randomly selected and assigned to 2 dietary treatments. Pigs were kept in 10 pens (5 pens per treatment) balanced for BW. The control (**CTR**) group received a commercial diet, and the antioxidant mixture (**AOX**) group received the commercial diet supplemented with the AOX, containing vitamin E and verbascoside from Verbenaceae extract.

The diets were isoenergetic and isolipidic and were formulated to meet the requirements for all nutrients (NRC, 2012). The composition of the experimental diets is reported in Table 1. Pigs were fed a corn-based diet twice daily and were rationed according to 9% of metabolic weight. Liquid feed was produced fresh each morning with a water:concentrate ratio of 3:1. The AOX supplement was supplemented in the experimental diets and provided a daily amount of about 150 mg vitamin E and 15 mg verbascoside (Rossi et al., 2014). The animals received the antioxidant supplement for 45 d before slaughter. The AOX supplement was composed of a water-soluble extract of Verbenaceae (*Lippia* spp.) leaves, prepared on an industrial scale using a standardized procedure including ultrasonic extraction with 60% aqueous ethyl alcohol followed by spray-drying with maltodextrins as an excipient. The phenylpropanoid glycosides and benzoic acid content of the feed supplement, was 1.75 ± 0.07 g/kg gallic acid, 0.45 ± 0.04 g/kg 3,4-dihydroxybenzoic acid, 1.91 ± 0.09 g/kg methyl gallate, 0.43 ± 0.04 g/kg isoverbascoside, and 4.47 ± 0.08 g/kg verbascoside.

To define the quantitative analysis of the phenolic compounds, HPLC-UV–diode array detector methods were used (Piccinelli et al., 2004). Microencapsulation technology with a protective matrix of hydrogenated vegetable lipids (spray-cooling technology) was used to protect the supplement from oxidative processes (Sintal Zootecnica, Isola Vicentina, Vicenza, Italy).

Animal BW was recorded at the beginning and at the end of the trial. Feed intake was recorded, and ADG and G:F were calculated.

Blood Samples

At the beginning and end of the trial, fasting blood samples were collected from 10 randomly selected females pigs per treatment (2 pigs/pen) by cranial vena cava puncture before the morning feeding. The blood samples were collected in 10-mL vacutainer glass tubes containing EDTA (Venoject; Terumo Europe N.V., Leuven, Belgium) and immediately stored at 4°C. Analyses were performed within 24 h of collection.

Carcass Traits and Sampling

Pigs were slaughtered in a commercial slaughterhouse (Hauser Carni S.p.a., Mezzocorona, Trento, Italy) at 130.1 ± 1.5 kg BW. Pigs were electrically stunned, and following exsanguination, the carcasses were scalded, de-haired, and eviscerated. Hot carcass weight was recorded and dressing percentage (HCW/live weight at slaughter) was calculated. Carcasses were stored at 2°C for 24 h, and then the left LD muscle was randomly selected from 10 pigs per treatment (2 pigs per pen) and excised from each carcass. The LD samples were weighed and their length was measured (cm), and then the samples were vacuum-packed, maintained under refrigerated conditions, and transported to a commercial packaging factory.

Kit Radicaux Libres Test

The total antiradical activity of whole blood and red blood cells (**RBC**) for each pig was evaluated using the Kit Radicaux Libres (**KRL**) biological test (Laboratoires Spiral s.a., Couteron, France). 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 2,2'-azobis(2-amidinopropane) dihydrochloride (Kirial International, Dijon, France). Hemolysis 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 KRL values, which represent the time, in minutes, required to reach

Table 1. Ingredients of experimental diet (as-fed basis)

Item	CTR ¹	AOX ²
Ingredient, g/kg of feed		
Corn	440.2	440.2
Soybean meal, 44% CP	168.8	168.8
Barley	140.3	140.3
Wheat middlings	99.7	98.7
Wheat bran	80.5	80.5
Beet pulp	29.5	29.5
Calcium carbonate	14.0	14.0
Trace element and vitamin premix ³	7.8	7.8
Dicalcium phosphate	8.2	8.2
Animal fat	4.9	4.9
NaCl	2.1	2.1
L-Lysine HCL	1.0	1.0
AOX supplement ⁴	—	1.0
DL-Methionine	0.8	0.8
L-Threonine	0.2	0.2
Calculated composition, g/kg		
ME, kcal/kg	3,155	3,155
CP, %	156.1	156.1
EE, ⁵ %	34.0	34.0
Lys, %	11.2	11.2
Met + Cystine, %	5.1	5.1
Thr, %	6.9	6.9
Trp, %	2.8	2.8
Ca, %	8.1	8.1
P, %	6.2	6.2

¹CTR = control.²AOC = antioxidant mixture.

³Vitamin-trace mineral premix provided the following nutrients per kilogram of diet: 3 mg all-*trans* retinol, 0.049 mg cholecalciferol, 15 mg vitamin E, 3.0 mg riboflavin, 0.015 mg cyanocobalamin, 10 mg pantothenic acid, 150 mg Fe as iron sulfate, 0.5 mg I as Ca(IO)₃, 0.3 mg Se as Na₂Se, 12 mg Cu as CuSO₄, 100 mg Zn as ZnO₂, and 50 mg Mn as MnO₂.

⁴The AOX supplement contained, per kilogram, 50 mg vitamin E and a water-soluble extract of Verbenaceae (*Lippia* spp.) leaves, titrated in verbascoside, to obtain 5 mg verbascoside.

⁵EE = ether extract.

50% of maximal hemolysis, representing whole blood resistance to free-radical attack. Intra- and interassay CV of the KRL test were 2.5 and 4%, respectively.

Pork Packaging and Storage

The LD samples were aseptically cut into steaks (slices of 7.5 mm thickness and 60 g average weight) and placed in low oxygen permeable ($< 1 \text{ cm}^3/\text{m}^2 \cdot 24 \text{ h}$ at standard temperature and pressure) polystyrene/ethylvinylalcohol/polyethylene trays (2 slices per trays). Trays were then flushed with 70:20:10% O₂:CO₂:N₂ using a vacuum-sealing unit (VS 100; Gustav Müller & Co. KG, Homburg, Germany) equipped with a gas mixer (Witt-Gasetechnik GmbH & Co. KG, Witten, Germany). Trays were covered and heat-sealed using a low oxygen permeable ($3 \text{ cm}^3/\text{m}^2 \cdot 24 \text{ h}$ at standard temperature and

pressure) laminated barrier film with a polyolefin heat-sealable layer. After packaging, the samples were placed in a refrigerator at $3 \pm 1^\circ\text{C}$ for up to 21 d. The temperature was monitored using a temperature data logger (Testo 175 T1; Testo SpA, Settimo Milanese, Milan, Italy). At the different time points, 2 trays per LD sample (4 slices) were removed from refrigerator and the samples were analyzed for physicochemical parameters, lipid oxidation, and microbiological analyses.

Physicochemical Parameters

All the analyses were performed on Days 0, 6, 12, 15, 18, and 21 of refrigerated storage at 4°C . For the pH determination, pork samples (10 g) were minced and combined with 90 mL distilled water and homogenized at 10,000 rpm for 1 min. The pH measurements were assessed using a pH meter (334-B; AMEL S.r.l., Milan, Italy). The pH was calibrated using 2 buffers (pH 4.0 and 7.0), and maintenance of calibration was monitored between samples.

Instrumental L*, a*, and b* color evaluations were recorded, using a CR-300 ChromaMeter (Minolta Camera Co., Ltd., Osaka, Japan), to assess color stability. The instrument was calibrated on the CIE L*, a*, and b* color space system using a white calibration plate (Calibration Plate CR-A43; Minolta Camera Co., Ltd.). The colorimeter had an 8-mm measuring area and was illuminated with a pulsed Xenon arc lamp (illuminated C) at a 0° viewing angle. Reflectance measurements were obtained at a viewing angle of 0° and the spectral component was included. To allow blooming, the trays were opened, and after 30 min, the data were collected. Each value was the mean of 6 measurements measured on the surface of the meat.

The color parameters were used to calculate total color changes (ΔE ; Boakye and Mittal, 1996) at each sampling, according to the following equation: $\Delta E = [(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2]^{1/2}$, in which L* is the lightness value; L₀* represents the lightness value at time zero; a* is redness value, where positive and negative values represent red and green color, respectively; a₀* represents redness value at time zero; b* is the yellowness value, where positive and negative values represent yellow and blue color, respectively; and b₀* represents the yellowness value at time zero.

Water-holding capacity (WHC) was measured on duplicate samples in line with the centrifugation method described by Jauregui et al. (1981). Two pieces of Whatman number 3 filter paper were folded around 1 piece of grade 410 filter paper to form a thimble. Approximately 1.5 g of muscle was inserted into the filter paper thimble and then into a 50-mL centrifuge tube. The sample was submitted to centrifugation at $30,000 \times g$

for 15 min at 4°C. Water-holding capacity was expressed as percentage weight loss from the original sample.

Determinations of moisture (method 985.41), ash (method 920.153), fat (method 960.39), and CP (method 928.08) content were made in duplicate on the samples of LD muscle according to the methods published by the AOAC International (AOAC, 2000).

Measurement of Lipid Oxidation

All the analyses were performed in duplicate on Days 0, 6, 12, 15, 18, and 21 of refrigerated storage at 4°C. Lipid oxidation was determined using the thiobarbituric acid reactive substances (TBARS) method as described by Lorenzo et al. (2014). The TBARS values were calculated from a standard curve of malonaldehyde (MDA) and expressed as milligrams MDA per kilogram sample.

Microbiological Analyses

All the analyses were performed in duplicate on Days 0, 6, 12, 15, 18, and 21 of refrigerated storage at 4°C. Two 4- to 5-mm-thick slices of 4 by 5 cm each were excised from the surface of all muscles using a stainless steel, sterile, rectangular template. A similar procedure was followed on opening the containers after the different storage times. For microbial counts, each sample were homogenized in 90 mL of diluent solution (0.85% NaCl and 0.1% peptone), and serial 10-fold dilutions were performed in sterile saline. Total viable count was determined according to ISO (2003a). The number of Enterobacteriaceae was determined according to ISO (2004). *Escherichia coli* counts were determined according to ISO (2001) and coagulase-positive *Staphylococcus* were determined following AFNOR (2004). *Pseudomonas* spp. counts were performed on Pseudomonas Agar Base with the addition of Pseudomonas CFC Supplement (Oxoid S.p.A., Milan, Italy), incubated at 30°C for 48 h. *Lactobacillus* were enumerated on de Man–Rogosa–Sharpe agar (Oxoid S.p.A., Milan, Italy). Plates were incubated at 30°C for 48 h under anaerobic conditions (AnaeroGen; Oxoid S.p.A., Milan, Italy). *Brochothrix thermosphacta* was enumerated according to the ISO (1996) method. A spore count of sulfite-reducing Clostridia was performed, following ISO (2003b), after pasteurization of the dilutions. *Salmonella* spp. detection was performed following ISO (2012). Detection and enumeration of *Listeria monocytogenes* were performed according to AFNOR methods (AFNOR, 1998). Microbiological data were transformed into logarithms of the number of cfu (cfu/g).

Sensory Shelf Life

The sensory shelf life is designed to validate the length of time that a product will have the same “acceptable quality” over its lifespan. Changes in sensory properties cause consumer reject, because they are very susceptible to products sensory quality. The sensory shelf life was assessed according to the sensory quality system (Beckley and Kroll, 1996), using the difference from control method that evaluates visual and aromatic attributes (color and global smell). A reverse storage design was implemented using freezing to halt all deterioration processes (Lawless and Heymann, 2010). The differences from the control method were used to rate each visual and aromatic attribute relative to a gold standard. The gold standard referred to the CTR samples at the beginning of storage (Day 0), as a reference for fresh meat. To halt all the deterioration processes, the CTR samples were stored at –20°C until the sensory evaluation. All the meat slices (CTR vs. AOX) were stored at 4°C for 6, 12, 15, 18, and 21 d and then frozen at –20°C to have the same conditions as the CTR sample. On the evaluation day, the samples were defrosted (at 4°C for 24 h) and removed from the trays. The evaluation was stopped when the sensory shelf life end point was archived (global smell and color between samples and gold standard were significantly different; $P < 0.05$).

The sensory panel was composed of 18 members who were familiar with meat and sensory procedures. The mean age of the assessors was 36 (24–46 yr old). All were either students or employees of the University of Milan (Italy). All assessments were performed in a sensory laboratory according to ISO (2007) recommendations.

The LD samples were evaluated by the panel in 3 sessions. Within each session, the design was balanced for order and carryover effects (MacFie et al., 1989) and the samples were randomized considering sampling storage. The sessions were conducted considering the set of defrosted samples (CTR vs. AOX). The panelists evaluated 4 samples per session (gold standard versus 6, 12, and 15 d of storage). The meat slices (1 slice per panelist) were individually presented in dishes. The panelists were not informed about the experimental approach, and the samples were blind-coded with random 3-digit numbers. Panelists were requested to evaluate the intensity of each attribute using a 10-cm unstructured line scale with 3 anchors: –5, 0, and +5. The gold standard was assigned “0” on the scale, and those samples that were less intense in terms of each attribute compared with the standard were rated less than 5, whereas those that were more intense were rated greater than 5 (Pecore and Kellen, 2002).

Statistical Analysis

Statistical analyses of the data were performed using SPSS (SPSS/PC Statistics 21.0; SPSS Inc., Chicago, IL). The data on growth performance, antioxidant status, and physical and chemical meat parameters were analyzed using 1-way ANOVA with dietary treatment as main effects. Sex was excluded from the model because the difference was not significant ($P > 0.05$). The microbiological data were assessed using 2-way ANOVA, to evidence the effects of treatment, time, and their interactions. The data on TBARS and color indices during storage time were assessed using 1-way repeated measures ANOVA to evidence the effects of treatment, time, and their interactions. Linear regression was used to evaluate correlation between muscle TBARS and redness values. The sensory data for each attribute were submitted to 1-way ANOVA for treatment effects. The significance of these effects was tested with *t*-tests. Means were compared according to Duncan's test. Pen was the experimental unit for the growth performance parameter. Each pig was considered the experimental unit for antioxidant status and meat quality parameters. Data are presented as means $\pm$ SEM, and a value of $P \leq 0.05$ was used to indicate statistical significance.

RESULTS AND DISCUSSION

Growth Performance

No significant effect of dietary treatments on pig performance was detected. The average BW of the pig at slaughter was 130.7 ± 1.6 kg. There was no significant effect ($P > 0.05$) of dietary treatment on ADG (839 ± 28 g/d for the CTR vs. 791 ± 26 g/d for the AOX) and G:F (0.27 ± 0.08 for the CTR vs. 0.25 ± 0.08 for the AOX). The present data are in agreement with the literature. In fact, previous studies have reported that dietary supplementation with dietary antioxidants containing polyphenols did not affect growth performance in pigs (Rossi et al., 2013b; Ranucci et al., 2015).

Antioxidant Status

In the present study, dietary supplementation with the AOX improved ($P < 0.05$) the total antioxidant status of the animals, expressed as a KRL value (94.4 ± 2.81 for the CTR vs. 124.8 ± 3.74 for the AOX). No differences ($P > 0.05$) in RBC between dietary treatments were observed. In the AOX group, these data suggest that the increased amount of dietary polyphenols and vitamin E may protect the endogenous antioxidants involved in the balance between pro- and antioxidants, improving pig blood antioxidant status.

In the literature, the prevention and/or reduction of oxidative stress with natural antioxidant supplementation has been already reported (Costa et al., 2010; Pastorelli et al., 2013). The ability of water-soluble extracts of Verbenaceae to strengthen the endogenous antioxidant system was confirmed in agreement with previous studies in many livestock species fed the same extract (Palazzo et al., 2011; Casamassima et al., 2012, 2015; Rossi et al., 2013b). Dietary supplementation with natural antioxidants has been found to not only reduce oxidative stress markers but also improve pork quality (Lahucky et al., 2010; Rossi et al., 2014).

The mechanism of action of verbascoside are still not fully clarified. Actually, it is hard to obtain final data on the bioavailability and bioactivity of a single phenolic compound, due the synergistic activity of polyphenol mixtures contained in plant extracts. Some studies reported that these molecules are partly absorbed in the gut, transferred into the circulatory stream, and transported to the target tissues (Wu et al., 2006; Funes et al., 2009). Previous study in rats fed natural extracts containing verbascoside reported that it was partially absorbed and the main compounds found in plasma are verbascoside and isoverbascoside, whereas minor metabolites were hydroxytyrosol, caffeic acid, and ferulic acid and its glucuronic and homoprotocatechuic acids (Quirantes-Piné et al., 2013). These compounds are able to modulate the enzymatic activation of superoxide dismutase, glutathione peroxidase, and catalase activities, positively affecting the endogenous antioxidant system (Alipieva et al., 2014). Moreover, a recent in vitro study reported that verbascoside is an activator of nuclear factor E2-related factor 2 (*Nrf2*) and induce the expression of heme oxygenase-1 (*HO-1*), which is a cytoprotective gene, in response to oxidative damage (Wang et al., 2012).

Carcass Characteristics

The data on carcass and LD muscle characteristics are reported in Table 2. Dietary supplementation with the AOX positively affected ($P < 0.01$) the dressing percentage. In a previous study, we observed a higher carcass and LD muscle weight in pigs fed the same AOX (Rossi et al., 2014). Recently, Hanczakowska et al. (2015) and Ranucci et al. (2015) reported no effects of dietary supplementation with natural antioxidants on carcass characteristics, which is probably related to the different dietary supplementation.

By what means verbascoside can improve carcass characteristics is difficult to clarify, but different metabolic pathways should be considered. The antioxidant activity, which protects the intestinal mucosa from damage, and the antibacterial activity against pathogens could both improve gut health and thereby in-

Table 2. Carcass and longissimus dorsi (LD) characteristic in pig fed pigs fed the control (CTR) or the antioxidant mixture (AOX) supplemented diet¹

Item	CTR	AOX	<i>P</i> -value
Carcass weight, kg	103.35 (1.51)	104.6 (1.25)	0.359
Dressing percentage, %	79.02 (0.22) ^a	80.10 (0.11) ^b	0.006
LD weight, kg	4.06 (0.09)	4.22 (0.10)	0.281
LD length, cm	51.7 (1.03)	52.5 (0.94)	0.584

^{a,b}Means within a row with different superscripts differ ($P < 0.05$).

¹Data are reported as mean (SEM).

crease the availability and absorption of nutrients and, in particular, AA. The antiinflammatory activity that decreases the catabolic effect of protein is activated primarily by proinflammatory cytokines. Moreover, recently, Valenzuela-Grijalva et al. (2017) reported that polyphenols might have an anabolic activity by modulating animal metabolism in a way comparable to the action of β -adrenergic agonist compounds.

Physical and Chemical Parameters

The pH values at 24 h and during storage were stable during the storage time, with the mean values ranging from 5.63 to 5.67, without significant differences ($P > 0.05$) between the dietary treatments.

Water-holding capacity is an important property that influences the quality of pork for fresh consumption. The data showed that WHC was not affected ($P > 0.05$) by the dietary treatment (Fig. 1). The values observed in the present study were in agreement with previous data obtained from similar pork cuts and fall within the range observed by Brøndum et al. (2000). No previous studies have reported the WHC values of pork steaks for fresh consumption stored in MAP at 4°C for 21 d. Hayes et al. (2010) reported that in raw minced pork patties stored in MAP at 4°C for 12 d, the addition of lutein, sesamol, ellagic acid, and olive leaf extract did not affect WHC. In contrast, we found a significant effect ($P < 0.001$) of storage time on WHC. A decrease in water loss from time 0 and the other sampling times followed by a stable trend was observed. This could be due to an initial loss of water due to natural leakage from cut surfaces as stated by Van Moeseke and De Smet (1999).

Figures 2A, 2B, and 2C show the changes in color indices measured at the surface of the LD muscle in relation to dietary treatment during 21 d of refrigerated storage in MAP. The lightness (L^*) values were not affected by dietary treatment ($P > 0.05$), in agreement with O'Grady et al. (2008) in raw meat stored in a modified atmosphere pack at 4°C from pigs fed grape seed extract and bearberries. Storage time affected ($P < 0.01$) the L^* values, in agreement with Muhlisin

Figure 1. Water-holding capacity in longissimus dorsi muscle from pigs fed the control (CTR) or the antioxidant mixture (AOX) supplemented diet in relation to storage time at 4°C under modified atmosphere packaging. Data are reported as mean $\pm$ SEM. Time effect, $P < 0.001$; treatment effect, $P = 0.694$.

et al. (2014), who reported an increase in L^* values of LD samples packed in a modified atmosphere.

The redness (a^*) values were higher ($P < 0.001$) in the LD muscle in pigs fed the AOX than in pigs fed the CTR. The same result was obtained by Lorenzo et al. (2014) in porcine patties with the addition of grape extract and packed in a modified atmosphere. In fact, the color-stabilizing effect of dietary antioxidants has already been reported (Jia et al., 2012; Rossi et al., 2014). The red color stability in the LD muscle from the AOX group indicated a high, persistent color stability during refrigerated storage. This has a positive influence on the consumers' purchasing decision, because it represents an indicator of meat freshness (Brewer et al., 2002).

As expected, a decrease ($P < 0.001$) in redness values in relation to storage time was observed.

No interactions between storage time and treatment were observed ($P = 0.154$). These data are in agreement with previous studies that show that meat discoloration is mostly due to a marked decrease in redness. In addition, a negative correlation ($R = -0.634$, $P < 0.001$) between redness and TBARS values was observed, in agreement with Schmidt et al. (2016). In fact, Mancini and Hunt (2005) reported a decrease in redness in meat due to myoglobin oxidation and metmyoglobin accumulation.

The b^* values were significantly affected by only storage time ($P < 0.001$). No interactions between time and treatment were detected ($P = 0.260$). The yellowness values showed an initial decrease and then an increase from 12 d of storage, not only in the control samples but also in the AOX samples.

The overall trends of total color changes were comparable in both experimental groups, with mean values of 4, but in the CTR group, the changes took place more rapidly. In addition, LD muscle samples from pigs fed the AOX-supplemented diet exhibited a slight ΔE variation, even if not significant. Only at 18 d of refrigerated storage under MAP were the ΔE values higher

($P < 0.05$) in the CTR muscle than in the AOX samples (6.2 ± 0.2 for the CTR vs. 4.3 ± 0.2 for the AOX).

The chemical composition of the LD muscle from pigs fed the CTR and AOX-supplemented diet showed no differences ($P > 0.05$) between experimental groups, with a mean value of $72.4 \pm 0.16\%$ for moisture, $23.01 \pm 0.16\%$ on a wet-weight basis for CP, $1.84 \pm 0.12\%$ on a wet-weight basis for ether extract, and $1.16 \pm 0.01\%$ on a wet-weight basis for ash. In agreement, previous studies reported that dietary supplementation with antioxidants did not affect meat chemical composition (Lahucky et al., 2010; Kołodziej-Skalska et al., 2011; Ranucci et al., 2015). The mean values of intramuscular fat were lower than those reported in our previous work (Rossi et al., 2014) in medium-heavy pigs, which is probably related to the different pig genotype.

Microbiological Parameters

The microorganisms usually involved in meat spoilage are the lactic acid bacteria, *B. thermosphacta*, *Pseudomonas* spp., and Enterobacteriaceae (Pennacchia et al., 2011). The microbial growth is shown in Fig. 3. Total viable count of the LD muscle exhibited a constant and gradual increase ($P < 0.001$) in loads from 3 log cfu/g at 0 d to 7 log cfu/g at 21 d, in agreement with Lorenzo et al. (2014), who observed the same trend in pork patties stored under MAP for 20 d. As expected, during refrigerated storage in MAP, the microbial populations in raw meat increase following the characteristic microbial growth pattern (Garcia-Lopez et al., 1998). No significant difference ($P = 0.149$) was observed in microbial counts between the CTR and the AOX groups. However, considering the value of the total viable count of 7.0 log cfu/g as a threshold limit to determine the end of the shelf life (Ercolini et al., 2011; Tang et al., 2013), in the CTR group, this was exceeded after 15 d, whereas in the AOX muscle, it was reached after 21 d.

Lactic acid bacteria loads increased ($P < 0.001$) during storage (from 1.98 to 4.46 log cfu/g in the CTR and from 1.00 to 4.88 log cfu/g in the AOX), without significant differences ($P = 0.770$) between treatments. A study in beef sirloin stored in MAP reported that the population of lactic acid bacteria reached a level of 4.3 to 6.6 log cfu/g in the acceptable samples from a sensorial point of view (Pennacchia et al., 2011).

Brochothrix thermosphacta, a psychotropic spoilage microorganism usually isolated from raw meat products packaged in different conditions, was characterized by a rapid increase ($P < 0.001$) from time 0 up to the end of the trial (7.24 and 6.78 log cfu/g at time 21 in the CTR and AOX samples, respectively). After 15 d, the samples from both experimental groups exceeded the level of 5 log cfu/g, recognized as the

Figure 2. Color parameters of longissimus dorsi muscle in relation to storage time at 4°C under modified atmosphere packaging from pigs fed the control (CTR) or the antioxidant mixture (AOX) supplemented diet. Data are reported as mean $\pm$ SEM. (A) Lightness (L^*) values: time effect, $P = 0.003$; treatment effect, $P = 0.255$; and time $\times$ treatment, $P < 0.001$. (B) Redness (a^*) values: time effect, $P < 0.001$; treatment effect, $P = 0.017$; and time $\times$ treatment, $P = 0.154$. (C) Yellowness (b^*) values: time effect, $P < 0.001$; treatment effect, $P = 0.782$; and time $\times$ treatment, $P = 0.260$.

Figure 3. Changes in microbial counts of longissimus dorsi muscle under modified atmosphere packaging from pigs fed the control (CTR) or antioxidant mixture (AOX) supplemented diet and stored at 4°C for 21 d as affected by natural antioxidants. (A) Total viable count. (B) Lactic acid bacteria (LAB). (C) *Pseudomonas* spp. (D) *Brochothrix thermosphacta* counts. Data are reported as mean $\pm$ SEM. For the total viable count: time effect, $P < 0.001$; treatment effect, $P = 0.149$; and time $\times$ treatment, $P = 0.618$. For the LAB: time effect, $P < 0.001$; treatment effect, $P = 0.770$; and time $\times$ treatment, $P = 0.095$. For *Pseudomonas* spp.: time effect, $P < 0.001$; treatment effect, $P = 0.011$; and time $\times$ treatment, $P = 0.282$. For *B. thermosphacta*: time effect, $P < 0.001$; treatment effect, $P = 0.071$; and time $\times$ treatment, $P = 0.439$.

limit above which an alteration in smell is likely, described as a cheese–dairy or sewage-like odor. The *B. thermosphacta* loads tended to be lower in the AOX samples ($P = 0.071$) compared with the CTR samples.

Pseudomonas spp. are mainly involved in the deterioration of refrigerated meat (Ercolini et al., 2007). A gradual increase in this parameter ($P < 0.001$) was observed from the first sampling time, without exceeding the threshold limit indicating the spoilage according to Good Manufacturing Practices criterion (Moberg, 1989). The same parameter was significantly lower ($P = 0.011$) in the AOX group compared with the CTR group. The present data seem to indicate that dietary supplementation with the AOX containing vitamin E and verbascoside can have inhibitory properties on *Pseudomonas* spp., a specific spoilage organism in raw pork. To the best of our knowledge, this is the first experimental study to report the antimicrobial activity of verbascoside against spoilage and foodborne pathogen-

ic bacteria. This result suggests that dietary verbascoside did exert antimicrobial activities in the LD muscle.

Exactly how verbascoside acts on microbial populations in meat is still unknown. In fact, polyphenols and their metabolites are able to be transferred in the tissues, but their storage within specific tissues needs additional studies (Manach et al., 2004). However, it is possible that secondary metabolites of verbascoside possess an antimicrobial activity against the growth of microorganisms during meat storage. On the other hand, as previously observed in *in vitro* studies, a natural extract from Verbenaceae, containing verbascoside, inhibited Gram-positive and Gram-negative bacteria growth (Oliveira et al., 2007). In fact, the antimicrobial activities of several natural extracts containing polyphenols have been reported when they are mixed with minced meat or meat products (Georgantelis et al., 2007; Lorenzo et al., 2014; Baldin et al., 2016).

Escherichia coli, Enterobacteriaceae, and Clostridia were below the detection limit (2 log cfu/g)

for all the periods considered. These microorganisms could be considered specific indicators of fecal contamination during slaughter or during processing. Finally, no *Salmonella* spp. and *L. monocytogenes* were detected in any of the samples examined.

Oxidative Stability

Figure 4 shows the oxidative stability of the LD muscle in relation to dietary treatment and storage time. The mean values are in agreement with the data reported by Apple et al. (2007) in pig muscles. The TBARS values were significantly ($P < 0.001$) affected by storage time and dietary treatment. The oxidative stability was higher in the LD muscle from the AOX group than in those from the other group. As expected, storage time negatively affected oxidative stability in the LD muscle. A significant interaction between storage time and treatment was also observed ($P < 0.001$). The TBARS value in the AOX group was lower than the threshold level of fresh meat (0.5 mg MDA/kg muscle) up to 15 d of refrigerated storage (Lanari et al., 1995).

Conversely, in the CTR group, the MDA content reached these values at 6 d. The TBARS limiting threshold for meat acceptability is 2 mg MDA/kg sample, which was not exceeded in the AOX samples (Campo et al., 2006). The LD muscle from the AOX group also showed slight changes during the 21 d of refrigerated storage, in agreement with data reported by Lorenzo et al. (2014). A high protection from lipid oxidative phenomena, due to the antioxidant properties of vitamin E and verbascoside, was observed in the LD from pigs fed the AOX.

This finding is in agreement with the literature reporting a lower TBARS concentration in the muscle of pigs fed plant antioxidants containing polyphenols (Rossi et al., 2014; Pastorelli et al., 2015). In contrast, O'Grady et al. (2008) did not find any improvement in oxidative stability in raw LD muscle under MAP on 12 and 16 d of storage when supplementing pig diets with grape seed extract and bearberries. Dietary supplementation with the AOX, containing vitamin E and verbascoside, during the last phase of pig fattening protects the muscle from oxidative decay, which leads to loss of both sensory and nutritional qualities.

Sensory Shelf Life

The most important overall characteristics for consumers are color, aroma, and flavor. Color deterioration and the development of oxidative phenomena in fact decrease the attractiveness of meat for consumers (Grunert et al., 2004). All sensory traits, in particular those related to aroma and appearance, deteriorate during refrigerated

Figure 4. Oxidative stability of longissimus dorsi muscle during 21 d of refrigerated storage at 4°C under modified atmosphere packaging from pigs fed the control (CTR) or antioxidant mixture (AOX) supplemented diet. TBARS = thiobarbituric acid reactive substances; MDA = malonaldehyde. Data are reported as mean $\pm$ SEM. Time effect, $P < 0.001$; treatment effect, $P < 0.001$; and time $\times$ treatment, $P < 0.001$.

storage and reduce shelf life. Consumer preferences tested in relation to beef stored in different packaging conditions have shown that acceptance is mainly related to the preservation of a reddish color (Carpenter et al., 2001). In the present study, the panelists compared samples at different storage times with a gold standard (CTR samples at Day 0), considered a reference for fresh meat, to detect differences in color and aroma scores

The data on sensory shelf life are reported in Fig. 5. The sensory evaluation was stopped at 15 d because global smell and color between samples and gold standard were significantly different ($P < 0.05$). Therefore, the sensory shelf life end point was achieved.

At 6 d of refrigerated storage, no differences ($P > 0.05$) between dietary treatments were observed. At 12 d, the AOX samples showed a lower overall intensity in smell ($P < 0.05$) compared with the gold standard. At 15 d of refrigerated storage, the AOX samples showed a smaller difference ($P < 0.05$) in appearance and aroma from the gold standard compared with the CTR samples. This could be related to the MDA values in the CTR group, which, at 15 d of storage, were higher than 1 mg/kg muscle, thus matching the sensory detection of rancidity (Gray and Pearson, 1987). Overall smell tended to increase after 12 d of storage, and off-flavor intensity ratings followed the same pattern, as observed in the CTR group, which presented a high value compared with the fresh LD muscle. This data is in agreement with previous studies, which reported that off-flavors were reduced by the addition of a natural extract containing polyphenols in beef and pork patties and in cooked and minced chicken (Rojas and Brewer, 2007; Brannan, 2009; Lorenzo et al., 2014).

Figure 5. Sensory shelf life of longissimus dorsi muscle from pigs fed the control (CTR) or antioxidant mixture (AOX) supplemented diet and stored at 4°C under modified atmosphere packaging. NS = $P > 0.05$; * $P < 0.05$.

Conclusions

Overall, the results suggest that dietary supplementation with the AOX containing vitamin E and verbascoside in the last phase of pig fattening improves the in vivo oxidative status and carcass dressing percentage. The AOX improves the color and lipid stability in the LD muscle and reduces *Pseudomonas* spp. growth. An improvement in sensory shelf life during refrigerated storage under MAP was also found.

Dietary AOX supplementation is, therefore, a promising antioxidant and antimicrobial for enhancing the shelf life of raw pork under commercial conditions.

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