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The effects of dietary verbascoside on blood and liver oxidative stress status induced by a high *n*-6 polyunsaturated fatty acids diet in piglets[1,2]

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ABSTRACT: Twenty-four weaned female Hypor piglets (10.9 ± 0.1 kg mean BW) were used to evaluate the antioxidant effect of a natural extract, titrated in verbascoside, on blood and liver oxidative status in relation to a high intake of *n*-6 PUFA, inducing oxidative stress. Piglets were assigned to 1 of 3 experimental groups; the first group was fed a diet with 9% sunflower oil (T1) and the second received the sunflower oil diet supplemented with 5 mg of verbascoside/kg feed from Verbenaceae extract (*Lippia* spp.; T2). The third group was fed a control diet (CTR), in which an isoenergetic replacement of oil by starch was done. Blood samples were collected at the beginning and the end of the trial (30 d). At the end of the trial, the animals were slaughtered and the liver specimens were collected. Oxidative stress markers, including total antiradical activity, superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT) activities, were determined in blood samples. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and γ-glutamyl transferase (GGT) plasma levels were also evaluated. Immunohistochemistry and western blot analyses were performed in liver to evaluate heat shock protein (Hsp) 70, Hsp90, and Kupffer and Ito cell activation. Liver activities of SOD, GPX, and CAT were also determined. Total antiradical activity in blood and red blood cells were affected ($P < 0.01$) by dietary treatments. The *n*-6 PUFA supplementation at a high dosage for 30 d induced oxidative stress, decreasing total antiradical activity in blood and red blood cells (CTR vs. T1 + T2; $P < 0.01$) and plasma CAT activity (CTR vs. T1 + T2; $P = 0.088$) and increasing ALT value (CTR vs. T1 + T2; $P < 0.01$). Also, in liver, the CAT and GPX activities tended to be lower in pigs fed *n*-6 PUFA diets than pigs fed a control diet (CTR vs. T1 + T2; $P = 0.090$ and $P = 0.085$, respectively). The liver samples presented a normal architecture and no Ito and Kupffer cell activations were observed. In liver, the SOD activity tended to be lower in the T1 group ($P = 0.064$) than in the CTR and T2 groups. Moreover, the level of Hsp70 was higher ($P < 0.01$) in the T1 group than the CTR and T2 groups. These data suggest that the dose of dietary verbascoside partially restores the antioxidant status of the liver without affecting the systemic responses to oxidative stress induced by a high-fat diet.

Key words: immunohistochemistry, liver, oxidative stress, piglet, verbascoside

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INTRODUCTION

The reactive oxygen species have essential biological functions but their accumulation lead to cellular damage (Klaunig et al., 2010). In healthy animals, reactive oxygen species production is counterbalanced by antioxidant defenses and an imbalance between their generation and inactivation leads to oxidative stress (Bashan et al., 2009; Avery, 2011). Some authors have reported that a high-fat diet increased

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reactive oxygen species production, causing hepatic damage (Cardoso et al., 2013, Kikugawa et al., 2003). Moreover, Patterson et al. (2012) reported that a diet rich in *n*-6 PUFA–induced liver oxidative stress, stimulating Kupffer and Ito cell activation. Recently, Lee et al. (2013) observed that the heat shock proteins (**Hsp**) were revealed to be defensive factors against oxidative stress and that Hsp70 and Hsp90 are induced in response to environmental and physiological stresses (Watanabe et al., 2004; Kalmar and Greensmith, 2009).

In farm animals, oxidative stress was involved in pathological disorders, including those relevant to health and production (Zhu et al., 2012). In fact, the antioxidant status declines after weaning, increasing morbidity and mortality (Sauerwein et al., 2005; Yin et al., 2014).

Many plants compounds, such as polyphenols, display interesting antioxidant activities (Korkina, 2007). They are able to integrate endogenous antioxidant defenses, which are depressed after weaning (Bouayed and Bohn, 2010). There is growing evidence that verbascoside, a water-soluble derivate of phenylpropanoids, is a powerful antioxidant (Afanas'ev, 2005). It is characterized by caffeic acid and hydroxytyrosol bound to a β-(D)-glucopyranoside, with a rhamnose in sequence (1–3) to the glucose molecule.

A previous study showed that dietary verbascoside decreased reactive oxygen metabolites in healthy weaned piglets (Pastorelli et al., 2012) and reduced nitrosative stress in the small intestine of swine (Di Giancamillo et al., 2013). The aim of this study was to assess the effects of dietary supplementation of verbascoside on blood and liver oxidative status in weaned piglets exposed to *n*-6 PUFA–induced oxidative stress.

MATERIAL AND METHODS

Procedures involving animals were performed in accordance with the European Union Guidelines (86/609/EEC 1986) and approved by the Italian Ministry of Health (Legislative Decree 116/92).

Animal

A total of 24 weaned female piglets (Hypor) were purchased and transported to the Experimental Zootechnical Center of the University of Milan (Lodi, Italy) where they were weighed and individually housed. The animals were divided into 24 pens (1 piglet/pen) and reared in an environmentally controlled room (average temperature 26°C and relative humidity 60%). Pens (1 by 1 m) contained a dry feeder and 1 nipple waterer. During the period of adaptation (7 d), the piglets received a commercial diet (Ferraroni Mangimi SpA, Bonemerse, Italy) for ad libitum consumption.

Design

After 7 d of adaptation (10.9 ± 0.1 kg average BW), the piglets were assigned to 1 of 3 dietary treatments (8 piglets/group). The composition and chemical analysis of experimental diets are reported in Table 1. The first group was fed a diet with 9% sunflower oil (**T1**; *n*-6 PUFA high dosage and oxidative stress inducing). The second group was fed with T1 diet supplemented with an extract of Verbenaceae (*Lippia* spp.) leaves titrated in verbascoside to obtain 5 mg verbascoside/kg feed (**T2**; *n*-6 PUFA high dosage plus antioxidant). The third group was fed a control diet (**CTR**), in which an isoenergetic replacement of oil by starch was done. The NE ratio between sunflower oil and corn starch was 2.43. The dose of plant extract in the feed was chosen on the basis of our previous study in piglets (Corino et al., 2007). The composition of Verbenaceae extract, according to a certificate of analysis provided by the manufacturer, 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 avoid oxidation in the feed, the supplement was microencapsulated within a protective matrix of hydrogenated vegetable lipids using spray cooling (Sintal Zootecnica, Isola Vicentina, Vicenza, Italy). Animals were restrictively fed 2.7 times the maintenance requirements for energy (NRC, 1998). To highlight the possible effects of the induced dietary oxidative stress condition, the rations had the same protein and energy content by the adoption of an appropriate ratio between the "oil" groups (T1 and T2) and the "nonoil" group (CTR; 1:1.13). A restrictive feeding regimen was adopted to guarantee the total intake of feed (energy, nutrients, and antioxidant). Piglets were individually weighed at the beginning and at the end of the trial. Amounts of feed offered and refused were recorded daily to estimate feed intake of each piglet. These data were used to calculate ADG and G:F of each pen. The trial lasted 30 d and at the end, the piglets were slaughtered.

Blood and Tissue Samples

At the beginning and at the end of the trial, blood samples were collected by cranial vena cava puncture before the morning feeding (total number of specimens = 24). The blood samples were collected in 10-mL vacutainer glass tubes containing EDTA (Venojectl Terumo Europe N.V., Leuven, Belgium), which were immediately transported to the laboratory pending analysis. Plasma samples, obtained from blood samples by centrifugation (3,500 × *g* for 15 min at 4°C), were stored at –80°C to determine the enzyme activities related to oxidative stress. At the end of the trial, the pigs were slaughtered and liver samples were immediately

Table 1. Ingredients and chemical analysis of experimental diets basal diet (as fed basis)[1]

Item	Control	Sunflower oil	Sunflower oil + verbascoside
Starch	19.5	–	–
Barley	14.1	15.9	15.9
Steam rolled wheat	11.5	13	13
Wheat	9.2	10.4	10.4
Steam rolled corn	8.8	9.9	9.9
Sunflower oil		9.1	9.1
Soybean meal, 44% CP	6.6	7.5	7.5
Soy protein concentrate (Soycomil-R[2])	4.4	5	5
Dried whey	4.4	5	5
Corn	4.4	5	5
Wheat bran	3.5	4	4
Fish meal, herring	3	3.4	3.4
Lactose	2.7	3.1	3.1
Dextrose	2.2	2.5	2.5
Plasma AP 820[3]	1.8	2	2
Citric acid	0.82	0.9	0.9
Dicalcium phosphate	0.56	0.64	0.59
Calcium carbonate	0.56	0.64	0.59
Trace element and vitamin premix[4]	0.37	0.42	0.42
Natural extract[5]	–	–	0.1
Sodium butyrate	0.25	0.28	0.28
NaCl	0.18	0.2	0.2
Benzoic acid	0.13	0.15	0.15
L-Lys HCL	0.44	0.5	0.5
DL-Met	0.19	0. 21	0. 21
L-Thr	0.3	0.34	0.34
L-Trp	0.08	0.09	0.09
Enzyme (Ronozyme WX[6])	0.01	0.02	0.02
Phytase (Ronozyme MP[6])	0.01	0.02	0.02
Analyzed composition			
NE,[7] Mcal/kg	2,076	2,700	2,700
CP, %	16.0	18.3	18.0
Crude fat,%	1.93	10.87	10.92
Lys, %	1.26	1.43	1.45
Met + Cys, %	0.65	0.72	0.74
Thr, %	0.92	1.2	1.1
Trp, %	0.27	0.34	0.36
Ca, %	0.68	0.79	0.81
P, %	0.63	0.72	0.74

[1]The control and sunflower oil diets had the same protein and energy content by the adoption of an appropriate daily ration (1 sunflower oil vs. 1.13 control diet).

[2]ADM, Chicago, Illinois, U.S.

[3]APC Europe, S.A. Granolles, Barcelona, Spain

[4]Each kilogram of vitamin premix provided 3,300,000 IU of vitamin A, 340,000 IU of vitamin D_3, 20,300 IU of vitamin E, 2,100 mg of thiamine, 6,200 mg of riboflavin, 6,200 mg of vitamin B_6, 300 mg of vitamin B_{12}, 4,000 mg of vitamin K, 120 mg of biotin, 72,000 mg of niacin, 2,100 mg of folic acid, 21,000 mg of pantothenic acid, and 620,000 mg of choline. Each kilogram of vitamin premix provided 206,000 mg Fe as iron carbonate, 2,070 mg Mn as manganous oxide, 206,000 mg Zn as zinc oxide, 41,000 mg Cu as copper sulfate, 3,100 mg I as potassium iodide, and 400 mg Se as sodium selenite.

[5]Water soluble extract of Verbenaceae (*Lippia* spp.) leaves, titrated in verbascoside, to obtain 5 mg verbascoside/kg feed.

[6]DSM Nutritional Products Ltd, Kaiseraugst, Switzerland.

[7]Net energy values were calculated using NRC (1998) values.

excised from each pig (total number of specimens = 24). The samples were vacuum packed and stored at –80°C. Western blot analyses and ELISA were performed on the samples within 2 wk of collection.

For the microanatomical analyses (histology and immunohistochemistry), additional small (1 cm^3) liver samples were removed from each pig. These samples (total number = 24) were promptly fixed in 4% paraformaldehyde in 0.01 *M* PBS, pH 7.4, for 24 h at 4°C, dehydrated in a graded series of ethanol, cleared with xylene, and embedded in paraffin.

Diet Nutrient Composition

Feed samples were collected, frozen at –20°C, and analyzed for DM (method 934.01; AOAC, 2006), CP (method 984.13; AOAC, 2006), crude fat (method 920.39; AOAC, 2006), AA (method 982.30; AOAC, 2006), Ca (method 985.01; AOAC, 2006), and P (method 985.01; AOAC, 2006).

Blood and Plasma Analyses

Blood. The analyses on the total antiradical activity of whole blood and red blood cells were performed within 24 h of sample collection. The total antiradical activity was evaluated using the Kit Radicaux Libre (**KRL**) biological test based on free radical-induced hemolysis (Prost, 1992). The KRL test allows the ex vivo dynamic evaluation of the overall antioxidant defense potential of an individual. Whole blood and red blood cell samples were diluted (1:25 and 1:50 vol/vol, respectively) with a KRL buffer (300 mOsm/L), and the whole blood or red blood cell suspension samples were submitted to organic free radicals produced at 37°C under air atmosphere from the thermal decomposition of 2,2-azobis (2-amidinopropane) dihydrochloride. The kinetics of sample resistance to hemolysis was recorded using a 96-well microplate reader by monitoring the changes at 620 nm absorbance at 37°C (Laboratoires Spiral, Dijon, France). Results are expressed as the time required to reach 50% of maximal hemolysis (half hemolysis time [$\mathbf{HT_{50}}$]) and are expressed in minutes. A control blood, with a known HT_{50}, was used as an internal control. The intra- and interassay coefficients of variation of the KRL test were 2.5 and 4%, respectively.

Plasma. Plasma samples were analyzed to evaluate the activity of superoxide dismutase (**SOD**), catalase (**CAT**), and glutathione peroxidase (**GPX**). The activity of SOD was measured using Cayman's Superoxide Dismutase Assay Kit (Cayman Chemical Company, Ann Arbor, MI). The kit uses a tetrazolium salt to detect the superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD is defined as

Table 2. Primary antisera used in the immunohistochemical studies of the liver

Primary antibody	Code/source[1]	Dilution[2]	Incubation	Antigen retrieval
Heat shock protein 70 (Hsp70), monoclonal	N27F3-4/Enzo LifeSciences	1:100 IHC 1:1,000 WB	Overnight at room temperature	Heat
Heat shock protein 90 (Hsp90), monoclonal	AC88/Enzo LifeSciences	1:100 IHC 1:1,000 WB	Overnight at room temperature	Heat
Desmin clone D33, monoclonal	H7094/Dakocytomation	1:50 IHC 1:500 WB	Overnight at room temperature	Heat
Lysozyme EC3.2.1.17, polyclonal	A0099/Dakocytomation	1:400 IHC 1:1,500 WB	Overnight at room temperature	Proteinase K

[1]Enzo LifeSciences, Farmingdale, New York, U.S.; Dakocytomation, Milan, Italy.

[2]IHC = immunohistochemistry; WB = western blot.

the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. The SOD assay measures all 3 types of SOD (Cu\Zn, Mn, and Fe). The activity of CAT was measured using Cayman's Catalase Assay Kit (Cayman Chemical Company). The method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H2O2. The formaldehyde produced is measured colorimetrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald; Sigma-Aldrich, Milan, Italy) as the chromogen. Purpald specifically forms a bicyclic heterocycle with aldehydes, which oxidation changes from a coloress to a purple color. The activity of GPX was measured using Cayman's Glutathione Peroxidase Assay Kit (Cayman Chemical Company). The kit measures GPX activity indirectly by a coupled reaction with glutathione reductase. Oxidized glutathione is produced on the reduction of hydroperoxide by GPX and is recycled to its reductase state by glutathione reductase and the reduced form of nicotinamide adenine dinucleotide phosphate (**$NADP^+$**; **NADPH**). The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm. When the GPX activity is rate limiting, the rate of decrease is directly proportional to the GPX activity in the sample.

Plasma alanine aminotransferase (**ALT**), aspartate aminotransferase (**AST**), and γ-glutamyl transferase (**GGT**) were determined by an Automated Clinical Chemistry Analyzer (ARCO model; Biotech Instruments S.p.A., Rome, Italy).

Liver Analyses

Histology. Serial microtome sections (4 μm thick) were obtained from each paraffin-embedded liver sample and were stained with hematoxylin and eosin sequential staining to ascertain structural details. Other sections were used for immunohistochemistry.

Immunohistochemistry. Immunostaining of the liver sections was performed to detect Hsp70 and Hsp90 as markers of oxidative stress in situ. We also evaluated lysozyme and desmin immunoreactivities to identify the hepatic resident macrophages and, respectively, the hepatic stellate cells populations during possible activation concomitant with lipid peroxidation and oxidative stress. The immunohistochemical procedure applied is previously described by Di Giancamillo et al. (2013). Briefly, a preliminary antigen retrieval was performed either by heat, with a microwave treatment (for 5 min at 450 W in citrate buffer, pH 6), or by proteinase K treatment in PBS, pH 7.4, at room temperature for 5 min (lysozyme and desmin, respectively). Immunohistochemical stainings were performed using the Elite ABC KIT system (Vector Laboratories, Inc., Burlingame, CA). Before applying the primary antibodies, endogenous peroxidase activity was blocked by incubating the sections in 3% H_2O_2 in PBS. Nonspecific binding sites were blocked by incubating the sections in normal goat serum (Dakocytomation, Milan, Italy). Sections were then incubated with the primary antibodies (Table 2) overnight at 4°C.

After washing with PBS, sections were incubated with biotin-conjugated either anti-mouse or anti-rabbit IgG antibodies (Dakocytomation), washed with PBS, and reacted with peroxidase-labeled avidin–biotin complex (Vector Laboratories, Inc.).

The immunoreactive sites were visualized using a freshly prepared solution of 10 mg 3.3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) in 10 mL of a 0.5 *M* Tris buffer at pH 7.6 containing 1.5 mL of 0.03% H_2O_2. To ascertain structural details, sections were counterstained with Mayer's hematoxylin. Immunoreactive structures were assessed using an Olympus BX51 light microscope (Olympus, Milan, Italy) equipped with a digital camera.

The specificity of the immunostaining was verified by incubating in parallel other sections with 1) PBS instead of the specific primary antibodies (Table 2), 2) preimmune sera instead of the primary antisera, and 3) PBS instead of the secondary antibodies. The results of these controls were negative (i.e., staining was abolished).

Tissue Homogenization and Western Blot Analysis. Liver samples were homogenized in 2 mL of ice-cold lysis buffer (50 m*M* Tris HCl, pH 7.5, 0.2% Triton X-100, and protease inhibitor mixture) per 200 mg of tissue using an Ultra-Turrax homogenizer (IKA-Werke, Staufen, Germany). For each sample, the homogenate was then centrifuged at 20,000 × *g* for 20 min at 4°C to remove all insoluble material. The supernatant was collected, and total protein content was measured using a commercial protein quantification kit (Pierce, Rockford, IL) based on bicinchoninic acid colorimetric detection of the cuprous cation obtained by protein Cu^{2+} reduction in an alkaline medium. The optical densities were read at 562 nm against a calibration curve using BSA (Sigma-Aldrich) with a working range of 50 to 800 μg/mL. Total proteins (35 μg) were resolved by 10% SDS-PAGE and electrotransferred onto nitrocellulose membranes. After blocking, membranes were incubated overnight at 4°C with primary antibodies (see Table 2) and subsequently with a horseradish peroxidase-conjugated secondary antibody (1:5,000) at room temperature for 45 min. Immunoreactivity was detected by chemiluminescence autoradiography according to the manufacturer's instructions (Biorad, Milan, Italy), and the images were scanned. The optical intensities of the protein bands of interest were determined densitometrically using Scion Image software (Scion Corporation, Frederick, MA).

Each electrophoresis gel contained samples from treatment groups to reduce between-blot effects. The values were normalized to glyceraldehyde 3-phosphate dehydrogenase levels.

Tissue Homogenization and ELISA Analysis. Liver samples were homogenized in 1 mL of homogenization buffer (50 m*M* Tris HCl, pH 7.5, 5 m*M* EDTA, and 1 m*M* dithiothreitol) per 100 mg of tissue using an Ultra-Turrax homogenizer (IKA-Werke). The homogenate was then centrifuged at 10,000 × *g* for 15 min at 4°C. After collecting the supernatant, the analysis was applied in accordance with the manufacturer's protocol (Cayman Chemical Company). Liver samples were analyzed to evaluate the enzyme activity related to oxidative stress—SOD, CAT, and GPX—with the same kits used for plasma analyses, according to the manufacturer's instructions.

Statistical Analyses

All analyses were performed with SAS software (version 8.1; SAS Inst. Inc., Cary, NC), and a value of $P < 0.05$ was used to indicate statistical significance. One-way ANOVA was used to determine the effects of dietary treatments on the variables examined in blood and liver. Blood and plasma parameters values obtained at the beginning of the trial were inserted as a covariate in the model. Orthogonal contrasts of blood and plasma parameters and on liver enzyme activities on the second sampling time were conducted (CTR vs. T1 + T2) and reported only when a significant effect was detected. The individual piglet values were considered the experimental unit of all response variables. The data were presented as least squared means ± pooled SEM.

RESULTS

Growth Performance

No significant effect of dietary treatments on pig performance was detected, due to the restrictive feeding regimen (Table 3). The average BW of the piglets at slaughter was 22.0 ± 0.3 kg with an ADG of 382 ± 0.1 g/d. As expected, due to the different energy concentration of the diets, the G:F was higher ($P < 0.01$) in pigs fed oil-supplemented diets than in controls.

Blood Antiradical Activity

The total antiradical activity of the whole blood and red blood cells showed a significant effect of dietary treatment ($P < 0.05$; Table 4). In both blood and red blood cells, a difference ($P < 0.05$) between pigs fed a control diet and pigs fed the oil-supplemented diets (T1 and T2) was observed at the end of the trial. Moreover, no differences were observed between pigs fed the sunflower oil and pigs fed the sunflower oil plus verbascoside (T1 vs. T2).

Plasma Analyses

The CAT, GPX, and SOD plasma activities were evaluated (Table 4). No significant differences were observed between pigs fed control or sunflower oil or sunflower oil plus verbascoside diets (CTR vs. T1 vs. T2). At the end of the trial, plasma CAT activity tended to be higher ($P = 0.088$) in pigs fed a control diet than pigs fed the oil-supplemented diets (T1 + T2).

Plasma ALT, AST, and GGT values are also reported in Table 4. No significant differences were observed between pigs fed control or sunflower oil or sunflower oil plus verbascoside diets (CTR vs. T1 vs. T2). The orthogonal contrast, showed that ALT tended to be lower ($P = 0.060$) in pigs fed a CTR than pigs fed the oil-supplemented diets (T1 + T2) at the end of the trial.

Liver

Histology. All liver samples, irrespective of dietary treatment, presented a normal architecture of parenchyma and the anatomy of the hepatic lobule

Table 3. Piglet growth performances in relation to dietary treatments[1]

Item	Control	Sunflower oil	Sunflower oil + verbascoside	Pooled SEM	*P*-value
Initial weight, kg	10.9	10.7	11.0	0.101	0.566
Final weight, kg	21.6	21.9	22.5	0.311	0.494
ADG, g/d	366	385	396	0.008	0.332
G:F	0.56[A]	0.65[B]	0.67[B]	0.013	<0.001

[A,B]Within a row, means without a common superscript differ ($P < 0.01$).

[1]$n = 8$; data are reported as mean ± pooled SEM.

appeared regular with a normal central vein and portal area appearance (Fig. 1).

Immunohistochemistry. Hepatocyte nuclei were abundantly immunoreactive for both Hsp70 (Supplemental Fig. 1a, 1b, and 1c; see online version of the article at http://journalofanimalscience.org) and Hsp90 (Supplemental Fig. 1d, 1e, and 1f; see online version of the article at http://journalofanimalscience.org). Almost all the hepatocyte nuclei were immunopositive, irrespective of the treatment.

Lysozyme is a marker of inducible macrophage activation and identifies the resident liver macrophage population, that is, the Kupffer cells. Lysozyme-immunoreactive, irregularly shaped cells were present in perisinusoidal localizations. These were interpreted as Kupffer cells and were frequently observed in the liver parenchyma of all the experimental piglets (Supplemental Fig. 1g, 1h, and 1i; see online version of the article at http://journalofanimalscience.org).

Desmin, although with some species-specific differences, can be considered a marker of activated hepatic stellate cells or Ito cells. Desmin-immunoreactive, roundish cells were sparsely identified in the liver parenchyma of all the studied piglets (Supplemental Fig. 1l, 1m, and 1n; see online version of the article at http://journalofanimalscience.org). These cells were interpreted as hepatic stellate cells (or Ito cells), and their numbers did not change in relation to dietary treatments.

Western Blot. This was performed for an accurate immunochemical quantification of the immunohistochemical results. There was an increase in Hsp70 immunopositivity in the pigs fed the sunflower oil diet compared to pigs fed the control and sunflower oil plus verbascoside diets ($P < 0.01$; Fig. 2).

Enzyme-Linked Immunosorbent Assay Analyses. Liver samples were analyzed to evaluate enzyme activity: SOD, CAT, and GPX (Table 5). Liver CAT and GPX enzyme activities did not differ between pigs fed the control or sunflower oil or sunflower oil plus verbascoside diets (CTR vs. T1 vs. T2).

Liver SOD enzyme activity tended to be lower ($P = 0.062$) pigs fed the sunflower oil diet compared with pigs fed the control and sunflower oil plus verbascoside diets. Orthogonal contrast showed that liver CAT and GPX enzyme activities tended to be higher in pigs fed a CTR than pigs fed the oil-supplemented diets ($P = 0.090$ and $P = 0.085$ for T1 + T2, respectively).

DISCUSSION

We assessed both systemic and local responses to oxidative stress using verbascoside to decrease the response to stress induced by a high supplementation of sunflower oil. In our previous study, a comparison between verbascoside and trolox (water-soluble analog of vitamin E) has been performed (Rossi et al., 2009). The natural extract containing verbascoside showed an in vitro antioxidant activity equivalent to 38.19 mmol trolox per gram of active principle.

The induced nutritional oxidative stress did not affect the piglet growth performances, according to a previous study in pig exposed to *n*-3 PUFA-induced oxidative stress and fed sweet chestnut wood extract (Frankič and Salobir, 2011). The same result on G:F was observed. In disagreement with the present data, Boler et al. (2012) reported that dietary supplementation with oxidized corn oil for 56 d in barrows reduced

Table 4. Blood antiradical activity and plasma enzyme activities at the end of the trial in relation to dietary treatments[1]

Blood and plasma parameter[2]	Control	Sunflower oil	Sunflower oil + verbascoside	Pooled SEM	*P*-value
Blood total antiradical activity, HT_{50}	105.6[A]	88.1[B]	83.3[B]	2.816	0.001
RBC antiradical activity, HT_{50}	56.6[a]	52.8[b]	53.1[b]	0.634	0.021
Plasma CAT activity, $nmol \cdot min^{-1} \cdot mL^{-1}$	17.4	15.2	15.6	0.518	0.155
Plasma GPX activity, $nmol \cdot min^{-1} \cdot mL^{-1}$	64.3	64.0	63.4	0.527	0.797
Plasma SOD activity, units/mL	0.16	0.16	0.15	0.046	0.733
Plasma ALT, IU	140.5	155.2	150.9	3.141	0.502
Plasma AST, IU	92.7	97.8	90.6	0.387	0.648
Plasma GGT, IU	27.5	29.7	29.3	0.934	0.706

[A,B]Within a row, means without a common superscript differ ($P < 0.01$).

[a,b]Within a row, means without a common superscript differ ($P < 0.05$).

[1]$n = 8$; data are reported as mean ± pooled SEM.

[2]HT_{50} = half hemolysis time; RBC = red blood cells; CAT = catalase; GPX = glutathione peroxidase; SOD = superoxide dismutase; ALT = alanine aminotransferase; AST = aspartate aminotransferase; GGT = gamma-glutamyl transferase.

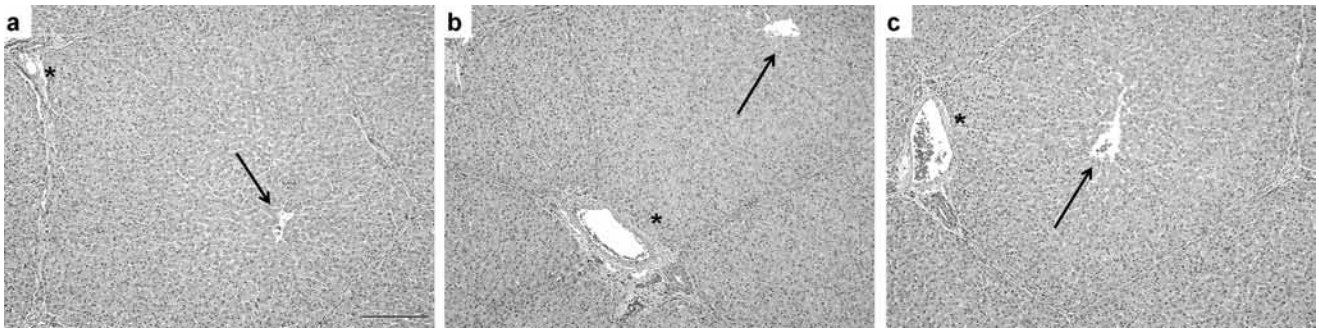
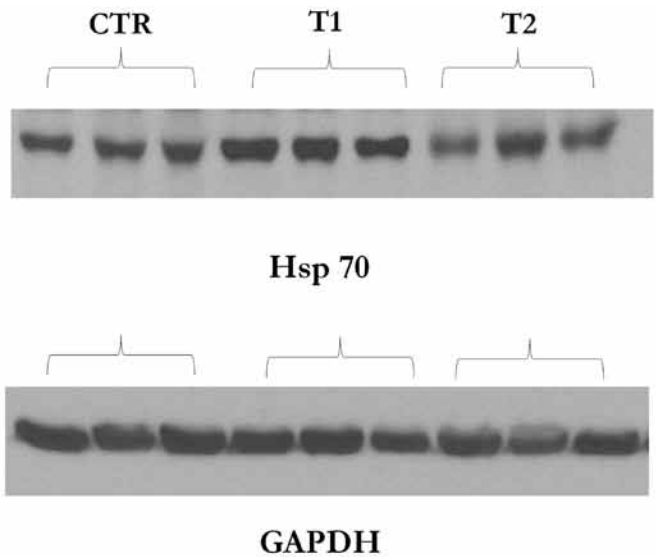


Figure 1. Histology of liver. All figures have the same scale bar as Supplemental Fig. 1: scale bar: 200 µm. The normal architecture of liver lobules is evident: note the presence of central veins (arrows) and portal areas (asterisks; a= control diet; b=sunflower oil diet, c=sunfllower oil plus verbascoside diet). 180x44mm (300 x 300 DPI)

ADG and BW. This should be related to the different length of dietary supplementation and substance inducing oxidative stress.

The oxidative stress induced by a high supplementation of sunflower oil influenced the systemic response through a decrease in total blood antiradical activity. The blood and red blood cell antiradical activity values are in line with the reference values for female piglets (Pastorelli et al., 2013). As previously observed, the KRL test is able to evaluate the total antioxidant activity in pigs in relation to dietary supplement (Rossi et al., 2013b). Our data showed the sensitivity of the KRL test in identifying nutritional oxidative stress; in fact, in piglets fed a CTR, the antiradical activity was higher than in piglets fed oil-supplemented diets. The present results revealed that verbascoside did not improve the systemic antioxidant status. In line with our data, Frankič and Salobir (2011) found no difference in plasma total antioxidant status of pigs exposed to *n*-3 PUFA-induced oxidative stress and fed sweet chestnut wood extract. Other studies have also reported no difference in plasma total antioxidant status in pigs fed a high dosage of linseed oil and *Calendula officinalis* extract or carvacrol, capsaicin, and cinnamaldehyde (Frankič et al., 2008; Frankič and Salobir 2011). The same results were obtained in broilers fed *n*-3 PUFA, sweet chestnut wood extract, and α-tocopherol (Voljč et al., 2013).

Regarding antioxidant enzymes, although several mechanisms protect the cells against reactive oxygen species (**ROS**)–mediated damage, CAT, GPX, and SOD play a major role. These enzymes have been detected in both plasma and liver, where they contribute to the antioxidant defense mechanisms (Lee et al., 2002). Catalase is a heme protein in all aerobic cells, which metabolizes H_2O_2 to oxygen and water (Decker et al., 2000). We found that plasma CAT activity tended to be lower in pigs fed the sunflower oil diets than in pigs fed the CTR, in accordance with Varma et al. (2004) in rats. Glutathione peroxidase plays an important role in the



	CTR	T1	T2
HSP 70	0.80 ± 0.11^{A}	1.40 ± 0.12^{B}	0.86 ± 0.10^{A}
HSP 90	0.15 ± 0.05	0.13 ± 0.06	0.14 ± 0.04
Lysozyme	0.44 ± 0.12	0.62 ± 0.14	0.48 ± 0.10
Desmin	0.25 ± 0.15	0.30 ± 0.13	0.34 ± 0.14

Figure 2. Western Blot analysis. Hsp 70 protein adducts increased in liver of T1 animals (P<0.01). Hsp 70 level are normalized and quantified to GAPDH. 180x93mm (300 x 300 DPI). CTR, control diet; T1 sunflower oil diet; T2 sunflower oil plus verbascoside diet; Hsp70 heat shock protein 70; GAPDH; glyceraldehyde 3-phosphate dehydrogenase. ^{A,B}Within a row, means without a common superscript differ (P < 0.01).

Table 5. Liver antioxidant enzymes activities in relation to dietary treatments[1]

Liver[2]	Control	Sunflower oil	Sunflower oil + verbascoside	Pooled SEM	*P*-value
CAT activity, units/mg protein	27.5	25.2	24.8	0.661	0.222
GPX activity, units/mg protein	77.3	77.1	76.8	0.102	0.183
SOD activity, units/mg protein	0.26	0.21	0.24	0.008	0.064

[1]n = 8; data are reported as mean ± pooled SEM.

[2]CAT = catalase; GPX = glutathione peroxidase; SOD = superoxide dismutase.

detoxification of xenobiotics in the liver and catalyses the reduction of H_2O_2 and hydroperoxides to nontoxic products (Hsu et al., 2008). No difference in plasma GPX activity was observed, in agreement with previous studies reported that dietary supplementation with a high dosage of linseed oil and antioxidant mixture did not influence plasma GPX activity (Frankič et al., 2008; Frankič and Salobir 2011). In disagreement with our data, Jiang et al. (2011) found a significant decrease in this enzyme in gilts fed for 18 d with a high dosage of zearalenone to induce oxidative stress. Superoxide dismutase catalyzed the decomposition of superoxide anions to hydrogen peroxide (H_2O_2; Decker et al., 2000). Plasma SOD activity was not influenced by dietary supplementation with sunflower oil, in agreement with Lauridsen et al. (1999), who used rapeseed oil. The same result was observed in chicken supplemented for 20 d with a high dosage of linseed oil and fed sweet chestnut wood extract and α-tocopherol (Voljč et al., 2013).

Moreover, we measured the effect of plasma AST, ALT, and GGT as indicators of hepatocyte damage; no differences in plasma AST and GGT were observed in relation to dietary treatment, in accordance with Frankič and Salobir (2011). Plasma ALT tended to be higher in pigs fed the sunflower oil diets than pig fed the CTR, which is a possible result of oxidative damage in the liver.

Considering the whole blood and plasma data, we observed that dietary verbascoside supplementation did not affect the systemic responses on oxidative stress status induced by sunflower oil. Our previous studies showed that the same dosage of verbascoside (5 mg/kg feed) decreases serum reactive oxygen metabolite (determination of reactive oxygen metabolites) production in healthy piglets (Pastorelli et al., 2012). The same effect was observed in Italian hares supplemented with verbascoside (Palazzo et al., 2011). The discrepancy of these results is related by the fact that in the present trial an oxidative stress status was induced by dietary supplementation of n-6 PUFA, while the previous studies regard healthy animals.

The liver plays a central role in the energy metabolism. A large amount of free fatty acids from dietary glucose and fat flow directly into the liver. A recent study of Akbiyik et al. (2004) found that liver increased fatty acid oxidation to compensate a high fat intake. This is also supported by Matsuzawa-Nagata et al. (2008), who reported that an upregulation of genes responsible for ROS production occurs in the liver of mice fed a high-fat diet. Also, Cardoso et al. (2013) reported that a high-fat diet in mice enhanced ROS release from liver mitochondria when fatty acids were used as substrates. Considering the abovementioned literature, we decided to investigate not only whole blood and plasma parameters but also liver as a target organ during oxidative stress induced by *n*-6 PUFA at a high dosage.

We histologically observed a normal parenchymal architecture of liver and the anatomy of hepatic lobule appeared to be regular in all dietary treatments. In addition, no qualitative and quantitative modifications within the liver resident macrophages and hepatic stellate cells were observed. Previous studies have reported that oxidative stress is able to stimulate Kupffer and Ito cell activation in the liver under pathological conditions (Tsukamoto et al., 1995; Fu et al., 2008). Our data showed that a short-term high-fat diet in piglets did not cause damage to hepatocytes to such a degree to lead to Kupffer and Ito cell activation. Moreover, the role of the Hsp in liver oxidative stress modulation was investigated and we found that piglets that underwent oxidative nutritional stress (sunflower oil diet) by dietary administration of 9% sunflower oil showed a higher hepatic level of Hsp70 in comparison with the piglets fed the control and sunflower oil plus verbascoside diets. Many studies have shown that Hsp70 protects cells from various stresses (Oyake et al., 2006; Zhong et al., 2009) and increases in stressful conditions. Kalmar and Greensmith (2009) have described the roles of Hsp upregulation in tissue and cell protection against oxidative stress. Negrato et al. (2013) reported that concentration of Hsp70 was higher in intensively reared pigs compared to extensive breeding. Moreover, Valros et al. (2013) observed a higher intestinal amount of Hsp70 in pigs with tail wounds, suggesting that it can be used as a tool to evaluate stressful conditions. Considering our results, piglets fed sunflower oil plus verbascoside diet presented a level of Hsp70 comparable to the control group, whereas piglets fed sunflower oil diet revealed a higher level of Hsp70 (+75 and +62% compared with

control and sunflower oil plus verbascoside diets, respectively), therefore suggesting the protective role of verbascoside in hepatic cells. In fact, as previously observed, many oxidizing agents have been shown to result in Hsp induction, and treatment of cells with antioxidants attenuates the response (Gorman et al., 1999; Padmini et al., 2012). Verbascoside are able to scavenge free radicals and reactive oxygen molecules such as superoxide anion, nitric oxide, and hydroxyl radical (Zhao et al., 2009) but the mechanism through which verbascoside acts as a protective agent for hepatocytes, decreasing the level of Hsp70, remain to be investigated.

Liver enzymes were also investigated because antioxidant defenses play a key role in the detoxification of free radicals. Our data showed that a diet rich in *n*-6 PUFA tended to decrease liver CAT and GPX activities. Liver SOD activity tended to be higher in the piglets fed the CTR and the sunflower oil plus verbascoside diets than piglets fed the sunflower oil diet. This indicates that verbascoside supplementation in a high-fat diet partially restores the antioxidant status of the liver, decreasing oxidative stress. Similar results have confirmed that natural antioxidants have a protective role in the reduction of oxidative stress and restore the activity of hepatic enzymes in rats (Huang et al., 2010; Khan et al., 2012). In addition, Schmatz et al. (2012) demonstrated that treatment with resveratrol prevents the decrease in activities of CAT and SOD, thus improving the antioxidant defense.

There is a growing interest in the role of complementary and alternative medicine in maintaining health and preventing diseases. Phytogenic feed additives as antioxidant substances are the subject of intense debate. Special attention has focused on verbascoside, which has been repeatedly shown to be a potent antioxidant in healthy animals (Corino et al., 2007; Casamassima et al., 2013; Di Giancamillo et al., 2013; Rossi et al., 2013a). In summary, we examined the effects of *n*-6 PUFA high dosage diet and verbascoside supplementation on blood and liver oxidative status in piglets. Our data showed that the *n*-6 PUFA high dosage diet increases blood and liver oxidative stress markers without stimulating Kupffer and Ito cell activation. The amount of dietary verbascoside is able to modulates oxidative damage in the piglets liver without affect the systemic responses to oxidative stress.

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