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Changes in nitrosative stress biomarkers in swine intestine following dietary intervention with verbascoside

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Summary. In farm animals, oxidative stress can be involved in several intestinal pathological disorders, and many antioxidant molecules, especially those of plant origin, can counteract free radicals, thus stabilizing the gut environment and enhancing health. The aim of the study was to investigate whether the use of verbascoside (VB), a polyphenol plant compound, in pig feeding could modulate oxidative and/or nitrosative stress in the gut. Eighteen male piglets (Dalland) were assigned to two groups, which were fed with either a control diet (CON) or a diet supplemented with 5 mg/kg of verbascoside (VB) for 166 days. At slaughter, duodenum and jejunum specimens were collected. Immunohistochemistry and Western blot analyses were performed on the samples to evaluate free radical adducts, including acrolein (ACR), 8-hydroxydeoxyguanosine (8-OHdg) and nitrotyrosine (NT). A KRL test was also used to assess the total blood antioxidant activity, and no difference was observed. Immunohistochemistry and Western blot showed that dietary treatment decreased the levels of nitrotyrosine in enteroendocrine cell populations ($P<0.05$). Characterization of the enteroendocrine cell typology was then performed, and serotonin-immunoreactive cells were revealed to be directly involved in decreasing the nitrosative stress status. This preliminary study demonstrates the important role of dietary VB in decreasing stress biomarkers in swine gut, thus highlighting a possible intervention aimed at building a large prospective for antioxidant dietary supplementation in food animal species.

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Introduction

Free radicals are highly reactive molecules produced under normal biological conditions, mainly during oxygen consumption in redox reactions required to generate energy and eliminate xenobiotic and pathogenic organisms. Organisms have their own natural protection in the form of enzymatic and chemical detoxification systems against excessive free-radical generation, which in turn is responsible for various degrees of cellular damage. Thus, under normal physiological conditions, a balanced state is established between free-radical production and antiradical factor interventions (Lehucher-Michel et al., 2001). Nevertheless, various lifestyle, as well as nutritional and environmental factors are able to alter this balanced state, thus resulting in oxidative stress. This in turn may cause an impairment of the individual's overall defence capacity, thus leading to the development of many diseases, especially chronic ones.

Many natural substances present in plants as secondary metabolites, such as phenolics and flavonoids, have the ability to directly neutralise free radicals. In fact, some of these substances inhibit chain reactions that lead to the formation of additional radicals, thus preventing cellular damage which is a frequent occurrence in both humans and other mammalian species. Some others activate antioxidant enzymes, such as glutathione peroxidase (Hu, 2011). At present, however, limited knowledge exists about the translation between the large body of evidence supported by *in vitro* studies and the scarce quantity of studies concerning *in*

vivo conditions (Fraga, 2007). From this point of view, the swine is an excellent candidate model to be examined, because it is either a widely reared food animal species or an extensively studied animal model for biomedical problems (Walters et al., 2011a,b).

At present pigs have fast growth rates, not only due to precise feed formulations and genetic improvement, but also through better than previous management, which tends to respect animal welfare (Bezkorovainy, 2001). To sustain faster growth, sub-therapeutical levels of antibiotics have long been used by farmers as growth promoters. However, the long-term use of antibiotics as feed additives causes antibiotic resistance in pathogens (Bach Knudsen, 2001) and may select for antibiotic-resistant bacterial strains, which may then be transferred to other bacteria (Aarestrup, 1999). European Union (EU) laws against the use of antibiotics in animal feed have been in effect since January 1, 2006 (Regulation EC no 1831/2003). As a consequence, it is crucial to find alternative and healthier feed strategies which are able to limit microbial resistance (Daglia, 2012), as well as to stabilize the gut environment, enhance health, sustain food animal species performance, and improve the quality of the products derived.

The dietary use of a polyphenol such as verbascoside, a phenylpropanoid glycoside, has been suggested as being efficient in preventing or attenuating oxidative stress and hence improving animal health (Stevenson and Hurst, 2007; Virgili and Marino, 2008; Daglia, 2012). The antioxidant activity of verbascoside on cellular systems and several organs has been demonstrated in humans (Li et al., 1999; Chen et al., 2002; Vertuani et al., 2011). In animal species, the antioxidant properties of verbascoside have been demonstrated in rabbits (Liu et al., 2003), weaned piglets (Corino et al., 2007), and hares (Palazzo et al., 2011). In addition, Bruins et al. (2006) found that polyphenol tea extracts may inhibit net fluid and electrolyte losses involved in secretory diarrhoea from enterotoxigenic *Escherichia coli* in animal models. Sehm et al. (2007) reported that dietary polyphenol showed a positive effect on the structure of the gastrointestinal tract of post weaning piglets and can improve pig health. In addition Lien et al. (2007) found an improvement in average daily gain and immune response parameters in weaned piglets fed with plant polyphenols.

No data on the long-term nutrition with natural extracts, titrated in verbascoside, in pigs are available in the literature. We examined how a long-term dietary supplementation with verbascoside could influence the intestinal structure of pigs, and we investigated whether the use of verbascoside in pig nutrition could modulate oxidative and/or nitrosative stress biomarkers in the intestine.

Material and methods

Animals and diet

Eighteen male pigs (Dalland) weighing 7.1±0.2 kg and aged 25±2 days, were randomly selected and assigned to two dietary groups: a control group (CON=9) and an experimental group fed with a diet supplemented with 5 mg/kg of verbascoside (VB=9). The amount of VB in the feed was chosen on the basis of our previous studies on pigs (Corino et al., 2007; Pastorelli et al., 2011). On arrival the piglets were clinically healthy. The diet compositions were fortified to meet or exceed nutrient requirements (NRC 1998) for all nutrients in the growth phases. The animals were housed in individual cages under environmentally controlled conditions and given *ad libitum* access to water and diet. The antioxidant supplement contained a water-soluble extract of *Verbenaceae* (*Lippia* spp.) leaves (Consorzio Powerfeed, Costa de Nobili, Pavia, Italy). To avoid oxidation in the complete feed, the supplement was microencapsulated within a protective matrix of hydrogenated vegetable lipids using spray-cooling technology (Sintal Zootecnica, Isola Vicentina, Vicenza, Italy).

The trial lasted 166 days and the pigs were slaughtered at an average live weight of 109.6±2.2 kg.

All animals were treated in accordance with both the policies and the principles of laboratory animal care in compliance with European Union guidelines (86/609/EEC), which were approved by the Italian Ministry of Health (Law 116/92).

Blood and tissue samples

At the beginning of the trial and at 150 days, blood samples were collected by vena cava puncture before the morning feeding. The blood samples (total number of specimens=36) were collected in 10-mL vacutainer glass tubes containing EDTA (Venoject, Terumo Europe N.V., Leuven, Belgium), which were immediately transported to the laboratory pending analysis.

At the end of the trial (166 days), the pigs were slaughtered by approved procedures (Italian Ministry of Health; DL .333/1998), and samples of proximal duodenum (5 cm after the pylorus) and proximal jejunum (5 cm after the beginning of the jejunum) were immediately excised from each pig (total number of specimens=36). The samples were vacuum-packed and stored at -80°C. Western blot analyses were performed on the samples within two months of collection.

For micro-anatomical analyses (histology, histomorphometry, immunohistochemistry, and double immunofluorescence), additional full thickness (approximately 1 cm^3) samples were removed at the same above-mentioned localizations from each pig. These samples (total number=36) were promptly fixed in 4% para-formaldehyde in 0.01 M phosphate-buffered saline (PBS) pH 7.4 for no longer than 24 h at 4°C, dehydrated in a graded series of ethanol, cleared with xylene, and embedded in paraffin (see below).

Blood analysis

The analyses on the total antioxidant activity of

whole blood were performed within 24 hours of sample collection. The total antioxidant activity of the whole blood was evaluated using the KRL biological test based on free radical-induced haemolysis (Prost, 1992). The KRL test allows the *ex vivo* dynamic evaluation of the overall antioxidant defence potential of an individual. Whole blood was diluted (v:v 1:25) with KRL buffer (300 mosm/l), and 50 μl of whole blood suspension were submitted to organic free radicals produced at 37°C under air atmosphere from the thermal decomposition of 2,2azobis (2-amidinopropane) dihydrochloride (AAPH). The kinetics of sample resistance to haemolysis were recorded using a 96-well microplate reader by monitoring the changes at 620 nm absorbance, at 37°C (Laboratoires Spiral, France). Results were expressed (in minutes) as the time required to reach 50% of maximal haemolysis (half-haemolysis time, HT50).

Micro-anatomical analyses of the gut

Histology and histomorphometry

The full-thickness intestinal specimens were divided into two parts, oriented on a corkplate with the mucosa downwards, and fixed as already described. Following fixation, the specimen couples were embedded in paraffin: one for transversal and one for horizontal sectioning. The intestinal wall was transversally evaluated at three different levels (superficial, middle and deep) of the tissue blocks, each containing three groups of serial sections (Di Giancamillo et al., 2010). In the paraffin blocks used for the transversal sectioning, histomorphometrical analyses were performed. In addition, the horizontal sectioning of the middle layer enabled us to observe as much of the intestinal crypts as possible.

The serial and semi-serial microtome sections (4 μm-thick) obtained were stained with Hematoxylin-Eosin (HE) sequential stain to ascertain structural details. Other sections from both the duodenum and jejunum were utilized as follows.

For the histomorphometry, on HE-stained sections the height of intestinal villi (V) (10 villi measured per section), the depth of intestinal crypts (C) (10 crypts measured per section), and the ratio of villi and crypts values (V:C ratio) were measured and calculated using an image analysis software (Image pro Plus 6.3 Media Cybernetics Inc., Silver Springs, USA). Other sections from both the duodenum and jejunum were used for immunohistochemistry and double immunofluorescence.

Immunohistochemical (IHC) analyses and cellular counts

Immunostaining of the gut sections was performed as described above in order to detect the following markers of oxidative-nitrosative stress *in situ*. Acrolein (ACR) is produced by hyperoxidation of lipids, and it was identified as a secondary product resulting from lipid peroxidation *in vivo*. 8-hydroxydeoxyguanosine (8-OHdg), an oxidized nucleoside of DNA, is a sensitive marker of DNA damage caused by increased cellular production of reactive oxygen species (ROS), part of the cell aerobic metabolism. Reactive nitrogen species (RNS) such as peroxynitrite, can nitrate specific amino acids, such as tyrosine, thus altering the protein function. Nitrotyrosine (NT) is widely used as a sensitive marker of this reaction, indicating *in vivo* nitrosative stress, very closely linked to oxidative stress. All the primary antibodies used in this study for identifying stress biomarkers are detailed in Table 1.

Immunohistochemical staining was performed using the Elite ABC KIT system (Vector Laboratories, Inc., California, USA). Before applying the primary antibodies, endogenous peroxidase activity was blocked by incubating the sections in 3% H_2O_2 in PBS. Non-specific binding sites were blocked by incubating the sections in normal goat serum (Dakocytomation, Italy). Sections were then incubated with the primary antibodies overnight at 4°C. After washing with PBS, sections were incubated with biotin-conjugated anti-mouse Ig antibodies (Dakocytomation), washed with PBS and reacted with peroxidase-labelled avidin-biotin complex (Vector Laboratories). The immunoreactive sites were visualized using a freshly-prepared solution of 10 mg of 3.3'-diaminobenzidine tetrahydrochloride (DAB, Sigma, Italy) in 15 ml of a 0.5M 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 haematoxylin.

The specificity of the immunostaining was verified by incubating sections with: (i) PBS instead of the specific primary antibodies ; (ii) preimmune sera instead of the primary antisera; (iii) PBS instead of the secondary antibodies; (iv) antisera which were pre-absorbed with an excess of respective synthetic peptides

Table 1. Primary antibodies used to identify oxidative/nitrosative stress biomarkers.

Target protein	Antibody	Species	Dilution	Characterization	Immunizing antigen/source
Nitrotyrosine (NT)	Anti-NT	Mouse monoclonal	1:1000 (IHC) 1:3000 (WB)	Immunohistochemistry Western blot	3-(4-hydroxy-3-nitrophrnylacetamido) propionic acid conjugated to bovine serum albumin (BSA)/GeneTex, Inc
Acrolein (ACR)	Anti-ACR	Mouse monoclonal	1:500 (IHC) 1:1000 (WB)	Immunohistochemistry Western blot	Acrolein modified keyhole-lympet hemocyanine/ Abcam
8-Hydroxy-2'-deoxyguanosine (8-OHdG)	Anti-8-8-8-OHdg	Mouse monoclonal	1:1000 (IHC) 1:3000 (WB)	Immunohistochemistry Western blot	Chemical/ Small Molecule: 8-Hydroxy-2'-deoxyguanosine conjugated Keyhole Limpet Hemocyanin/Abcam

(3 μg/μl) before incubation with sections. The results of these controls were negative (i.e. staining was abolished).

Immunoreactive cells were counted by image analysis software in 10 fields using an Olympus Bx51 light microscope (Olympus, Italy) equipped with a digital camera (at x400 each field represented a tissue section area of about 0.036 mm^2) (Di Giancamillo et al., 2008). The observer was not aware of the origin of the sections.

Double immunofluorescence

Because with cellular counting only NT-immunoreactive mucosal cells were revealed to be influenced by VB (see below), we opted to characterize the cellular typology involved, using a double immunofluorescence procedure. The dewaxed and re-hydrated sections were incubated with the first-step primary antibody, 1:10 goat anti-mouse nitrotyrosine, for 24 h at 18-20°C, then washed in TBS (Tris-buffered saline solution 0.05 M Tris/HCl, 0.15 M NaCl pH=7.6), and subsequently treated with the Avidin-Biotin blocking kit solution (Vector Laboratories Inc.). The sections were then washed in TBS for 10 min and incubated with a solution of goat biotinylated anti-rabbit IgG (Vector Laboratories Inc.) 10 μg/ml in TBS for 1 h at 18-20°C. After rinsing twice in TBS, the sections were treated with Fluorescein-avidin D (Vector Laboratories Inc.), 10 μg/ml in $NaHCO_3$, 0.1 M, pH 8.5, 0.15 M NaCl for 1 h at 18-20°C. The sections were then washed in TBS and incubated with rabbit IgG (Vector Laboratories Inc.) for 2h to inhibit binding of the second primary antiserum to the goat anti-rabbit IgG used in the first sequence. For the second step of the double immunofluorescence procedure, the slides were treated with somatostatin, gastrin or serotonin antibodies (polyclonal rabbit anti-somatostatin-14, AB1976; polyclonal rabbit anti-gastrin, AB930; polyclonal rabbit anti-serotonin, AB938, all from Abcam, Italy). Sections were then rinsed in TBS for 10 min and incubated with 10 μg mL^{-1} goat biotinylated anti-rabbit IgG (Vector Labs.) for 1 h at 18-20°C. The sections were then washed twice in TBS, and treated with Rhodamine-Avidin D (Vector Laboratories Inc.), 10 μg mL^{-1} in $NaHCO_3$, 0.1 M, pH 8.5, with 0.15 M NaCl for 1 h at 18-20°C. Finally, slides with tissue sections were embedded in Vectashield Mounting Medium (Vector Laboratories Inc.) and observed using a Confocal Laser Scanning Microscope (FluoView FV300, Olympus, Italy). The immunofluororeactive structures were excited using Argon/Helio-Neon-Green lasers, with excitation and barrier filters set for fluorescein and rhodamine. Images containing the superimposition of fluorescence were obtained by sequentially acquiring the image slice of each laser excitation or channel.

Tissue homogenisation and Western blot analysis

Tissue specimens were homogenised in 2 ml of an ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 0.2% Triton X-100, and protease inhibitor mixture) per 200 mg of tissue using an Ultra-Turrax homogeniser (IKA-Werke, Staufen, Germany). The homogenate was then centrifuged at 20000 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, USA) based on the bicinchoninic acid (BCA) 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 a bovine serum albumin (SIGMA, Italy) with a working range of 50-800 μg/ml. Total proteins (35 μg) were resolved by 10% SDS-PAGE and electro-transferred onto nitrocellulose membranes. After blocking, membranes were incubated overnight at 4°C with primary antibodies (see Table 1) and subsequently with a horseradish peroxidase-conjugated secondary antibody (1:5000) at room temperature for 45 min. Immuno-reactivity was detected by chemiluminescence autoradiography according to the manufacturer's instructions, and the images were scanned. The optical intensities of the protein bands of interest were determined densitometrically using Scion Image software. Each electrophoresis gel contained samples from both treatment groups in order to reduce between-blot effects. The values were normalized to GADPH levels.

Statistical analyses

Statistical analysis of the quantitative data was performed using the general linear model of the SAS package (version 8.1, Cary Inc., NC, USA). Blood analyses were submitted to one way ANOVA and the baseline measure of that variable (weaning values) was used as a covariate. Histomorphometrical analyses (cells counts) were conducted by ANOVA using the PROC MIXED procedure of the SAS package. The mixed model included the fixed effect of treatment and the random effect of the pig. The individual pig values were considered to be the experimental unit of all the response variables. The data were presented as least squared means ± SEM. Differences between means were considered significant at $P<0.05$.

Results

Blood antioxidant activity

Dietary supplementation with VB did not affect the blood total antioxidant activity in pigs (Fig. 1).

Micro-anatomical analyses

Histology and histomorphometry

The histological and structural aspects of both duodenum and jejunum in VB-supplemented animals

was judged to be fully normal. Moreover, histomorphometrical analysis revealed no differences between the controls and treated animals (Table 2).

Immunohistochemistry and cell counts

Immunostaining of duodenum and jejunum with NT antibody revealed that in both control and treated animals nitrotirosine was present in small, roundish endocrine cells (Fig. 2a) and lymphocytes of the diffuse lymphatic tissue. We subsequently quantified NT-immunoreactivity by cell counts, and found that NT was significantly lower in the duodenal endocrine cells of the treated animals, but not in the mucosal lymphocytes (Table 3). Jejunum on the other hand, showed no significant differences among the groups (Table 3). Immunoreactivity to ACR was present in small endocrine cells (Fig. 2b) and lymphocytes of the diffuse lymphatic tissue in both controls and treated animals. ACR quantification by cell counts revealed no differences among the groups in either the duodenum or jejunum (Table 3).

Finally, we evaluated the expression of 8-OHdg in VB pigs compared with controls. We noted that immunoreactivities were present in the nuclei of endocrine cells and mucosal lymphocytes of the diffuse lymphatic tissue in both duodenum and jejunum, but also in the mucous cell of the Brunner gland of the duodenum in both control and treated animals (Fig. 2c). Counts of the immunoreactive structures did not show any differences among the treatment groups in either duodenum or jejunum (Table 3).

Double immunofluorescence

Because with cellular counting only anti-NT immunoreactive endocrine cells were revealed to be influenced by VB, we decided to characterize the cellular typology involved. NT revealed no co-localization with somatostatin (Fig. 2d) and gastrin (immunostaining only for duodenum; Fig. 2e), whereas some co-expression was present with serotonin in the duodenum (Fig. 2f), but not in the jejunum (Fig. 2g) in VB animals.

Western blot analyses

The antibodies used in this study recognized adducts with two specific molecular weights: 50 and 20kDa in

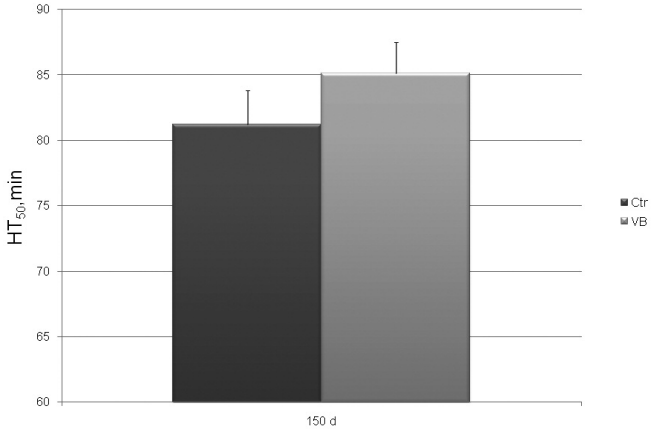


Fig. 1. KRL test. Total antioxidant activity of whole blood in pigs fed control diet or diet supplemented with verbascoside (Treatment effect P=0.287).

Table 2. Effects of dietary VB on villi height (V), crypt depth (C), V:C ratio in the duodenum and jejunum1

Measure[1]	CON	VB	P values
Duodenum			
Villi height (V), μm	380.15±8.42	392.55±26.31	0.385
Crypts depth (C), μm	502.44±15.91	505.68±26.31	0.885
V:C ratio	0.75±0.06	0.78±0.06	0.983
Jejunum			
Villi height (V), μm	406.27±6.33	401.22±8.72	0.560
Crypts depth (C), μm	461.18±6.11	453.49±14.79	0.713
V:C ratio	0.88±0.05	0.88±0.05	0.932

[1]: Values are least squared means ± SEM; n / treatment=9.

Table 3. Effects of dietary VB on histomorphometrical analyses related to immunohistochemistry (oxidative/nitrosative stress biomarkers) of duodenum and jejunum[1].

Measure[1]	CON	VB	P values
Anti-NT			
Duodenum			
Endocrine cells	10.95±0.29	8.95±0.26	<0.001
Lymphocytes	5.00±0.25	4.90±0.20	0.664
Jejunum			
Endocrine cells	14.70±0.14	14.30±0.20	0.123
Lymphocytes	4.95±0.19	5.05±0.21	0.628
Anti-ACR			
Duodenum			
Endocrine cells	16.75±0.30	16.50±0.33	0.586
Lymphocytes	4.75±0.17	4.45±0.19	0.109
Jejunum			
Endocrine cells	15.10±0.36	14.90±0.35	0.710
Lymphocytes	3.10±0.09	3.05±0.07	0.709
Anti-8OH-dg			
Duodenum			
Endocrine cells (nuclei)	17.10±0.32	17.45±0.30	0.587
Lymphocytes (nuclei)	73.89±0.74	73.20±0.88	0.514
Brunner glands (nuclei)	90.20±1.24	89.10±0.91	0.480
Jejunum			
Endocrine cells (nuclei)	19.79±0.43	19.30±0.30	0.367
Lymphocytes (nuclei)	91.47±0.94	91.80±1.15	0.829

[1]: Values are least squared means ± SEM; n / treatment = 9.

both duodenum and jejunum, with the exception of 50 and 30kDa for ACR jejunum. Comparison of the relative densities using the anti-ACR or anti-8OHdg revealed no statistical differences among the groups in either duodenum or jejunum, whereas anti-NT showed a statistically lower signal of both the isoforms in the

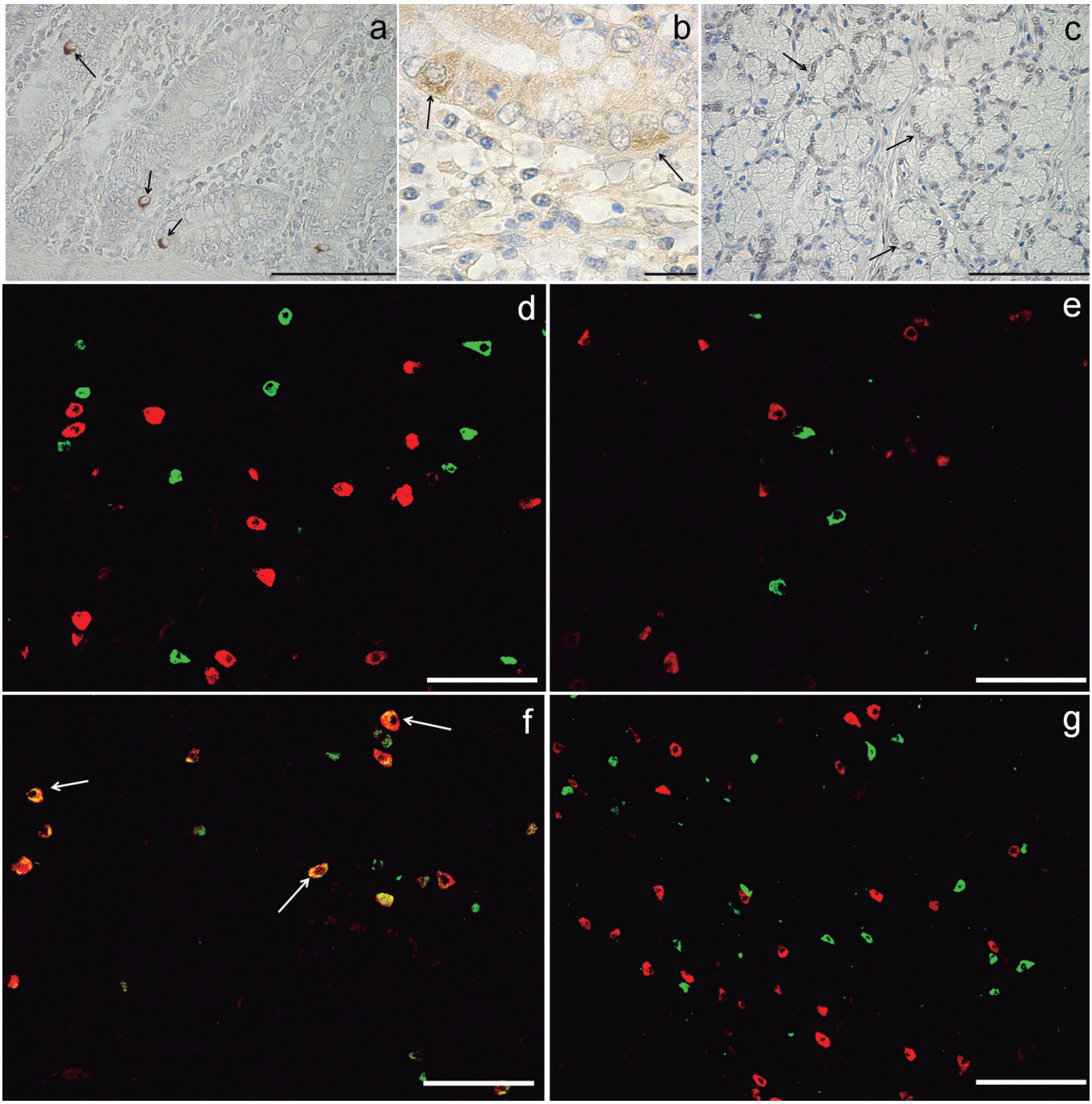


Fig. 2. Micro-anatomical analyses in VB supplemented animals. Immunohistochemistry for **(a)** nitrotyrosine in small roundish endocrine cells of the duodenal crypts (arrows), **(b)** acrolein in endocrine cells of the duodenal crypts (arrows), **(c)** 8-OHdg in some nuclei of duodenal Brunner glands (arrows). Double immunofluorescence for **(d)** nitrotyrosine and somatostatin in duodenum with no co-localization (green and red respectively), **(e)** nitrotyrosine and gastrin in duodenum with no co-localization (green and red respectively), **(f)** nitrotyrosine and serotonin in duodenum with co-expression (green and red respectively, and yellow for co-expression), but not in jejunum **(g)** (green and red respectively). Scale bar: a, c, 100 μm; b, 10 μm; d-g, 20 μm.

duodenum of VB animals (Fig. 3).

Discussion

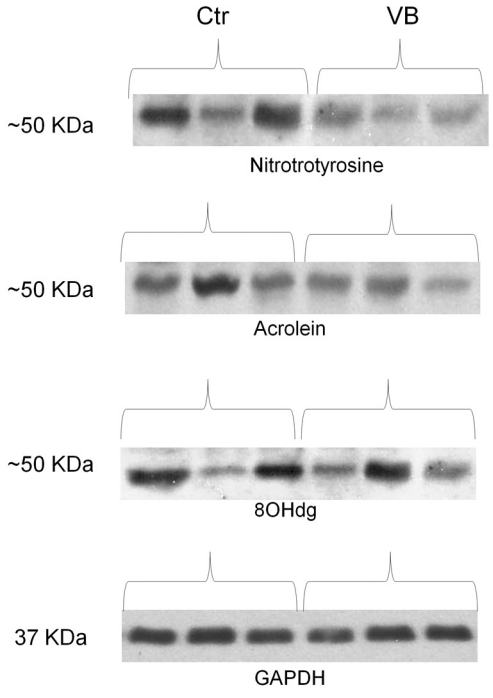
In recent years there has been an increased scientific interest in the application of antioxidants to dietary interventions due to findings linking oxidative stress to the development of chronic and degenerative diseases. Phenolic compounds that are commonly present as secondary metabolites in both edible and nonedible plants have been studied in recent years studied because they are able to display several biological effects, including antioxidant activity (Hu, 2011). This is of a special importance considering that, when in excess, the reactive oxygen species (ROS) and the reactive nitrogen species (RNS), which are products of normal metabolic cellular pathways, can lead to the development of chronic pathological conditions (Finkel and Holbrook, 2000). As previously observed, acute and chronic stress can induce gastrointestinal oxidative stress and mucosal injury (Manashi et al., 2000; Chen et al., 2007). Quite recently, antioxidant therapies have been hypothesized as effective in contrasting some human degenerative diseases (Firuzi et al., 2011). Additionally, nutritional epigenetics is now being debated concerning human age-related diseases (Park et al., 2012) and some types of malignancies (Shen et al., 2012).

Our results show that biomarkers of oxidative and nitrosative stress are detected in the mucosal components of both the duodenum and jejunum, especially in the endocrine cells that play a fundamental role in the control of the gut.

To our knowledge, this is the first time that epithelial cells belonging to the diffuse endocrine system have been correlated to oxido-redox balance. Less surprising is the presence of stress biomarkers in immuno-competent cells belonging to diffuse lymphatic tissue, as also observed in fish (Pascoli et al., 2011).

We focused our attention on the gut environment, because in farming systems several stress factors (disease pressure, transportation, vaccination, feed transitions, long-term periods of farming, mixing and other environmental factors) play a major role in disturbing the gut. There are essentially two ways by which stress negatives impact on gut health: i) by causing disturbances in gastrointestinal motility and feed intake patterns, and ii) by leading to imbalances between resident microflora and microbial pathogens, thus increasing the risk of infections (Rostagno, 2009). These in turn result in systemic changes in the animal, such as oxidative stress and inflammation, leading to the loss of gut barrier integrity (Zhou and Zhong, 2012) and consequently diarrhoea and intestinal infections. The end result is increased use of drugs, higher veterinary costs, poorer efficiency and daily gain, higher mortality rate and consequent financial loss.

In this study, NT was found to be influenced by VB dietary intervention with a significant decrease, measured by NT-immunopositive endocrine cell count and Western blot in the duodenum, but not in the



	Ctr	VB	P *values*
NITROTYROSINE			
Duodenum			
High isoform (~50KDa)	0.72 ± 0.09	0.57 ± 0.02	<0.001
Low isoform (~20KDa)	0.32 ± 0.01	0.22 ± 0.01	<0.001
Jejunum			
High isoform (~50KDa)	0.67 ± 0.01	0.64 ± 0.01	0.099
Low isoform (~20KDa)	0.41 ±0.01	0.41 ± 0.01	0.942
ACROLEIN			
Duodenum			
High isoform (~50KDa)	0.76 ± 0.02	0.73 ± 0.02	0.507
Low isoform (~30KDa)	0.53 ± 0.01	0.54 ± 0.01	0.692
Jejunum			
High isoform (~50KDa)	0.79 ± 0.01	0.77 ± 0.01	0.420
Low isoform (~30KDa)	0.59 ± 0.02	0.58 ± 0.01	0.588
8OHdg			
Duodenum			
High isoform (~50KDa)	0.77 ± 0.04	0.74 ± 0.02	0.668
Low isoform (~20KDa)	0.33 ± 0.02	0.32 ± 0.02	0.737
Jejunum			
High isoform (~50KDa)	0.46 ± 0.02	0.44 ± 0.01	0.325
Low isoform (~20KDa)	0.25 ± 0.01	0.27 ± 0.01	0.329

Fig. 3. Western blot. Nitrotyrosine protein adducts decreased in duodenum of VB animals (P<0.05), but not in jejunum; Acrolein and 8-OHdg revealed no significant differences within the groups of treatment. Nitrotyrosine, acrolein and 8-OHdg levels were normalized and quantitated to GAPDH.

jejunum. This may be explained by considering that the duodenum is more exposed than jejunum to the chemical stress of the acidic contents of the stomach. Secondarily, the duodenum is the first intestinal site in which the microencapsulated VB can affect the intestinal mucosa, and in which, consequently, the studied molecule may display (and possibly exhaust) its antioxidant properties. Considering this, a gradual release in the active principle could be commercially performed in the encapsulation procedure in such a way as to reach the intestinal tracts further and ensure a more extended antioxidant activity.

We also performed double immunofluorescence tests for co-localization studies. Endocrine cells that were immunoreactive for serotonin were found to be the only ones directly involved in relieving nitrosative stress. In the gut, serotonin is synthesized by the enterochromaffin cells and intramural neurons (Gershon, 2003), and the gut is one of the major sites of serotonin synthesizing and releasing. Besides being a neurotransmitter in the central nervous system, serotonin has also emerged as a key mediator of various biological processes in peripheral tissues, such as the regulation of bowel motility and secreting processes, cell proliferation and differentiation, as well as visceral sensitivity (Gershon and Tack, 2007). In addition, serotonin is a pivotal signalling molecule in the brain-gut axis, both in health and disease (Crowell and Wessinger, 2007). Because enterochromaffin cells were shown in this study to be less subject to nitrosative stress after VB supplementation, we would suppose that a reduction in the serotonin nitrosative stress status may indirectly influence the efficiency of the gut barrier, modulating the motility and the secretory processes of the mucosal cells.

Our data on total antioxidant activity of whole blood failed to reveal any difference between dietary treatments, as in a recent study of our group (Rossi et al., 2011) upon post-weaning piglets. In contrast, other authors (Liu et al., 2003), have reported the antioxidant action of verbascoside on plasma TBARS values (malondialdheyde measure) of immobilized rabbits fed with 0.8 mg/kg. We suggest that the VB effect after long-term dietary supplementation may exert only a local (duodenum) and not a systemic antioxidant activity, due to the VB dosage used, which is possibly entirely metabolized by duodenal mucosa.

To date, considerable evidence of oxidative stress has been obtained as causing a number of diseases in farm animals. Taking into account the great amount of oxidative stress-mediated conditions involved in all the farming phases (weaning, growing and fattening phases), there is a great potential for dietary antioxidant supplementation in farm animals (Lykkesfeldt and Svendsen, 2007; Lallès et al., 2011), although further studies to evaluate the optimal dose and length of the studied dietary supplementation and a better understanding of its mechanism of action are needed. Finally, considering the antioxidant activity of verbascoside, it would also be interesting to investigate its possible influence on porcine meat quality and to assess its possible use as a substitute for synthetic vitamin E (alpha-tocopheryl acetate), which is usually present in feedstuff.

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