USE OF POLYPHENOLS EXTRACTED BY OLIVE MILL WASTEWATER AS DIETARY SUPPLEMENTS IN PIG: EFFECTS ON GUT ASSOCIATED LYMPHOID TISSUE, BLOOD LEUKOCYTES AND ALVEOLAR MACROPHAGES.

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Abstract: The polyphenols are able to reduce the amount of free radicals and stimulate the immune response. In this study, polyphenols extracted from olive mill wastewaters, were fed to pigs for three months. Morphological, inflammation and immunological parameters were evaluated. Histological samples of gastrointestinal tracts were analyzed for immunodetection of cyclooxygenase-2; superoxide anion production in primary cell cultures of blood leukocytes and alveolar macrophages was measured. COX-2 immunopositivity was present mainly in the control pigs, while superoxide anion production was lower in pigs fed polyphenols. In the light of these results, we could suggest the possible re-use of agri-food industry wastes as feed additives for farmed animals.

Keywords: *feeding, adult pig, polyphenols, immune response, antioxidant effect.*

1. INTRODUCTION

Polyphenol**s** have already found application in animal nutrition, in particular as feed additives to reduce free radicals in a wide variety of animal species. Several studies carried out on diets supplemented with additives containing natural antioxidants show their capability to improve the productive performance, immune response and health of livestock besides reducing the risks of various animal diseases such as cancer and other degenerative diseases [1].

Agricultural by-products are a rich source of bioactive molecules, including polyphenols. Olive meal wastewater (OMWW), adequately treated, represent an important source of these molecules, in particular manner of oleuropein, hydroxytyrosol and tyrosol.

These compounds are able, in vitro, to inhibit the production of various inflammatory substances such as leukotriene B4 (LTB4) from endogenous arachidonic acid as well as cyclooxygenase-2 (COX-2) and i-NOS gene expression [2,3].

One of the important anti-inflammatory mechanisms is the inhibition of eicosanoids generating enzymes including phospholipase A2, cyclooxygenase, and lipoxygenase thereby reducing the concentration of prostanoids and leukotrienes.

Arachidonic acid is released by membrane phospholipids through phospholipase A2 (PLa2) cleavage; it can be metabolized by cyclooxygenase (COX) pathway into prostaglandins (PGs) and thromboxane A2 (TXA2), or by lipoxygenase (LOX) pathway to hydroperox-yeicosatetraenoic acids (Hpetes), hydroxyeicosatetraenoic acids (Hetes) and leukotrienes (Lts).

Cyclooxygenase exists in two major isoforms (COX-1 and COX-2) and one variant (COX-3) [4-5].

In this note, we reported the use of polyphenols extract by OMWW as dietary supplement in adult pig. In detail, we detected cyclooxygenase-2 immunolocalization in the gut associated lymphoid tissue and the production of anion

superoxide in primary blood leukocytes and alveolar macrophages cell cultures.

2. MATERIAL AND METHODS

2.1 Experimental design

This study was carried out on 13 month old pigs (genetic type autochthonous Casertana) housed at a local farm (Mastrofrancesco Farm, Morcone, Benevento, Italy). Twenty-four animals were divided into two experimental groups (treated and control group). In the control group twelve animals of both sexes were fed with a standard diet for the finishing stage (wheat 20%, barley 20%, corn 20%, soy 10%; bran 20%; fava beans 10%). In the treated group twelve animals were fed with polyphenols extracted from olive mill wastewaters (OMW) (0,33mg/Kg/day) added to the standard diet. Slaughter took place at 16 months of age. The main compounds contained in the extract from OMWW were represented by hydroxytyrosol and its derivated.

2.2 Sampling

Pigs were killed 120 days after the treatment commencement, slaughtering took place at local slaughterhouses (Santa Croce del Sannio and Vitulano, Benevento, Italy). Internal organ sampling started soon after death, through a horizontal incision along the abdominal midline. Blood, alveolar macrophages and gastrointestinal tract were collected from all animals. Tissue samples of $1cm^2$ of length were taken of the central part of the following gut compartments: gastric fundus, duodenum, jejunum, ileum, caecum, and colon. Samples were washed three times in 09% NaCl solution and fixed in 10% buffered formaldehyde. To extract alveolar macrophages, lungs were washed with 200 ml of sterile PBS.

The pulmonary regions (caudal lobes) were gently massaged and then the liquid was slowly collected in sterile tubes on ice. Blood was collected in chilled tubes containing EDTA and placed on ice until further use.

2.3 Immunohistochemistry

Cyclooxygenase-2 (COX-2) expression in the gastrointestinal tract was performed by avidin-biotin immunohistochemical technique. Goat polyclonal antibodies raised against COX-2 [(Cox2 (k-20): sc-23984. (Santa Cruz Biotechnology, Inc.)] diluted 1:250. , were applied on histological sections overnight at 4°C. The other components of the immunological reaction were contained in the Vectastain Elite ABC kit (PK-6105 goat) from Vector Laboratories. The final staining was performed using a solution of 3-3' diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO) of 10 mg in 15 mL 0.5 M Tris buffer, pH 7.6,containing 0.03% hydrogen peroxide.

2.4 Superoxide anion assay

Both cultured pig alveolar macrophages (at a density of 60,000) and leukocytes (at a density of 120,000) were seeded in a 96-well microplates, in triplicate. Cells were incubated with 100 µl PBS 1× (Lonza) containing NBT (1 mg/ml, Sigma) and

zymosan A (2000 µg/ml, Sigma) for 90 min. Control samples were incubated only with NBT. Following incubation, peripheral leukocytes and macrophages were washed in PBS 1× twice, to remove all residual NBT solution, leaving only a cell pellet containing formazan. To quantify the formazan product, the intracellular formazan was dissolved in 120 µl 2 M KOH and 140 µl dimethylsulfoxide (DMSO, Sigma), and the resulting color reaction was measured with a microplate reader (Model 680 Biorad) at 620nm.

2.4 Microscopically observations

Both cytological (peripheral blood smear, leukocytes isolated from peripheral blood and alveolar macrophages) and histological slides were observed using a microscope Nikon E600.

2.5 Statistical analysis

Statistical analysis was carried out using GraphPad Prism version 5.0 (GraphPad, San Diego, CA). Results are expressed as means ± standard error of the mean (SEM). Two tailed, paired *t* tests were performed and statistical significance was claimed between two groups where $p<0.05$.

3. RESULTS

3.1 Immunohistochemistry

COX-2 immunopositive cells were identified mainly in the gastrointestinal tracts of the control group. In particular, immunopositivity was detected in the lamina propria and intra-epithelial leukocytes of caecum (Fig. 1d), and colon tracts (Fig. 1a).

Immunopositivity was also present in the effector sites of gut associated lymphoid tissue (GALT) represented by ileum solitary follicles and Peyer's patches (Fig. 1h).

Immunopositive cells were absent in the caecum and ileum tracts, and in the solitary follicles of treated animals (Fig. 1f). COX-2 immunopositive cells with weak intensity of staining, were present in the colon tract (Fig. 1b). Negative controls did not show any positivity (Fig. 1c).

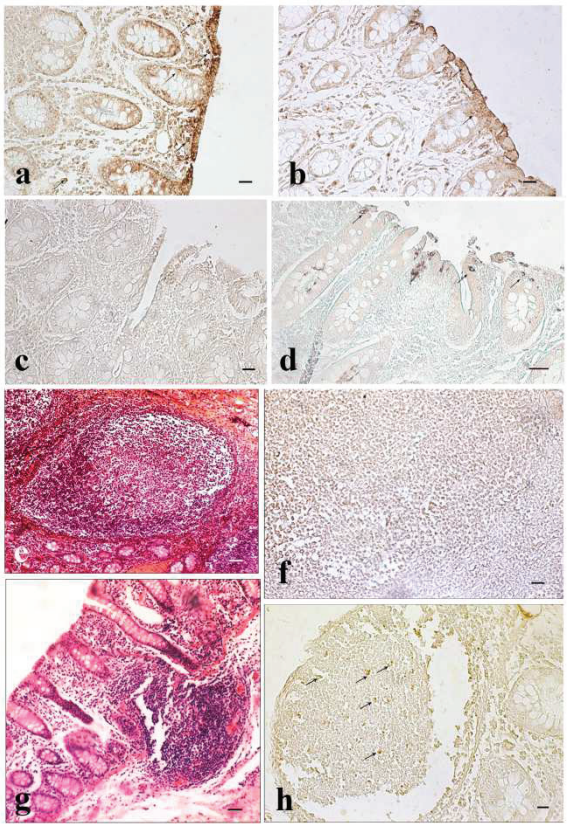

Fig.1: Immunohistochemistry; cyclooxygenase-2: a) Immunopositive leukocytes (arrows) in the colon mucosal layer of control pig; b) Immunopositive cells with weak intensity of staining (arrows) in the colon mucosal layer of treated pig; c) negative control of colon tract of control pig d) Immunopositive intra-epithelial leukocytes (arrows) in the epithelium of caecum tract of control pig. e) Hematoxylin-eosin staining of a ileum solitary follicle of treated pig; f) Absence of immunopositivities in the same ileum follicle solitary showed in the previous picture (histological section consecutive to the previous one); g) Hematoxylin-eosin staining of a Peyer's patch of caecum control pig; h) various immunopositive cells (arrows) located in the same Peyer's patch showed in the previous picture (histological section consecutive to the previous one). Scale Bars: 20µm (a-e, h); 10µm (f); 50µm (g).

3.2 Superoxide anion assay

The OMW polyphenols were tested for anti-oxidant effects on pig blood leukocyte enriched

fraction and alveolar macrophage fraction by superoxide anion assay. Peripheral blood leukocytes of pigs belonging to the treated group showed lower levels of superoxide anion compared to control animals after *in vitro* stimulation with zymosan (Fig. 2). Alveolar macrophages extracted from pig lungs of the control group showed higher superoxide anion levels with respect to treated animals (Fig. 2).

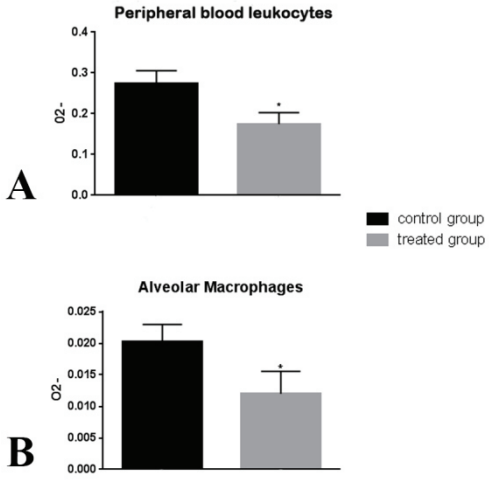

Fig.2: Superoxide anion assay; (A) Peripheral blood leukocytes: The anion superoxide levels were lower in pigs belonging to treated group compared with control animals. (B) Alveolar macrophages: The anion superoxide levels were lower in pigs belonging to treated group compared with control animals Each value represents the mean ± S.E.M. of three separate experiments; $p<0.05$.

4. CONCLUSIONS

The results described in this note could suggest that OMW polyphenols, added to the standard diet of farmed pigs, can improve their welfare, with particular reference to their immune and inflammatory response. Further investigations will be necessary to better understand the possible role of these compounds on animal health, but certainly, our results represent a starting point to could suggest the possible re-use of agri-food industry wastes as feed additives for farmed animals.

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