# USE OF POLYPHENOLS EXTRACTED FROM AGRICULTURAL WASTE BY-PRODUCT AS DIETARY SUPPLEMENT IN AQUACULTURE

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***Abstract*** – Agricultural waste by-products are a rich source of biomolecules with antimicrobial, immunostimulant, anti-tumoral and anti-oxidants properties. The use of polyphenols extracted from these by-products as dietary supplements in animal feed may improve the health of animals thus limiting the use of antibiotics. We used the polyphenols extracted from the skin of chestnuts, of which Campania is the main producer, in order to evaluate a possible immunostimulant activity on leucocytes isolated from blood and GALT of the rainbow trout *Oncorhynchus mykiss*.

***Keywords***: chestnut skin extracted, rainbow trout, blood and GALT leucocytes.

## 1. INTRODUCTION

Agricultural waste by-products are rich in biologically active molecules such as terpenes, terpenoids, alkaloids and phenolic substances [1]. Although agricultural waste represents a problem due to its disposal, it represents an inexhaustible source of substances that can be used in various fields, ranging from cosmetics to nutraceuticals.

An approach of waste valorisation, pointed at the recovery of the phenolic fraction to be employed for animal supplemental feeding, may represent an efficient approach to improve the health of animals thus limiting the use of antibiotics and vaccines.

In fact, although vaccination represents the most effective prophylactic measure of controlling fish deseases, it is too expensive and pathogen specific.

Therefore, reinforcement of the defense mechanism of fish through administration of immunostimulants is considered as a promising alternative to vaccines because of their broad spectrum activity, cost effectiveness, and eco-friendly disease preventive measure [2].

Natural immunostimulants such as alkaloids, flavanoids, pigments, phenolic compounds or polyphenols, terpenoids, steroids, and essential oils can modulate the innate immune response and their use in aquaculture is generally accepted [3].

In particular, the polyphenols represent a large group of at least 10,000 different compounds produced by plants as secondary metabolites in order to protect them against different types of stress such as reactive oxygen and nitrogen species, UV light, pathogens, parasites and plant predators [4, 5, 6].

They contain one or more aromatic rings with one or more hydroxyl groups attached to them [4,5].

Due to their antioxidative properties, all polyphenols offer numerous health benefits, alone or in combination with other molecules. They have antioxidant, anti-inflammatory, anti-carcinogenic and other biological properties, and may protect from oxidative stress and some diseases [7].

The Campania region is the main producer of chestnuts in Italy and in Europe.

The kernel of chestnut are usually consumed whole or used as ingredient in a variety of processed foods.

Several studies reported the presence of bioactive antioxidant molecules, not only in edible kernels of chestnut [8,9,10] but also in chestnut waste [11,12] resulting from the agricultural and industrial processing of these plant foods.

Therefore, we have extracted polyphenols from chestnut skins, characterized classes of polyphenols present in these wastes and evaluated a possible immunostimulant activity on lymphocytes isolated from blood and lymphocytes extracted from GALT of the rainbow trout *Oncorhynchus mykiss*.

## 2. MATERIAL AND METHODS

### *2.1* Animals

Adult rainbow trout (*Oncorhynchus mykiss*), weighing 500–600 g, were used for this study. Fish were obtained from a local dealer (Di Mella, Santacroce del Sannio, Benevento, Italy) and allowed to acclimate in recirculating water system tank at 12 ◦C for 24 h before sacrifice. Fish were euthanized by immersion in MS-222 (MP Biomedicals, LLC, Aurora, OH, USA) 80 mg/L (LC50 >200 mg/L).

Fish used in this study were treated in accordance with the European Commission recommendation 2007/526/EC and 2010/63/UE on revised guidelines for the accommodation and care of animals used for experimental and other scientific purposes. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Biogem Consortium, Ariano Irpino, Italy.

The protocol was approved by the Committee on the Ethics of Animal Experiments of the same Consortium. Water parameters and environmental conditions were as stated in the European Commission recommendation 2007/526/EC and 2010/63/UE. All efforts were made to minimize fish suffering.

### 2.2 Extraction and characterization of polyphenols from chestnut shell

The polyphenols extracted from chestnut shell were recovered by vacuum filtration and the solvent was evaporated in rotary evaporator (Mod. Hei VAP Value; Heidolph, Schwabach, Germany). The residue was placed in a drier and weighed up to constant value and the extraction yield was calculated as the percentage weight loss of the starting material.

Polyphenol analysis was performed by LC-4000 Series Integrated HPLC Systems (JASCO, Japan). All solvents were filtered through a 0.45-μm filter disk (Millipore Co., Bedford MA, USA). The mobile phase composed by water–formic acid (99.80:0.20, v/v) (solvent A) and methanol (solvent B) was used. For chestnut polyphenol extract analysis the gradient elution was according to Azaizeh et al. [14] with some modifications. The temperature was maintained at 30°C. The specific elution conditions were: 0–5 min, 20% B; 5–10 min, 20–70% B; 10–21 min, 70–80% B; 21–25 min, 80% B, 30-32 min, 80-20% B; 32-40 min, 20% B.

Each run was followed by 5 min of washing with 100% B. The system was equilibrated between runs for 10 min using the starting mobile phase composition. Each sample was analyzed at least twice. The flow-rate was 0.8 ml/min. The injection volumes was 20 μl. Polyphenols extracted were identified by comparing retention times of the detected peaks and UV-VIS spectra with those of pure commercial standards and with literature data.

### 2.3 Total phenols content and Antioxidant activity

Total phenols content extracted from chestnut shell was determined by the Folin-Ciocalteu method [13]. The total phenols content was calculated as gallic acid equivalent from the calibration curve of gallic acid standard solutions (2–40_g/mL) and expressed as mg gallic acid equivalent (GAE)/mg of extract (on a dry basis). The analyses were done in triplicate and the mean value was calculated.

The antioxidant activity of the polyphenols extracted from chestnut shell was evaluated as antiradical activity by using the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•), according to Barreira et al. [8] 100 mg of extract were added to 2.4 mL of 0.0004% DPPH• in methanol and the absorbance was measured at 517 nm until the reaction reached a plateau (1 h). The anti-radical activity was expressed as a percentage of inhibition (%I) of the sample (As) compared to the initial concentration of DPPH• (Ac) according to the equation: $\%I = [(Ac - As)/Ac] \times 100$. The analyses were done in triplicate and the mean value was calculated.

### 2.4 Isolation of leucocytes from rainbow trout blood

Isolation of leucocytes from blood was carried out according to Mariano et al. [15]. Briefly blood was withdrawn from the caudal vein using a syringe previously rinsed with heparin. Leucocytes were isolated [16] with few modifications. Blood was diluted 1:5 with RPMI (Lonza 1640) and centrifuged at 300 × g for 10 min at 4°C. The pellet was diluted 1:50 with RPMI and layered onto Histopaque (1.077 g/l)(Sigma–Aldrich, St. Louis, MO, USA) and then centrifuged at 300 × g for 25 min at 4°C. Leucocytes were harvested from the interface and washed with RPMI by centrifugation at 300 × g for 10 min at 4°C. Isolated leucocytes were counted with cells counter (Casy)

**2.5 Extraction of intraepithelial leucocytes ( IEL) from rainbow trout GALT**

The mucosa-associated lymphoid tissues in teleost fishes include the gut, skin and gills. These tissues with their layer of mucus and array of nonspecific immune defences are exposed to the external environment and form the initial barrier to invasion by pathogens[17]. Intra-epithelial leucocytes are present in the gut but most leucocytes are present in the lamina propria of the gut folds and luminal to the stratum compactum [18]. In most teleost species examined, a layer (‘stratum granulosum’) consisting of one or a few rows of mast cells/granule cells is located between the stratum compactum and the circular muscle layer [19]

Extraction of IEL from GALT was carried out as fallow. Post cecal intestine (from the small intestine to the rectum) was withdrawn, cleaned from fat, cut longitudinally and washed with PBS(-) Ca Mg free containing 200U/ml penicillin and 200microg/ml streptomycin (stock solution Pen/Strep 5000U/ml-500microg/ml). Afterwards, the intestine was scraped on ice and cut into small pieces with a diameter of 0.5- 1 cm, incubated with 25 ml HBSS(-), containing 1mM DTT (0.004gr in 25ml) 1mM EDTA (0.009 in 25 mL) pH 7.4, onto a shaker at 120 rpm for 1 hour at 20°C. The supernatant was collected and filtered with a 100 micron nylon filter (millipore ) and centrifugated at 400 × g for 10 min at 4°C. Isolated leucocytes were resuspended in 2 ml RPMI.

To generate T and B enriched lymphocyte subpopulations of high purity, Nylon Wool Fiber Columns (Polysciences,Inc. Chemistry beyond the ordinary) were used according to the following protocol.

The column, composed by syringe with stopcock and plunger, was washed with RPMI 10% FBS at 37°C for 1 hour , and gently inverted until wool was wet and free of air bubbles. After 1 hour the stopcock was opened to let all the media drain, and closed. 1-2 x$10^8$ viable cells per column, in a volume of 2ml of RPMI, were added until entered the packed wool, stopcock was then closed. Then the top of the column was washed with 2ml of RPMI and allowed wash to enter the packed wool. The stopcock was closed. 2-5ml of RPMI were added to column to ensure that the top of the wool was covered with media and incubated for 1 hour at 37°C. After that, non-adherent T-cells were collected by using two washes without plunging, while adherent B-cells were collected by adding media to fill column which was then knocked to dislodge cells. Collected cells were spun down at 1200 rpm for 10 minutes and supernatant was discarded. Cell pellet was resuspended in approximately 10 ml of L15 and counted with CASY (ROCHE) automated cellular counter.

**2.6 Superoxide anion**

Superoxide production was determined as the reduction of nitroblue tetrazolium (NBT) according to Mariano et al. [15] with some modifications. Briefly both Leucocytes from GALT and blood (at a density of 400.000 cells/well) were cultured with both gallic acid (three concentrations: 1 µ/ml -10 µ/ml -100 µ/ml) and chestnut skin extracted polyphenols (five concentrations :10 µ/ml -50 µ/ml -100 µ/ml 500 µ/ml - 5000 µg/ml) and incubated at 18–20◦C for three hours before zymosan stimulation was done.

Afterwards, leucocytes were incubated with PBS 1× (Lonza) containing NBT(1 mg/ml, Sigma) and zymosan A (2000 µg/ml, Sigma) for 90 min. Following incubation, leucocytes were washed and centrifuged at500 × g, for 10 min at 4◦C 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 dis-solved in 120 µl 2M KOH and 140 µl dimethylsulfoxide (DMSO, Sigma), and the resulting color reaction was measured with a microplate reader (Model 680 Biorad) at a wavelength of 620nm.

### 2.7 Phagocytosis assay

The phagocytosis assay measures leucocytes phagocytic activity by zymosan stimulation, an inflammatory fluorescent molecule.

Cells (1 million cells/well) were plated in a 96-well microplate and treated with gallic acid (1 µg/ml-10µg/ml -100 µg/ml) and chestnut skin extracted polyphenols (10 µg/ml -50 µg/ml -100 µg/ml -500 µg/ml - 5000 µg/ml) for 3hours.

Then, to stimulate phagocytosis, the cells were incubated with fluorescent zymosan particles (Zymosan A S. Cerevisiae, 0.5 mg/ml) for 30 minutes. Cells phagocitic activity was measured by flow cytometry.

## 3. RESULTS

Our results indicated that polyphenols showed an high antiossidant activity, the major component of polyphenols extracted from chestnut shell was represented by Gallic Acid.
The polyphenolic extract showed any cytotoxic activity.

Superoxide anion production increased significantly in both the blood and GALT leucocytes incubated in vitro with chestnut skin polyphenol extracts compared to the control.

Likewise, leucocytes incubated first with chestnut skin polyphenol extracts and then with fluorescein-labelled zymosan particles showed an increase of phagocytosis quantified by recording their fluorescence by a flow cytometric (FCM) assay.

Polyphenol extracts used in our experiments, were also able to modulate immune-related gene expression of the pro-inflammatory cytokine TNF-α and anti-inflammatory cytokine IL-10 in the rainbow trout leucocytes.

## 4. CONCLUSIONS

Our preliminary results, show that polyphenols may improve the immune response of fish, at least under the conditions employed.

There is strong evidence that polyphenols promote various activities like antistress, growth promotion, appetite stimulation, tonic and immunostimulation, and to have antimicrobial properties in finfish and shrimp larviculture [3].

Polyphenols 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 demonstrated 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. Such activities could be attributed to their powerful antioxidant, immunomodulatory and anti-inflammatory effects by preventing free radicals from interacting with cellular DNA and their ability to alter the intestinal microbiota, increased digestibility and absorbance of nutrients [20].

Formerly, this study suggests the possible re-use of agri-food industry wastes as feed additives for farmed animals.

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