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Olive mill wastewater-enriched diet positively affects growth, oxidative and immune status and intestinal microbiota in the crayfish, *Astacus leptodactylus*

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ABSTRACT

In the present study, the effects of polyphenols extracted from olive mill wastewaters (OMWW) were investigated in the narrow clawed *Astacus leptodactylus* in a 24 week-feeding trial. 0.5 and 5.0 g of OMWW polyphenolic extract were mixed with 1 kg of basic diet to generate OMWW-LC (Low Concentration) diet and OMWW-HC (High Concentration) diet. Growth performances, antioxidant and immunological parameters, microbiota, and FA composition were evaluated. Growth performances (specific growth rate; feed conversion ratio; protein, lipid and carbohydrate efficiency ratio) and survival rate significantly improved in crayfish fed OMWW-enriched diets, in a polyphenol concentration-related manner. The activities of the oxidative enzymes GP, GR and CA were evaluated in the hepatopancreas. GP activity did not statistically changed. The increase in GR activity was statistically significant only in the OMWW-HC group. The increase in CA activity was statistically significant in both OMWW-LC and OMWW-HC groups. The immunological parameters (total haemocytes, phenoloxidase activity) significantly increased in OMWW-LC and OMWW-HC groups with respect to the control group. Superoxide anion production in the hemocytes statistically decreased only in the OMWW-HC group. No pathological changes in the midgut and hindgut after OMWW-enriched diets were detected by histological analysis. In crayfish fed on OMWW-enriched diet, total intestinal microbiota decreased, with the exception of anaerobes and yeasts. A significant increase in PUFA and decrease in MUFA was registered in the hepatopancreas. The emerging picture indicates that diets enriched with polyphenols extracted from OMWW had beneficial effects on crayfish growth and health and hence could be successfully employed in aquaculture and considered as a novel strategy of development to the feed industry sector.

1. Introduction

Since 2006 the EU has banned the use of antibiotics and other chemicals in farmed animals, as a consequence of the raising concerns about the risk to humans of chemical residues in food and of antibiotic resistance being passed on to human pathogens. Ever since, scientists have been looking at natural alternatives to chemical compounds as potential alternatives for enhancing productivity and the immune defenses (Makkar et al., 2007). Dietary supplements with growth promoting ability such as alkaloids, flavanoids, pigments, polyphenols, terpenoids, starch, steroids and essential oils have been employed in aquaculture (Citarasu, 2010). In recent years, the use of dietary supplements in feeds, such as probiotics, prebiotics and even herbal compounds has received a great deal of attention not only for the immune stimulating functions but also for the growth promoting effects on fishes and shrimps (Ganguly et al., 2010; Radhakrishnan et al., 2013). Studies carried out on crayfish show that diets enriched with L-carnitine or prebiotics such as MOS and FOS, positively affect growth performance, nutritional efficiency indices, immune response as well as the antioxidant status (Safari et al., 2014, 2015).

Phenolic compounds or polyphenols are a large class of natural compounds derived from plants. Epidemiological, clinical and nutritional studies strongly support the evidence that dietary phenolic compounds

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are effective in the prevention of common diseases, including cancer, neurodegenerative diseases, gastrointestinal disorders and others. Moreover, a large number of studies suggest immunomodulatory and anti-inflammatory properties of these compounds in humans (Scalbert et al., 2005; Pandey and Rizvi, 2009; Zhang and Tsao, 2016). Some studies report the positive effect of polyphenol-rich diets on the health of pigs and chickens (Brenes et al., 2016). The addition of polyphenols from grapes had positive effects on health and growth in the shrimp *Litopenaeus vannamei*, (Niyamosatha et al., 2015). However, polyphenol application in aquatic animal nutrition is a field largely unexplored.

The olive oil industry is very important in the Mediterranean countries, among which Spain is the main producer, followed by Italy, Greece, Turkey, Syria and Tunisia. Olive mill wastewaters (OMWW) are the main pollutant from three-phase extraction systems and traditional mills. The main characteristic of OMWW is the presence of organic compounds that turn OMWW into phytotoxic materials, representing a great environmental hazard when it is not managed properly (Federici et al., 2009). Luckily, OMWW contain valuable resources such as polyphenols, that can be as high as 10% of the dry weight (Fernández-Bolaños et al., 2012) and could be recycled and employed for several applications such as animal feeding.

In the present study, the effects of diets enriched with polyphenols extracted from OMWW were investigated in the narrow clawed *Astacus leptodactylus*. The effects were evaluated on growth performances, antioxidant and immunological parameters, intestinal microbiota and FA composition. Our results indicate that OMWW-enriched diets had beneficial effects on crayfish growth and antioxidant parameters. Moreover, OMWW-enriched diets enhance the immune status and decrease the intestinal microbiota, with the exception of yeasts, and hence they could play an important role in preventing disease outbreaks in aquaculture systems, posing the employment of polyphenols as a novel strategy of development to the feed industry sector. These results can be of benefits for the freshwater crayfish farming sector and for all those interested in developing a sustainable and advanced model of crayfish farming.

2. Materials and methods

2.1. Animals and husbandry

Adult crayfish *A. leptodactylus* of 26.85 ± 5.0 g. were imported by "LPA live seafood" (L.P.A. Pesca Srl via dell'Industria, 8-47843 Misano Adriatico–RN, Italy) from Lake Sevan (Armenia) and declared, according to the European Community Law, in good health and disease-free. All animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, or the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). Crayfish were held under natural photoperiod in 100 cm × 100 cm × 50 cm tanks (20 crayfish/tank) in a semi-recirculating system. Temperature and pH (pHmeter GLP 21 Crison), dissolved oxygen, total ammonia nitrogen, nitrite nitrogen, nitrate nitrogen, phosphorus, phosphate, total chlorine and firmness ($CaCO_3$) were measured twice a week with a Hanna Instruments Photometer C200. Temperatures ranged between 20 °C and 22 °C, pH between 7.9 and 8.4. Dissolved oxygen between 4 and 5 mg/l, total ammonia nitrogen between 0 and 0.35 mg/l, Nitrite nitrogen between 0.02 and 0.07 mg/l, Nitrate nitrogen between 7.5 and 11.3 mg/l, Total chlorine between 0.04 and 0.20 mg/l, Phosphates between 0.33 and 1.7 mg/l and Phosphorus between 0.2 and 2.2 mg/l.

2.2. Diet preparation

The basic diet was formulated according to Safari et al., 2014 with minor differences. The OMWW polyphenolic extract was obtained by membrane separation of OMWW according to Cassano et al. (2013). The main phenolic compounds identified by HPLC according to (Azaizeh et al., 2012), were: Hydroxytyrosol, Tyrosol, Caffeic acid, Verbascoside, Ferulic acid, Oleuropein. 0.5 and 5.0 g of OMWW polyphenolic extract were mixed with 1 kg of basic diet to generate OMWW-LC (Low Concentration) diet and OMWW-HC (High Concentration) diet, respectively. Pellet manufacturing was carried out according to Volpe et al. (2008, 2012). Composition and proximate analysis of the experimental diets are shown in Table 1.

2.3. Experimental design

Prior to beginning with the experiment, all experimental animals were weighed individually and randomly assigned to treatments. 60 animals divided into three groups of 20 animals/each were fed on the basic diet (control group); 60 animals divided into three groups of 20 animals/each were fed on pellets enriched with 0.5 g/kg of diet of OMWW extract (OMWW-LC group); 60 animals divided into three groups of 20 animals/each were fed on pellets enriched with 5 g/kg of diet of OMWW extract (OMWW-HC group). The amount of feed administered was 5% of the total body weight. Animals were weighted every two weeks and the amount of feed adjusted accordingly. We did not measure the amount of feed really ingested, but according to our experience this amount was enough to guarantee an ad libitum feeding, while keeping uneaten feed at minimum. Animals were fed every day between 8:00 and 9:00 am. The feeding experiment lasted for 24 weeks.

2.4. Evaluation of growth performance

Survival Rate (SR), Specific Growth Rate (SGR), Feed Conversion Ratio (FCR), Protein Efficiency Ratio (PER), Lipid Efficiency Ratio (LER), Carbohydrate Efficiency Ratio (CER) and Hepatosomatic Index (HIS) were calculated according to Safari et al. (2014).

Table 1
Ingredients and proximate composition of the experimental diets. Values are reported as mean ± SEM. Three samples for each diet were analyzed.

Ingredients (gr kg^{-1})	Diets		
	Control	OMWW-LC	OMWW-HC
Fish meal (anchovy)	45	45	45
Krill meal	45	45	45
Soybean meal	140	140	140
Corn gluten	240	240	240
Wheat flour	265	265	265
Corn starch	40	40	40
Fish oil	40	40	40
Canola oil	40	40	40
Soy lecithin	50	50	50
Cholesterol	5	5	5
Glucosamine	10	10	10
Choline	15	15	15
Vitamin C	10	10	10
Vitamin premix[a]	20	20	20
Mineral premix[b]	15	15	15
Carboxymethyl cellulose	20	19.5	15
OMWW extract	–	0.5	5
Nutrient level (%)			
Umidity	8.2 ± 0.3	7.4 ± 0.2	7.7 ± 0.2
Proteins	18.0 ± 0.3	18.5 ± 0.3	17.9 ± 0.2
Carbohydrates	61.4 ± 1.0	60.9 ± 0.7	61.6 ± 0.8
Lipids	6.1 ± 0.1	6.3 ± 0.1	6.1 ± 0.1
Ash	6.3 ± 0.2	6.9 ± 0.3	6.7 ± 0.2

0.1; and antioxidant (BHT), 100. Vitamin premix contains (mg kg^{-1}) E, 30; K, 3; thiamine, 2; riboflavin, 7; pyridoxine, 3; pantothenic acid, 18; niacin, 40; folacin, 1.5; choline, 600; biotin, 0.7 and cyanocobalamin, 0.02.

[a] Zoofast, Italy.

[b] Mineral premix contains (mg kg^{-1}) Mg, 100; Zn, 60; Fe, 40; Cu, 5; Co, 0.1; I,

2.5. Tissue sampling

Hemolymph was obtained from ventral sinus using the anticoagulant buffer as reported in Smith and Soderhall (1983). The Hemolymph was centrifuged at 1000 *g* for 5 min at 4 °C. The supernatant was employed for DPPH assay, the hemocytes were employed to determine the phenoloxidase activity and the superoxide anion activity.

2.6. Oxidative parameters

Animals used in the feeding trial were assayed for enzymatic activity. Animals were sacrificed 24 h after the last feeding.) Glutathione Peroxidase (GP), Glutathione Reductase (GR) and Catalase (CA) were determined in the hepatopancreas (10 crayfish per treatment) using commercial kits (BioVision Inc., Milpitas, CA 95035 USA). According to the manufacturer instruction GP (Glutathione Peroxidase Activity Colorimetric Assay Kit) was measured using a NADPH standard curve. The production of NADP was read at 340 nm. One unit of GP is defined as the amount of enzyme that causes the oxidation of 1 μmol of NADPH to NADP per minute at 25 °C. GR activity (EnzyChrom™ Glutathione Reductase Kit) was measured using 5,5-Dithiobis (2-nitrobenzoic acid) (DTNB) to generate the standard curve. The production of TNB was measured at 405 nm. One unit of GR is the amount of enzyme that generate 1 μmol of TNB per minute at 25 °C. CA activity (Catalase Activity Colorimetric Assay Kit)was measured using a standard curve of H_2O_2 read at 570 nm. One unit of CA is the amount of enzyme that decompose 1.0 μmol of H_2O_2 per min at pH 4.5 at 25 °C. The kit could detect 1μU or less of catalase activity in the sample. A microplate reader (Model 680 Biorad) was employed. Total proteins were evaluated with the Bradford method using bovine serum albumin (BSA) as standard.

2.7. Immunological parameters

Phenoloxidase activities were performed according to Cárdenas and Dankert (1997) with minor modifications. 10 crayfish per treatment were employed. Hemocytes were obtained by centrifugation at 1000*g* for 5 min at 4 °C in rinsing buffer (10 mM sodium cacodylate, 0,25 M sucrose and 20 mM CaCl2, pH 7.0) and counted with an automated cell counter and analyzer system (Casy, model TT, Roche innovates AG, Basilea, Switzerland). Hemocytes were homogenized in homogenizer buffer (10 mM sodium cacodylate and 20 mM CaCl2, pH 7.0) centrifuged at 16000 *g* for 20 min at 4 °C and the supernatant (hemocytes lysate supernatant (HLS), was used for measuring phenoloxidase activity. Superoxide production was determined as the reduction of nitroblue tetrazolium (NBT) according to Mariano et al. (2013) on the hemocytes obtained as above and suspended in ice-cold PBS.

2.8. Histology

The digestive tract of crayfish is composed of a cuticle lined foregut (esophagus and stomach), a cuticle-free midgut with dorsal and ventral caeca (hepatopancreas) and a cuticle-lined hindgut or intestine (Vogt, 1996). Only hepatopancreas and intestine were analyzed in this study. Organs were treated using classic histological methods as reported in Varricchio et al. (2012). Histological slides stained by haematoxylin-eosin were observed using a microscope Nikon Eclipse TI-S and photographed with the program NIS-Elements BR 4.30.00 LO (build 1017 64-bit) Laboratory Imaging. The following parameters were considered for the hepatopancreas : i) general integrity of the hepatopancreas; ii) area of the tubule/the area of the internal lumen; iii) presence of hemocytes infiltration. For the intestine: i) height and width of the intestinal folds; ii) distance between the nucleus and the cell membrane; ii) presence of hemocytes infiltration.

2.9. Lipid analysis

Hepatopancreas and muscle from the tail were analyzed for fatty acids (FA) composition. Lipids were extracted with the Soxhlet method according to the Methods of analysis used for chemical control of food of the National Institute of Health, 1996 (Reports ISTISAN 96/34).

The fatty acid profile was determined by gas chromatography, as fatty acid methyl esters (FAMEs) by gas chromatographic analysis, according to method AOAC 996.06 (AOAC, 1997), as reported in Volpe et al. (2015).

2.10. Intestinal microbiota analysis

Total Viable Count (TVC) of colonies on agar plates was carried out by conventional microbiological methods according to the European Regulation 1441/2007. Four samples were analyzed for each group (200–250 mg), in triplicate. The intestinal content was homogenized in 2–2.5 ml of 0.1% peptone solution (Oxoid, S.p.a., Rodano, Milano, Italy) using a paddle peristaltic homogenizer Stomacher 400 Circulator (Seward LTD, UK) and an aliquot of appropriate serial dilutions was transferred to agar media (Oxoid, S.p.a., Rodano, Milano, Italy), in duplicate. The following agar media were used: BHI agar for aerobic mesophilic bacteria, MacConkey agar for gram negative bacteria, Columbia CNA agar for gram positive bacteria, Schaedler agar for anaerobic bacteria and Sabouraud agar for molds and yeasts. Plates were incubated under the appropriate conditions for 1–5 days, thereafter the count of viable colonies was performed. Results were expressed as colony forming units per gram of intestinal sample (CFU/g).

2.11. Statistical analysis

We used the parametric test of one-way analysis of variance (ANOVA) following confirmation of normality and homogeneity of variance. The Duncan's multiple range test as post hoc analysis was employed to determine whether there were any statistically significant differences between the experimental groups. Data were expressed as mean ± SEM. Any significant difference was determined at the 0.05 level. The analyses were carried out with the Statistica version 7.0 statistical package (Statsoft Inc., Tulsa, OK, USA). Microbiota data were analyzed and graphically reported by using "GraphPad Prism 4" software, validating the statistical significance by the *p*-value ≤ 0.05. The number of samples analyzed (n) is reported in Figures and Tables.

3. Results

3.1. Growth performance and nutrient efficiency indices

Parameters of growth performance, feed utilization, survival rate and nutritional status are reported in Table 2. SGR significantly increased in both OMWW-LC and OMWW-HC groups compared to the control group. The highest SGR values were observed in the OMWW-HC diet. FCR values were significantly higher in the control group

Table 2
Growth performance of *Astacus leptodactylus* fed for 24 weeks on control diet, OMWW-LC containing diet and OMWW-HC containing diet.
Values are reported as mean ± SEM ($n = 30$).

Diets			
	Control	OMWW-LC	OMWW-HC
SGR %	0.21 ± 0.01^{c}	0.24 ± 0.01^{b}	0.32 ± 0.02^{a}
FCR %	12.7 ± 0.5^{a}	10.6 ± 0.3^{b}	8.5 ± 0.4^{c}
PER %	0.44 ± 0.02^{c}	0.51 ± 0.02^{b}	0.65 ± 0.03^{a}
LER %	1.29 ± 0.07^{c}	1.49 ± 0.05^{b}	1.92 ± 0.07^{a}
CER %	0.13 ± 0.01^{c}	0.15 ± 0.01^{b}	0.19 ± 0.02^{a}
HSI %	4.7 ± 0.05^{c}	5.9 ± 0.06^{b}	6.4 ± 0.05^{a}
SR %	67.7 ± 1.5^{c}	80.6 ± 1.3^{a}	81.5 ± 1.4^{a}

Values with different letters are significantly different ($P < 0.05$).

Table 3
Oxidative parameters. Total antioxidant activity (%) of the hemolymph and oxidative enzyme activities in the hepatopancreas (U/mg total protein). Values are reported as mean ± SEM ($n = 10$).

Diets			
	Control	OMWW-LC	OMWW-HC
Total antioxidant activity	83.01 ± 2.64[b]	92.50 ± 1.61[a]	93.05 ± 2.05[a]
Glutathione peroxidase (GP)	1.45 ± 0.71[a]	1.54 ± 0.83 [a]	1.7 ± 0.73[a]
Glutathione reductase (GR)	17.44 ± 3.03[b]	17.62 ± 3.02[b]	28.75 ± 3.05[a]
Catalase (CA)	0.96 ± 0.18[b]	1.80 ± 0.17[a]	1.86 ± 0.19[a]

Values with different letters are significantly different ($P < 0.05$).

compared to both OMWW-LC and OMWW-HC groups. The lowest FCR value was observed in the OMWW-HC group. PER, LER and CER values were significantly higher in both OMWW-LC and OMWW-HC groups, with the highest values observed in the OMWW-HC group. HSI was significantly higher in both OMWW-LC and OMWW-HC groups, with the highest values observed in the OMWW-HC group. SR was statistically increased in both OMWW-LC and OMWW-HC groups compared to the control group.

3.2. Oxidative parameters

In Table 3 are reported the activity (U/mg total protein) of the oxidative enzymes GP, GR and CA in the hepatopancreas. GP activity did not statistically changed. The increase in GR activity was statistically significant only in the OMWW-HC group. The increase in CA activity was statistically significant in both OMWW-LC and OMWW-HC groups with respect to the control group.

3.3. Immunological parameters

Hemocyte total count is reported in Table 4. The total number of hemocytes significantly increased in OMWW-LC and OMWW-HC groups with respect to the control group. No statistically differences were detected between OMWW-LC and OMWW-HC groups although the highest number was reached in the OMWW-HC group. Phenoloxidase activity in the hemocytes statistically increased in both OMWW-LC and OMWW-HC groups with respect to the control group. No statistically differences were detected between OMWW-LC and OMWW-HC groups although the highest value was reached in the OMWW-LC group (Table 4). Superoxide anion production in the hemocytes, after zymosan probing, statistically decreased only in the OMWW-HC group (Table 4).

3.4. Morphological parameters

Histological methods remain the primary tools for the evaluation of pathological changes in tissues in toxicological studies. In order to verify the harmlessness of the OMWW presence in the diets, we analyzed the histology of both hepatopancreas and hindgut of *Astacus leptodactylus*. In Fig. 1 the histological sections of hepatopancreas from the control group and crayfish fed on OMWW-enriched diets are reported. The transversal sections of hepatopancreas tubules show that the tissue integrity is well preserved in crayfish fed OMWW-enriched diet. Both in the control and experimental groups, areas of well-organized glandular tubular shape alternate with areas of tubules loosely bound together with wider intertubular spaces and bulging vacuoles inside the tubule cells. The area of the tubules, expressed as the area of the internal lumen/the total area of the tubule (Table 5), did not show any significant variation among groups. Tubule cells containing small vacuoles were more abundant in the crayfish fed on OMWW-enriched diets (Fig. 1).

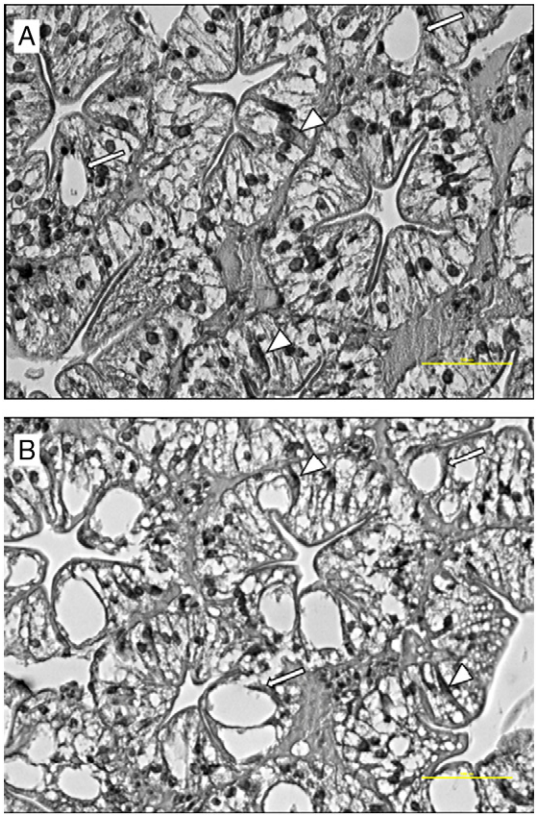

Fig. 1. Haematoxylin-eosin staining of hepatopancreas of *Astacus leptodactylus* control (A) and fed on OMWW-HC diet (B). Arrow head shows F-cells. Arrows point at the big vacuole contained in the B-cells. The remaining cells are R-cells with small vacuoles well evident in (B). Scale bars = 100 μm; hepatopancreas of OMWW-LC fed crayfish gave results similar to OMWW-HC fed crayfish (B).

The adluminal epithelium in the intestine of *A. leptodactylus* is a simple columnar epithelium rising in folds and laying on dense irregular connective tissue. A cuticle appears on the apical surface of the adluminal epithelium. Circular and longitudinal muscle fibers are visible within the connective tissue (data not shown). Neither the intestine folds morphology (height and width), nor the thickness of the adluminal epithelium (measured as nucleus-cuticle distance) showed any statistically significant variation in the crayfish groups (Table 5). No hemocyte infiltration was evident neither in the hepatopancreas, nor in the intestine.

Table 4
Immunological parameters. Total haemocytes, phenoloxidase activity and superoxide anion production in haemocytes. Values are reported as mean ± SEM ($n = 10$).

Diets			
	Control	OMWW-LC	OMWW-HC
Haemocytes (millions/ml of hemolymph)	3.57 ± 0.13[b]	4.22 ± 0.22[a]	4.62 ± 0.21[a]
Phenoloxidase activity (U/mg total protein)	0.113 ± 0.003[b]	0.132 ± 0.002[a]	0.128 ± 0.003[a]
Superoxide Anion (OD)	2.64 ± 0.11[a]	2.55 ± 0.09[a]	2.17 ± 0.08[b]

Values with different letters are significantly different ($P < 0.05$).

3.5. Fatty acid composition of hepatopancreas and tail muscle

In Table 6 is reported the FA composition of the tail muscle. SFA accounted for 33.27%, 33.55% and 32,46% in the control group, OMWW-LC group and OMWW-HC group respectively. MUFA accounted for 22.47% 23.45% and 23.34% in the control group, OMWW-LC group and OMWW-HC group respectively. PUFA accounted for 35.43%, 35.46% and 35.23% in the control group, OMWW-LC group and OMWW-HC group respectively. There were no statistically

Table 5
Histological parameters of hepatopancreas and intestine. Values are expressed as mean ± SEM. Values are not significantly different. Four animals for each group were analyzed. Three cross-sections/sample and 12 cross-sections/treatment were analyzed.

	Diets		
	Control	OMWW-LC	OMWW-HC
Hepatopancreas			
Lumen area/total tubule area	0.116 ± 0.054	0.094 ± 0.043	0.103 ± 0.057
Intestine			
Adluminal epithelium (nucleus-cuticle distance (μm)	23.81 ± 8.25	18.84 ± 6.58	21.31 ± 7.48
Intestinal folds height (μm)	134.24 ± 56.52	105.06 ± 46.76	137.39 ± 55.97
Intestinal folds width (μm)	271.42 ± 111.15	219.27 ± 77.32	252.89 ± 119.01

significant differences in SFA, MUFA and PUFA among groups, although some FA shown differences among groups, such as Myristic, Arachidic acid and Tricosanoic acid (only in OMWW-HC) that were statistically lower in crayfish fed on OMWW-enriched diets. Pentadecanoic and Lignoceric were instead higher in OMWW-HC diet group.

In Table 7 is reported the FA composition of the hepatopancreas. SFA accounted for 24.46%, 23.27% and 23.04% in the control group, OMWW-LC group and OMWW-HC group respectively. MUFA accounted for 32.36% 32.19% and 31.01% in the control group, OMWW-LC group and OMWW-HC group respectively. PUFA accounted for 31.94%, 33.54% and 35.26% in the control group, OMWW-LC group and OMWW-HC group respectively. There were no statistically significant differences in SFA, MUFA and PUFA among groups, with the exception of MUFA which was significantly lower in OMWW-HC and PUFA which was significantly higher in OMWW-HC with respect to OMWW-LC and control groups. Consequently, in the OMWW-HC group the PUFA/MUFA ratio was significantly higher than in the other groups. Among SFA, Myristic acid was statistically higher in both OMWW-LC and OMWW-HC groups with respect to the control group. Among MUFA, Elaidic acid was statistically lower in both OMWW-LC and OMWW-HC groups with respect to the control group. Among PUFA, cis-11,14-Eicosadienoic C20:2 and cis-11,14,17-Eicosatrienoic C20:3 n3 acids were statistically lower, while Linolenic was higher in both OMWW-LC and OMWW-HC groups with respect to the control group.

3.6. Intestinal microbiota

TVC of colonies in the intestine of *A. leptodactylus* was carried out by conventional microbiological methods. The count of the intestinal microbiota, reported as colony forming units per gram of intestinal sample (CFU/g) is shown in Fig. 2. The CFU/g of total bacteria significantly decreased in crayfish fed on OMWW-LC, with respect to the control and OMWW-HC groups $P < 0.05$). In particular, the CFU/g of

Table 6
FA composition of tail muscle. Values are reported as mean ± SEM. Five samples for each group were analyzed. Values with different letters are significantly different ($P < 0.05$).

Fatty acid	Diets		
	Control	OMWW-LC	OMWW-HC
Butyric C4:0	–	–	–
Caproic C6:0	–	–	–
Caprylic C8:0	–	–	–
Capric C10:0	–	–	–
Undecanoic C11:0	–	–	–
Lauric C12:0	–	–	–
Tridecanoic C13:0	–	–	–
Myristic C14:0	0.50 ± 0.01^{a}	0.05 ± 0.01^{c}	0.32 ± 0.11^{b}
Myristoleic C14:1	–	–	–
Pentadecanoic C15:0	0.05 ± 0.01^{b}	0.05 ± 0.01^{b}	0.44 ± 0.15^{a}
cis-10-Pentadecenoic C15:1	–	–	–
Palmitic C16:0	15.87 ± 0.31^{a}	16.54 ± 1.64^{a}	15.71 ± 1.43^{a}
Palmitoleic C16:1	2.58 ± 0.32^{a}	2.61 ± 0.31^{a}	2.73 ± 0.94^{a}
Heptadecanoic C17:0	0.89 ± 0.10^{a}	0.90 ± 0.06^{a}	0.79 ± 0.30^{a}
cis-10-Heptadecenoic C17:1	0.45 ± 0.05^{a}	0.44 ± 0.19^{a}	0.28 ± 0.04^{a}
Stearic C18:0	7.64 ± 0.26^{a}	7.25 ± 0.27^{a}	7.22 ± 0.59^{a}
Elaidic C18:1 n9t	0.85 ± 0.31^{a}	0.75 ± 0.29^{a}	0.73 ± 0.09^{a}
Oleic C18:1 n9c	17.98 ± 0.57^{a}	18.61 ± 2.19^{a}	18.53 ± 1.0^{a}
Linolelaidic C18:2 n6t	–	–	–
Linoleic C18:2 n6c	8.51 ± 0.45^{a}	7.96 ± 1.16^{a}	8.51 ± 0.43^{a}
Arachidic C20:0	1.36 ± 0.23^{a}	0.82 ± 0.29^{b}	1.16 ± 0.12^{ab}
γ-Linolenic C18:3 n6	–	–	–
cis-11-Eicosenoic C20:1	1.04 ± 0.39^{a}	1.18 ± 0.41^{a}	1.15 ± 0.16^{a}
Linolenic C18:3 n3	0.59 ± 0.07^{a}	0.73 ± 0.12^{a}	1.07 ± 0.17^{a}
Heneicosanoic C21:0	–	–	–
cis-11.14-Eicosadienoic C20:2	1.60 ± 0.06^{a}	1.67 ± 0.17^{a}	1.47 ± 0.14^{a}
Behenic C22:0	0.49 ± 0.01^{a}	0.39 ± 0.13^{a}	0.59 ± 0.13^{a}
cis-8,11,14-Eicosatrienoic C20:3 n6	–	–	–
Erucic C22:1 n9	–	–	–
cis-11,14,17-Eicosatrienoic C20:3 n3	–	–	–
Arachidonic C20:4 n6	–	–	–
Tricosanoic C23:0	7.17 ± 0.40^{a}	7.92 ± 0.27^{a}	6.19 ± 0.46^{b}
cis-13,16-Docosadienoic C22:2	–	–	–
Lignoceric C24:0	0.05 ± 0.01^{b}	0.05 ± 0.01^{b}	0.82 ± 0.33^{a}
cis-5,8,11,14,17-Eicosapentanoic C20:5 n3	21.51 ± 2.40^{a}	21.54 ± 2.89^{a}	20.60 ± 2.30^{a}
Nervonic C24:1	–	–	–
cis-4,7,10,13,16,19-Docosahexaenoic C22:6 n3	3.42 ± 0.48^{a}	3.91 ± 0.36^{a}	3.58 ± 0.94^{a}
Σ-SFA	33.43 ± 0.46^{a}	33.55 ± 1.33^{a}	32.46 ± 2.14^{a}
Σ-MUFA	22.58 ± 1.12^{b}	23.45 ± 2.49^{b}	23.34 ± 1.88^{b}
Σ-PUFA	35.48 ± 2.35^{a}	35.56 ± 2.10^{a}	35.23 ± 3.31^{a}
Σ-PUFA/Σ-SFA	1.07 ± 0.09^{a}	1.06 ± 0.10^{a}	1.09 ± 0.15^{a}
Σ-PUFA/Σ-MUFA	1.58 ± 0.18^{a}	1.53 ± 0.25^{a}	1.52 ± 0.21^{a}

Table 7
FA composition of hepatopancreas. Values are reported as mean ± SEM. Five samples for each group were analyzed. Values with different letters are significantly different ($P < 0.05$).

Fatty acids	Diets		
	Control	OMWW-LC	OMWW-HC
Butyric C4:0	–	–	–
Caproic C6:0	–	–	–
Caprylic C8:0	–	–	–
Capric C10:0	–	–	–
Undecanoic C11:0	–	–	–
Lauric C12:0	–	–	–
Tridecanoic C13:0	–	–	–
Myristic C14:0	0.74 ± 0.17^{b}	1.78 ± 0.35^{a}	1.91 ± 0.24^{a}
Myristoleic C14:1	–	–	–
Pentadecanoic C15:0	0.47 ± 0.12^{a}	0.38 ± 0.06^{a}	0.44 ± 0.09^{a}
cis-10-Pentadecenoic C15:1	–	–	–
Palmitic C16:0	16.04 ± 2.03^{a}	15.48 ± 1.70^{a}	15.72 ± 1.23^{a}
Palmitoleic C16:1	6.61 ± 1.66^{a}	9.12 ± 1.32^{a}	7.64 ± 1.86^{a}
Heptadecanoic C17:0	0.40 ± 0.25^{a}	0.31 ± 0.11^{a}	0.26 ± 0.04^{a}
cis-10-Heptadecenoic C17:1	0.58 ± 0.43^{a}	0.25 ± 0.12^{a}	0.29 ± 0.07^{a}
Stearic C18:0	2.75 ± 0.38^{a}	2.54 ± 0.51^{a}	2.40 ± 0.35^{a}
Elaidic C18:1 n9t	0.36 ± 0.09^{a}	0.15 ± 0.04^{b}	0.20 ± 0.05^{b}
Oleic C18:1 n9c	23.81 ± 0.43^{a}	21.10 ± 1.46^{a}	21.58 ± 1.96^{a}
Linolelaidic C18:2 n6t	0.05 ± 0.01^{a}	0.58 ± 0.15^{a}	0.32 ± 0.13^{a}
Linoleic C18:2 n6c	18.94 ± 3.77^{a}	20.02 ± 2.56^{a}	23.08 ± 3.04^{a}
Arachidic C20:0	0.28 ± 0.07^{a}	0.27 ± 0.05^{a}	0.16 ± 0.04^{a}
γ-Linolenic C18:3 n6	0.05 ± 0.01^{b}	0.36 ± 0.15^{a}	0.26 ± 0.20^{ab}
cis-11-Eicosenoic C20:1	0.97 ± 0.15^{a}	1.68 ± 0.55^{a}	1.46 ± 0.26^{a}
Linolenic C18:3 n3	2.64 ± 0.76^{a}	3.19 ± 0.85^{a}	3.23 ± 0.82^{a}
Heneicosanoic C21:0	–	–	–
cis-11,14-Eicosadienoic C20:2	1.69 ± 0.41^{a}	1.04 ± 0.19^{b}	0.97 ± 0.16^{b}
Behenic C22:0	0.15 ± 0.05^{a}	0.11 ± 0.01^{a}	0.05 ± 0.01^{a}
cis-8,11,14-Eicosatrienoic C20:3 n6	0.27 ± 0.09^{a}	0.12 ± 0.03^{a}	0.24 ± 0.06^{a}
Erucic C22:1 n9	0.05 ± 0.017^{a}	0.06 ± 0.02^{a}	0.05 ± 0.01^{a}
cis-11,14,17-Eicosatrienoic C20:3 n3	0.32 ± 0.05^{a}	0.05 ± 0.01^{b}	0.07 ± 0.02^{b}
Arachidonic C20:4 n6	–	–	–
Tricosanoic C23:0	3.73 ± 0.48^{a}	2.54 ± 1.21^{a}	2.19 ± 0.39^{a}
cis-13,16-Docosadienoic C22:2	0.17 ± 0.06^{a}	0.18 ± 0.02^{a}	0.25 ± 0.06^{a}
Lignoceric C24:0	–	–	–
cis-5,8,11,14,17-Eicosapentanoic C20:5 n3	6.16 ± 0.79^{a}	6.52 ± 1.32^{a}	5.44 ± 1.25^{a}
Nervonic C24:1	–	–	–
cis-4,7,10,13,16,19-Docosahexaenoic C22:6 n3	1.99 ± 0.48^{a}	2.16 ± 0.42^{a}	2.05 ± 0.42^{a}
Σ-SFA	24.46 ± 2.48^{c}	23.27 ± 1.52^{c}	23.04 ± 1.01^{c}
Σ-MUFA	32.36 ± 2.18^{ab}	32.20 ± 0.74^{ab}	31.01 ± 0.49^{b}
Σ-PUFA	31.94 ± 2.91^{ab}	33.54 ± 2.15^{ab}	35.26 ± 1.31^{a}
Σ-PUFA/Σ-SFA	1.32 ± 0.25^{a}	1.45 ± 0.17^{a}	1.53 ± 0.07^{a}
Σ-PUFA/Σ-MUFA	0.99 ± 0.15^{b}	1.04 ± 0.09^{b}	1.14 ± 0.04^{a}

aerobic mesophilic bacteria was reduced of 2.8 folds and increased of 1.4 folds ($P < 0.05$) in the crayfish fed on OMWW-LC and OMWW-HC diets respectively, when compared to the control. The CFU/g of gram negative bacteria decreased of 2.5 folds ($P < 0.05$) in OMWW-LC group and increased of 2.3 folds ($P < 0.05$) in OMWW-HC group with respect to the control group. The CFU/g of gram positive bacteria decreased of 3 and 1.5 folds ($P < 0.05$) in OMWW-LC and OMWW-HC groups respectively, when compared to the control group. The CFU/g of anaerobic bacteria increased of 1.2 and 4.1 folds ($P < 0.05$) in OMWW-LC and OMWW-HC groups respectively, when compared to the control group. The CFU/g of yeasts increased of 1.5 folds ($P < 0.05$) and decreased of 1.2 folds ($P < 0.05$) in OMWW-LC and OMWW-HC groups respectively, when compared to the control group.

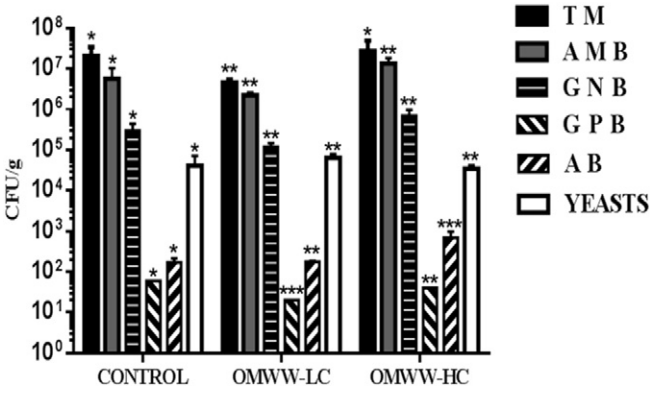

Fig. 2. Microbiological analyses of *Astacus leptodactylus* gut microbiota, subjected to different feeding regimes. Control = crayfish receiving the control diet; OMWW-LC = crayfish receiving the OMWW-LC diet; OMWW-HC = crayfish receiving the OMWW-HC diet. Total microorganisms (TM); aerobic mesophilic bacteria (AMB), gram negative bacteria (GNB), gram positive bacteria (GPB), anaerobic bacteria (AB), molds and yeasts. The values are mean ± SEM of 3 experiments. Different number of asterisks (*,**,***) for each microbial classification (e.g. TM, AMB, GNB, GPB, AB or Yeasts) indicates significant differences between different dietary groups (Control, OMWW-LC or OMWW-HC) ($p < 0.05$).

4. Discussion

In this study, the effects of polyphenols extracted from OMWW on *Astacus leptodactylus* are reported. Growth and nutritional indices were found to be significantly improved in crayfish fed on OMWW-enriched diets. We presume that OMWW-enriched diets improved digestibility of nutrients leading to higher feed efficiency and faster body weight gain. Although polyphenols have been used in aquaculture

for their growth promoting effects (Citarasu, 2010; Ganguly et al., 2010; Radhakrishnan et al., 2013), in the rainbow trout *Oncorhynchus mykiss* and in the seabream *Sparus aurata*, specific growth rate, feed conversion ratio and protein efficiency ratio were not improved by the OMWW inclusion in the diet (Sicuro et al., 2009, 2010). We can hypothesize that the use, by these authors, of crude olive water likely containing substances with antinutritional effects interfered with the growth (Gatlin et al., 2007). In this study, a rather pure OMWW extract was used whose polyphenol content reveals, according to the literature data (Azaizeh et al., 2012; Kaleh and Geißen, 2016), the predominance of hydroxytyrosol.

Hydroxytyrosol is a potent inducer of stress enzymes (Hamden et al., 2010; Cicerale et al., 2010). In this study, the activity of GR and CA statistically increased in the hepatopancreas of OMWW-enriched diet fed crayfish, although GR activity significantly increased only at high OMWW concentration. Polyphenols are believed to positively affect the oxidative status (Zhu et al., 2010; Zrelli et al., 2011; Ilavarasi et al., 2011). The enhancement in the activity of both GR and CA in the hepatopancreas of crayfish fed on OMWW-enriched diets suggests that the polyphenols added to the diet may protect the cells/tissues against the cytotoxic/genotoxic effects of peroxides and˙OH or that an increase in free radicals has taken place in crayfish fed on OMWW-enriched diets. Although speculative, the free radical production might take place as a consequence of the increasing metabolism as suggested by the presence of large glycogen and lipid storing vacuoles in the R-cells of the hepatopancreas of crayfish and by the improved growth performances that occurred in crayfish fed on OMWW-enriched diets. Although very little is known about the role played by polyphenols on lipid metabolism, the emerging picture indicates that polyphenols may act on both lipid synthesis and degradation (Priore et al., 2015). The OMWW-HC diet brought about a significant increase in PUFA and decrease in MUFA in the hepatopancreas. In particular, the differences were ascribable to the decrease of elaidic acid (MUFA) and the increase in linolenic acid (PUFA). Similar results were obtained in broiler chicken were a simultaneous decrease in MUFA and increase in PUFA, were determined by the inclusion of polyphenols in the diet and more interestingly, in the broiler chicken the concentration of α-linolenic acid in the breast meat increased in animal fed on diets integrated with polyphenols (Starčević et al., 2014). Linoleic (C18:2 n-6), linolenic (C18:3 n-3), eicosapentaenoic (EPA; C20:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3) are particularly important dietary ingredients for crustaceans (Wickins and Lee, 2002), thus the increase in linoleic acid induced by the OMWW-enriched diet may have interesting implications for aquaculture feeding industry. Indeed, according to our data, the inclusion of OMWW in the diet brought about an improvement in the PUFA/MUFA ratio in the hepatopancreas, without altering the FA profile in the tail muscle.

The hepatopancreas of crustaceans is the site of digestion, nutrient absorption, reserve storage and synthesis and secretion of digestive enzymes (Ceccaldi, 1989), thus can be considered a good indicator of the response to active substances in feeds (Schrenk, 2009). The hepatopancreas represents 2–6% of the total body weight and a change in its relative size is considered an indicator for chronic stress situations and it is used to evaluate differences in crayfish condition and nutritional status (Jussila and Mannonen, 1997). In the group fed on OMWW- enriched diet, HSI was about 25% higher in crayfish fed on OMWW-LC diet and 36% higher in in crayfish fed on OMWW-HC diet compared to control crayfish, suggesting better digestion and adsorption of food.

According to the histological analysis carried out in this study, the general structure of the hepatopancreas was not modified by the OMWW-enriched diets. In order to obtain a more objective evaluation, the ratio between the total area of the tubule wall on the area of the internal lumen, was measured and used as an indicator of tubule size. The ratio did not significantly change in the experimental groups with respect to the control, confirming the histological evidence. Moreover, no histopathological evidence have been detected in the hepatopancreas and hindgut, as indicated by the limited hemocytes infiltration in the OMWW-enriched diet fed crayfish. The histological analysis of the *Astacus leptodactylus* hepatopancreas and hindgut is in line with the digestive tract studies reported in literature for freshwater crayfish (Ceccaldi, 1989; Vogt, 1996; To et al., 2004; McGaw and Curtis, 2013). Both height and width of the internal ridges did not undergo any modification indicating that OMWW polyphenols did not affect the histomorphology of the hindgut, at least at the level of inclusion employed in this study.

This study suggests that OMWW-extracted polyphenols present in the diets, affect the microbial community of the *Astacus leptodactylus* hindgut. Particularly, the OMWW-enriched diet stimulate significantly the growth of anaerobic bacteria at the expense of other microbial groups. A possible explanation for the stimulatory effect of polyphenolic compounds on bacterial growth is that some microorganisms are able to use these compounds as nutritional substrates. In the particular case of lactobacilli, these bacteria possess the ability to metabolize phenolic compounds supplying energy to cells and positively affecting the bacterial metabolism (Cardona et al., 2013). It has also been suggested that prebiotics have beneficial effects in aquatic species such as in prawn *Penaeus semisulcatus* (Genc et al., 2007) supplemented with MOS. The effect can be additive and/or synergistic when prebiotics are provided with probiotics as on the European lobster (*Homarus gammarus L.*) (Daniels et al., 2010).

In this study, the effects on gut microbiota were assessed using culture-dependent method, due to the limited database of other more sophisticated methods such as MALDI-TOF (Popovic et al., 2014). In vitro research has shown that olive oil phenolic compounds have antimicrobial properties. Particularly, the phenolic compounds, oleuropein, hydroxytyrosol and tyrosol have demonstrated potent antimicrobial activity against several strains of bacteria responsible for intestinal and respiratory infections and hydroxytyrosol and oleuropein have also been shown to be cytotoxic to a large number of bacterial strains (Cicerale et al., 2010). The exact mechanism by which polyphenols exhibit antimicrobial activity is not clear. They could have bacteriostatic or bactericidal actions or act to inhibit adhesion of infection-causing bacteria within cells of the intestinal tract. While the dietary probiotic and prebiotic supplements have been reported widely to improve the growth and survival of many aquaculture species including lobsters (Rojas-Garcia et al., 2008; Salze et al., 2008; Daniels et al., 2010) also through the modulation of intestinal bacterial population. However, to date, the effects of dietary polyphenols supplements on intestinal microbial community of decapod crustaceans are poorly investigated.

The beneficial effect of OMWW polyphenols was also evidenced by the increase of both phenoloxidase activity and total hemocytes in animals fed on OMWW-enriched diets. Crustacean hemocytes play a key role in the immune response such as recognition, phagocytosis and melanization by releasing numerous molecules including antimicrobial peptides and prophenoloxidase after pathogen stimulation (Lee and Söderhäll, 2002). Phenoloxidase activity is an important component for increased resistance against an infection. Cellular haemolymph fraction is of specific interest because it appears to be strongly associated with defense against the pathogen and represents an indicator of crustacean health status (Sritunyalucksana et al., 2005). In fish and shellfish bioactive compounds from plants have been reported to enhanced innate immune parameter such as respiratory burst activity, total haemocytes and phenoloxidase (Harikrishnan et al., 2011).

4.1. Conclusions

In conclusion, the emerging picture, although not exhaustive of all possible effects of polyphenols, indicate that OMWW-enriched diets had beneficial effects on crayfish health and hence could play an important role in preventing disease outbreaks in aquaculture systems, posing the employment of polyphenols as a novel strategy of development to

the feed industry sector. Polyphenols extracted from renewable sources are cost-sensitive, thus such results can be of benefits for the freshwater crayfish farming sector and for all those interested in developing a sustainable, economical and advanced model of crayfish farming.

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