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Olive oil by-product as a natural antioxidant in gilthead sea bream (*Sparus aurata*) nutrition

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Abstract

The aim of this work was to evaluate the efficiency of a natural antioxidant substance in gilthead sea bream (*Sparus aurata*) feeds. An olive oil by-product, olive mill vegetation

water (VW), contains polyphenols, which have a strong antioxidant activity. A 147 days-long growth trial was conducted (monofactorial balanced; 4x3) with the diet as the experimental factor. Two diets (isonitrogenous CP= 40% and isoenergetic GE = 18Mj*kg^{-1} on DM) were formulated with 1% and 5% of VW inclusion level (VW1, VW5), against a control diet without VW. 600 juvenile gilthead sea breams (mean body weight 114.1±5.7g) were utilised. At the end of the growth trial the productive parameters and somatic indexes were calculated. Antioxidant activity in fish fillets was investigated using TBARS and DPPH assays. Some hematological parameters and digestive enzyme patterns were measured in fish in the middle and at the end of the experiment. The TBARS values showed slight delays in the development of oxidation in the fillet of fish fed with VW. No statistical differences appeared between the fish fed with the experimental diet and the control group, except for maltase activity which increased as the VW inclusion level was increased. The use of VW in a gilthead sea bream diet did not show any detrimental effects in gilthead sea bream production and physiological parameters and slightly improved the conservation of the fish fillets.

Keywords: natural antioxidants; fish nutrition; gilthead sea bream; TBARS; DPPH; olive oil phenols

Introduction

In aquaculture different antioxidants are used and they are classified on the base of their action mechanism. About 15 artificial antioxidants are actually used, among these, ethoxyquin (EQ), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and citric acid are the most utilized. In particular BHA is employed in fats and oils and EQ in fish meal, in order to preserve these from spontaneous oxidation. The function of EQ is

based on its ability to scavenge free radicals formed during lipid oxidation avoiding the rancidity of feeds that causes weight reduction, oxidative stress and vitamin E and C shortage. The upper limit both for BHA and EQ in fish feed, established in the European Union (EU), is 150 mg/kg feed, alone or in combination with butylated hydroxytoluene (BHT) [(Council Directive 70/524/EEC, replaced by Regulation (EC) No 1831/2003)]. The use of synthetic antioxidants in fish feed and subsequent carry-over to farmed fish fillets has received increasing attention from a food safety perspective in recent years (Berdikova Bohne et al. 2008, Petri et al. 2008), although a Maximum Residue Limits (MRLs) for synthetic antioxidants in food products from farm animals do not currently exist in the EU. Therefore, to avoid these undesirable side effects several natural compounds are investigated to find valid alternatives as partial substitute of synthetic antioxidants molecules. Until now there have been few researches on natural antioxidant use in gilthead sea bream feeding (Dimitrios 2006). The principal antioxidant compounds in the plant extracts are polyphenols and many by-products and wastes generated by agriculture contain phenols with potential application as natural antioxidants (Balasundram et al. 2006; Gokturk Baydar et al. 2007; Moure et al. 2001). Olive mill vegetation water (VW) is obtained by mean physical pressure during olive oil extraction and it is about the 50% of the initial weight of the olives. VW composition change mainly according olive cultivar and oil extraction techniques (Visioli et al. 1998a). Olives, olive oil and VW contain polyphenols that have been shown to have a strong antioxidant effect inhibiting human LDL oxidation and scavenging superoxide anions and hypochlorous acid (Visioli et al. 1998a, 1998b, 1999), free radical scavenging and antimicrobial, hypoglicemic and hypolipidemic actions (Perona et al. 2006; Yang et al. 2007). Furthermore, the positive effect of olive phenols on cardiovascular diseases has recently been demonstrated (Gonzalez-Santiago et al. 2006; Perona et al. 2006). VW is the main by-product from olive

oil extraction (Mulinacci et al. 2001). VW management and utilisation is actually a cost for olive farmers and its introduction into the aquafeed industry could transform this actual cost into a future benefit.

The effect of VW addition to fishmeal based diets was tested by evaluating productive parameters in a gilthead sea bream growth trial**;** furthermore, several fish physiology parameters were investigated and antioxidant activity analyses of the fish fillet were carried out.

Material and methods

Olive mill vegetation water analysis

The VW utilized in this study was purchased from a local oil mill (Lecce, SE Italy) and obtained by mechanical extraction from olive oil. The VW was chemically characterized and analysed for total organic compounds, total suspended solids (TSS), natural organic material (NOM) and pH. The total sugar content was determined using the spectrophotometric method at 550 nm (Bailey et al. 1992). The protein content was evaluated by the Kjeldahl Method. The total lipid content was measured using the gravimetric method (Delsal 1944). The Total polyphenols (TP) were measured using the Folin–Ciocalteau phenol reagent, as described by Box (1981). The TP values were expressed as phenol (C_6H_5OH) in g/L. The ortho-diphenol content was determined using a colorimetric method (Gutfinger 1981). The TSS measurements were performed according to a slightly modified technique (method 2540D) presented in APHA (1990). The NOM was analysed as the chemical oxygen demand with the standardised permanganate Kubel method (COD) (Hofmann 1965).

Diets

Two different diets were formulated with increasing levels of VW (VW1 and VW5, 1% and 5% v/w inclusion, respectively). These diets were tested against a control diet without VW. Fish feeds were prepared as dry pellets with a diameter of 3.5-mm by a private feed manufacturer. The diets were analyzed by proximate composition, according to standard methods (AOAC 1990), and the results showed that all the diets were isonitrogeonous (CP 40%) and isoenergetic (18 $MJ \cdot kg^{-1}$ DM) (Table 1).

Experimental plan and growth trial

This experimentation was carried out in the experimental centre of the faculty of Science at Lecce University. 600 juvenile sea bream (*Sparus aurata*) with an initial mean body weight of 114.1 ± 5.7g, were stocked randomly in twelve net tanks (1.00 m x 1.00 m x 1.50m), containing 50 fish each. Before to start the growth trial a palatability test, lasted 15 days, was performed on fish in order to investigate the VW feed acceptability on sea bream. This test was made because VW polyphenols could exert a detrimental effect on fish growth that could render the positive effects on the final product useless. The experimentation was started on June, 13th 2006 and finished on November 7th 2006. The experimental design was monofactorial, balanced with randomized blocks, 4 replicates per treatment (4x3), and the experimental factor was the diet. All the fish were weighted in bulk every 15 days in order to check the fish biomass gain and regulate the feeding ratio. The feeding ratio was 1.7% of the wet biomass. The feed was supplied 6 days per week, twice a day. The water temperature was 23.5 ± 4.2 °C, dissolved oxygen 6.3±1.0 ppm and salinity 32.9±2.2 ppt. These parameters were measured 3 times per week, resulting in the physiological range for this fish species.

Sampling and chemical analysis

At the end of the feeding trial, the fish were starved for 1 day, then the fish tanks were weighed for the final mean body weight. In order to determine the somatic indexes, 5 fish per tank, with a body weight close to the mean body weight, were sampled and killed by anaesthesia overdose. The gut and liver were separated from the rest of the body and weighed. The dorsal muscle tissues from the same fish body were sampled and frozen until the successive chemical determinations.

Growth performance and somatic indexes

At the end of the trial, the following mean individual growth performance indexes were calculated per treatment:

Biomass growth (BG), Specific growth rate (SGR), Feed Conversion Rate (FCR), Protein Efficiency ratio (PER) and Net Protein Utilization (NPU) (Table 2).

At the end of the trial, five fish were slaughtered from each experimental tank (20 fish per each diet), and viscero-somatic and hepato-somatic indexes (VSI and HSI) were calculated (Table 2).

Antioxidant analysis on the fish fillets

In order to determine the effects of the experimental diets on lipid oxidation, representative cutlets taken from the anterior portion of the dorsal muscle of each fillet were placed on ceramic dishes, wrapped in an oxygen permeable PVC and kept at 4 °C in a refrigerated chamber. Lipid oxidation was determined at 0, 24, 48, 72, 96, and 192 hours of storage using the 2-thiobarbituric acid method of Huang and Miller (1993) with slight modifications. The Thiobarbituric acid reactive substances (TBARS) were expressed as nmol malondialdehyde (MDA)$\cdot g^{-1}$ wet tissue.

DPPH radical scavenging activity

The free radical scavenging capacity was evaluated for the fish fillet methanol extracts according to the reported procedure (Brand-Williams et al. 1995) using the stable 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\cdot}$). The absorbance at 515 nm was measured against a blank of pure methanol at 10 min of the reaction and used to estimate the radical scavenging capacity of each antioxidant sample according to a standard curve. The kinetics of the $DPPH^{\cdot}$-antioxidant reaction was examined for each sample by measuring the absorbance at 515 nm against the methanol blank at 0, 5, 10, and 30 min of the reaction. The values were expressed as µmol TE (Trolox, vitamin E analogue, equivalent,) g^{-1} tissue.

Blood plasma parameters

Blood samples were collected from the caudal vein and inserted into heparinized tubes. The filled tubes were centrifuged at 1600 × g for 10 min to obtain plasma. The fresh plasma was frozen at -20 °C for later analyses. The osmolarity was measured using an automatic osmometer (5520 VAPRO, Delcon) and the protein concentration by the method of Lowry et al.(1985). Cortisol determination was performed with a commercially available immunoassays: EIA cortisol kit (Cayman Chemical, Ann Arbor, MI, USA). Considering the potential negative effect of phenols in this research, several physiological parameters (osmolarity, cortisol, alkaline phosphatase and Na/K ATPase) were investigated in the middle and at the end of the experiment.

Enzyme assays

Four fish for each condition were used for the enzymatic analyses. The intestines were separated and rinsed with cold distilled water. The mucose of each intestine was scraped and then homogenized in 1.1% NaCl, 0.5 mM PMSF (5 ml) using a hand held glass homogenizer. The liver was weighted just after removal, immediately minced in a homogenization buffer (1.1% NaCl, 0.5 mM PMSF) and then homogenized in the same buffer. All the enzyme assays

were conducted immediately after homogenate preparation. Leucine aminopeptidase and maltase were measured by using methods described previously (Storelli et al. 1986). Lipase determination was adapted from Albro et al. (1985). In order to establish the specific activities of the enzymes, protein concentrations were determined in the enzyme extracts using the method of Lowry et al. (1985), with bovine albumin as the standard. Enzyme and plasma analyses were conducted in the middle and at the end of the field experimentation.

Statistical analysis

The experimental design was balanced monofactorial with randomized blocks, three levels of treatment and four replicates (3x4). The fish diet was the experimental factor tested. The homogeneity of variance was tested for the individual fish weight with the Bartlett test at the beginning of the growth trial in order to assess similar intra group variability. All data were analysed by one-way ANOVA using R software (R release 2.5.0, 2007-04-23,). After the ANOVA, the differences among the means were determined by the Tukey multiple comparisons of means test, using the significance level of $P < 0.05$ (Venables and Ripley, 2002).

3 Results

VW is chemically characterized as follow: Sugars (g/l) 3.4 ± 0.9; Proteins (g/l) 3.2 ± 1.3; Lipids (g/l) 5.6 ± 1.6; Polyphenols (g/l) 4.99 ± 0.02; Ortho-diphenols (g/l) 0.46 ± 0.01; Total Suspended Solids (g/l) 44.26 ± 6.66; Natural Organic Material (g/l) 142.2 ± 21.3; pH 4.32 ± 0.39 (Values are expressed as Mean ± sd). The ingredients and the proximate composition of the diets are reported in table 1. The diet phenol content increased with VW inclusion and similarly the antioxidant DPPH activity in the diets has shown an increasing activity trend related to the VW inclusion (Table 1). At the beginning of the growth trial, the inter-treatment variances of the individual fish weights were homogeneus, Bartlett test ($B= 4.973$, $p< 0.05$),

thus all the fish were considered suitable for the growth trial. None of the considered performance parameters differed between the experimental treatments (Table 2) and there were no differences between the experimental treatments in the somatic indexes. Some of the productive traits are lower than other papers on gilthead sea bream (Sanchez-Muros et al. 2003; Gomez – Requeni et al. 2004; Sitjà-Bobadilla et al. 2005), and this fact is probably due to extruded fish feed used in these studies. The free radical scavenging activity (DPPH) in the fish fillets (Table 3) did not show any differences between the experimental groups, whereas the kinetic of lipid oxidation in refrigerated fish flesh fillets was investigated by the TBARS test (Fig.1) in order to evaluate their oxidative status. There was a statistically significant difference in the fillets examined with the TBARS test at 24 hours, and there was a trend in the following storage times, indicating the antioxidant effect of VW inclusion in fish feeds. The intestinal and hepatic enzymes (Table 4) were studied and they did not show any differences, except the maltase. Total amylases analyses of the intestines were carried out (unpublished results), and did not show any differences, whereas the intestinal maltase increased significantly with VW inclusion. The liver alkaline phosphatase and Na/K ATPase activities were not affected by the experimental diets. Regarding the plasma protein level, our results show that the protein level is not affected by antioxidant inclusion. The cortisol level was about 5 $ng \cdot ml^{-1}$ in the final samples; however, the intermediate sampling showed statistical differences between the groups fed with different diets, with a decreasing trend related to the increasing inclusion level of VW (Table 4).

Moreover, diet and fish fillet fatty acid profile was determined as well as the proximate composition of fish fillets. No differences were detected, therefore these results were not reported.

4 Discussion

The Mediterranean countries supply more than 95% of the world olive oil production, consequently a large volume of the effluent (800000 m^3/year in Italy alone) is produced within a period of only a few months (from November to February). The by-product used in this research, VW contains several phenolic compounds, such as hydroxytyrosol, tyrosol, oleuropein, and hydroxycinnamic acids (Mulinacci et al. 2001, Balasundram et al. 2006) that have antioxidant and nutritional properties, but at the same time there are aromatic molecules such as hydroxilated and methoxilated-benzoic, phenylacetic and phenyl propenoic acid that can originate through polymerisation of higher molecular weight compounds potentially responsible for antinutritional activity. Up to our knowledge, this research is the first case of VW natural polyphenol antioxidant utilisation in fish nutrition, for this reason low levels of VW inclusion were adopted in fish feed. Fish diet composition is similar in all the experimental treatments and an increase of antioxidant power was detected in fish feeds with VW inclusion. The growth trial conducted in this research, that was carried out in in order to investigate possible antinutrictional effects, did not show any negative effect on fish growth and the physiological parameters. Productive parameters and somatic indexes considered in the present study are comparable with those in other researches on gilthead sea bream under experimental conditions (Sanchez-Muros et al. 2003, Sadek et al. 2004). The absence of differences between experimental treatments in productive traits suggests the possibility of VW utilization in the sea bream feed. In an other study regarding VW inclusion in rainbow trout nutrition at the same level in fish feed (Sicuro et al., in press), some antinutritional effects of VW were registered. Sea bream seems to be more tolerant to high level of these polyphenols in fish feed and this could be an advantage for the future of aquafeed industry, considering the huge VW production in the Mediterranean countries, even if a polyphenols purification method must to be investigated. A large amount of literature exist concerning the antioxidant activities of some plants as natural antioxidants, Mourente et al.

(2002) showed that in gilthead sea bream, dietary oxidised oils influenced the activities of liver antioxidant defence enzymes, but these effects were partially restored by vitamin E. Moreover an other interesting aspect is that the antioxidant utilisation in fish feed can cause an improvement of final product quality (Ruff et al. 2002; Tocher et al. 2003). This fact is strictly related with the great attention of public opinion to fish farming products where artificial antioxidants, such as ethoxyquin, are stored in the fish fat tissues (He et al. 2000) and in other aquaculture products. Considering these facts, in the present research, the TBARS and DPPH analyses were carried out in order to evaluate the possible effects of VW antioxidants on fish flesh conservation. The TBARS test has already been utilised in researches on fish flesh quality (Menoyo et al. 2002; Mansour et al. 2006); the results of the kinetics of oxidation are comparable with those found by Menoyo et al. (2002) in Atlantic salmon, although the sea bream TBARS values are lower than the above cited results. Even if statistically significant differences only appeared at 24 hours, there is an improvement of TBARS values in fish fillet belonging to the VW groups. Is also evident that the antioxidant effect of VW inclusion on fish feed increased with the inclusion level. In the above mentioned study on rainbow trout nutrition with VW inclusion (Sicuro et al. in press), similar situation was registered in TBARS analysis, there was an improvement of TBARS in the fish fed with VW inclusion, but the difference was not evident in all the considered samples. Several physiological parameters have been investigated considering the possible negative effect of certain polyphenols. In the present study, the plasma protein level did not change with the inclusion of VW. Cortisol is considered the main fish hormone involved in the stress response in farming conditions. The cortisol levels in table 4 appear to be similar to normal farming conditions, that is about 5 $ng \cdot ml^{-1}$ (Rotllant et al. 2003). Cortisol level is approximately 50% lower in floating cages (Caruso et al. 2005). Cortisol decreases proportionally with VW inclusion in the first period, but these differences disappear at the end of the experiment. The serum total protein concentration is similar to other studies on sea bream

(Pavlidis et al. 1997; Sala-Rabanal et al. 2003). After haematological parameters, some digestive enzyme patterns have been investigated. Characterization of the main enzymes involved in the digestive process of sea bream is useful to reveal differences that may affect nutritional strategies utilized in farming (Alarcon et al. 2001; Venou et al. 2003) and the absence of an amylase inhibitor in gilthead sea bream (Venou et al. 2003) is confirmed in this study as the total amylases activities were not modified between the treatments. Only the activity of intestinal maltase significantly increased proportionally to the VW inclusion in the fish feeds, it is likely that the maltase activity is influenced by VW carbohydrates.

5 Conclusion

The results on the fish flesh shelf-life show that the antioxidant activity was slightly improved by the VW inclusion in fish feeds. The increased antioxidant activity in VW diets was detected and had an ameliorative effect on fish feed stability. Furthermore, VW inclusion in the fish feed did not negatively affect the fish growth and the greater part of the physiological parameters. On the light of this study, VW has an interesting prospective of utilisation, even if a purification and extraction system of useful polyphenols is necessary. Moreover, a clarification on the stability of VW antioxidants in extruded diets for seabream feeding is desirable. If these preliminary effects will be confirmed, VW could be considered as an available source of alternative antioxidant in fish feeds, rather than a simple effluent from olive oil extraction.

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Table 1. Ingredients and proximate composition of the experimental diets.

Ingredients (%)	Control	VW1*	VW5*
Herring fish meal	47.0	47.0	47.0
Wheat meal	20.4	20.4	20.4
Soybean extraction meal	18.6	18.6	18.6
Cod liver oil	10.0	10.0	10.0
Mineral mixture [1]	1.5	1.5	1.5
Vitamine mixture[2]	1.5	1.5	1.5
Liver-protector integrator[3]	1.0	1.0	1.0
Proximate composition (% DM)			
Moisture	10.0	9.2	10.8
Crude protein	39.8	39.3	39.8
Ether extract	11.8	11.0	11.6
Ash	8.3	7.5	8.1
Crude Fiber	2.0	2.4	1.7
Gross energy (Mj kg^{-1} DM)	18.4	18.0	18.0
Phenol content			
Polyphenols (g/Kg)	0.67±0.02	0.92±0.03	1.01±0.05
Ortho-diphenols (g/Kg)	0.07±0.03	0.07±0.04	0.01±0.05
Antioxidant activity			
(DPPH)	3.1	5.8	6.2

* Vegetation water is a liquid by-product of olive oil extraction.

[1] Mineral mixture (g or mg/kg diet): bicalcium phosphate 500 g, calcium carbonate 215 g, sodium salt 40 g, potassium chloride 90 g, magnesium chloride 124 g, magnesium carbonate 124 g, iron sulphate 20 g, zinc sulphate 4 g, copper sulphate 3 g, potassium iodide 4 mg, cobalt sulphate 20 mg, manganese sulphate 3 g, sodium fluoride 1g, (GrandaZootecnica, Cuneo, Italy).

[2] Vitamin mixture (IU or mg/kg diet): DL-a tocopherol acetate, 60 IU; sodium menadione bisulphate, 5 mg; retinyl acetate, 15000 IU; DL-cholecalciferol, 3000 IU; thiamin, 15 mg; riboflavin, 30 mg; pyridoxine, 15 mg; B12, 0.05 mg; nicotinic acid, 175 mg; folic acid, 500 mg; inositol, 1000 mg; biotin, 2.5 mg; calcium panthotenate, 50 mg; choline chloride, 2000 mg (GrandaZootecnica, Cuneo, Italy).

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Table 2 Performance and somatic indexes of the fish fed the experimental diets (mean ± S.D.).

	Control	VW1	VW5
Biomass gain[1]	143.7 ± 0.88$_{NS}$	141.2 ± 12.1 $_{NS}$	139.3 ± 8.1 $_{NS}$
SGR[2]	0.64 ± 0.04 $_{NS}$	0.64 ± 0.07 $_{NS}$	0.63 ± 0.03 $_{NS}$
FCR[3]	2.23 ± 0.20 $_{NS}$	2.10 ± 0.22 $_{NS}$	2.15 ± 0.21 $_{NS}$
PER[4]	1.13 ± 0.20 $_{NS}$	1.2 ± 0.11 $_{NS}$	1.17 ± 0.11 $_{NS}$
NPU[5]	0.23 ± 0.02 $_{NS}$	0.24 ± 0.02 $_{NS}$	0.23 ± 0.02 $_{NS}$
VSI[6]	2.48±0.59 $_{NS}$	2.78±0.61 $_{NS}$	3.06±0.58 $_{NS}$
HSI[7]	2.06±0.08 $_{NS}$	1.80±0.31 $_{NS}$	2.31±0.32 $_{NS}$

In the rows, different letters mean statistical difference at P≤0.05.

[1] Biomass gain (BG) (g) = final total weight – initial total weight;

[2] Specific Growth Rate SGR (%) = (ln final weight – ln initial weight)·100/feeding days

[3] Feed Conversion Rate (FCR) = total feed supplied (g of DM)/WG (g);

[4] Protein Efficiency Ratio (PER) = WG (g)/total protein fed (g);

[5] Net Protein Utilization (NPU) = utilised protein(%)/protein gain (%)

[6] VSI = (viscera/body weight) x 100

[7] HSI = (liver/body weight) x 100

Table 3. Free radical scavenging capacity (µmol TE / g tissue) (n=4).

Diet	% lb	% lb 10	% lb 30
Initial	0.171±0.01$_{NS}$	0.198±0.02 $_{NS}$	0.059±0.01 $_{NS}$
Control	0.184±0.03 $_{NS}$	0.213±0.04 $_{NS}$	0.061±001 $_{NS}$
VW1	0.159±0.02 $_{NS}$	0.209±0.02 $_{NS}$	0.058±0,01 $_{NS}$
VW5	0.159±0.03 $_{NS}$	0.183±0.03 $_{NS}$	0.058±0.01 $_{NS}$

^{NS} no statistical differences

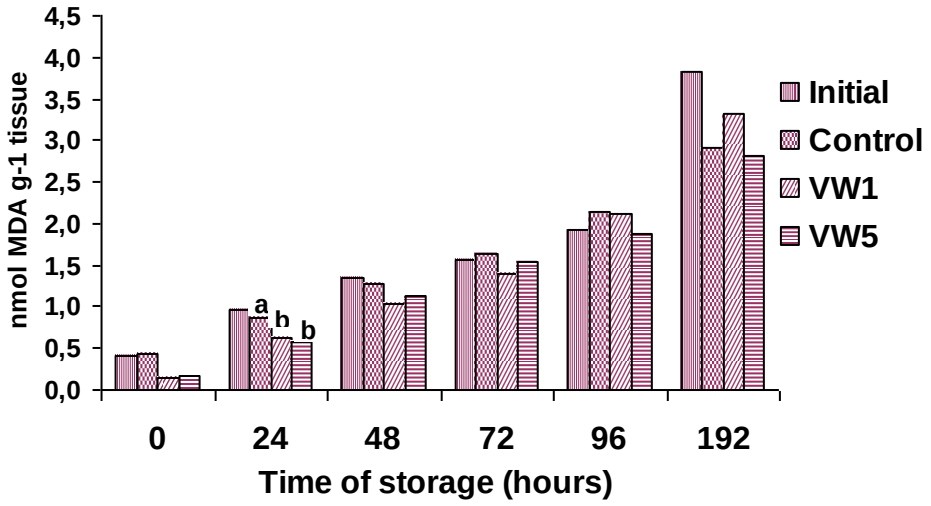
Table 4. Physiological parameters

	Intermediate sample			Final sample		
Intestinal enzymes	Control	VW1	VW5	Control	VW1	VW5
LAP (mU/mg)	22.2 ± 8.4 $_{NS}$	15.2 ± 3.5$_{NS}$	14.1 ± 3.5 $_{NS}$	90.0 ± 28.7 $_{NS}$	51.96 ± 11.9 $_{NS}$	66.04 ± 30.3 $_{NS}$
Lipase (mU/mg)	4.2 ± 2.4 $_{NS}$	3.9 ± 2.2 $_{NS}$	4.5 ± 2.8 $_{NS}$	4.5 ± 1.1 $_{NS}$	3.69 ± 1.6 $_{NS}$	4.1 ± 1.6 $_{NS}$
Maltase (mU/mg)	31.9 ± 8.5 $_{a}$	22.0± 5.9$_{a}$	87.0 ± 30.9 $_{b}$	48.2 ± 11.3 $_{a}$	94.32 ± 25.8 $_{b}$	139.34 ± 49.7 $_{b}$
Liver						
Na/K ATPasi (mU/mg)	9.8 ± 5.6 $_{NS}$	12.9 ± 5.6$_{NS}$	10.8 ± 5.2 $_{NS}$	15.5 ± 2.6 $_{NS}$	11.07 ± 2.2 $_{NS}$	12.33 ± 0.9 $_{NS}$
Alcaline Phosphatase (mU/mg)	28.8 ± 5.7 $_{NS}$	31.4 ± 2.7$_{NS}$	32.2 ± 4.9 $_{NS}$	65.6 ± 20.1 $_{NS}$	61.35 ± 26.2 $_{NS}$	77.39± 30.5 $_{NS}$
Plasma						
Osmolarity	420.5 ± 33.2 $_{NS}$	339 ± 82 $_{NS}$	414.5 ± 37.5 $_{NS}$	362.8 ± 3.9 $_{NS}$	354.0 ± 4.2 $_{NS}$	381.33 ± 52.4 $_{NS}$
Proteins (mg/ml)	53.6 ± 3.5 $_{NS}$	62.9 ± 8.4$_{NS}$	62.0 ± 6.0 $_{NS}$	58.6 ± 3.0 $_{NS}$	61.78 ± 10.7 $_{NS}$	56.59 ± 5.1 $_{NS}$
Cortisol (ng/ml)	10.0 ± 2.8 $_{a}$	6.7 ± 3.5 $_{b}$	3.0 ± 1.1 $_{b}$	4.7 ± 1.5 $_{NS}$	6.53 ± 3.6 $_{NS}$	6.67 ± 0.7 $_{NS}$

Separately by sample, means in the same row with different letters are significantly different ($P \leq 0.05$)

^{ns} no statistical differences

Figure 1: Kinetic of lipid oxidation in fish flesh fillets stored at 4°C



In the same group (hours of storage), different letters mean statistical difference at P≤0.05