Physiological effects of natural olive oil antioxidants utilization in rainbow trout (Onchorynchus mykiss) feeding

UNIVERSITÀ DEGLI STUDI DI TORINO

The olive oil by-product in 'rainbow trout, *Onchorynchus mykyss* (Walbaum)' farming: productive results and quality of product

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Abstract

The aim of this work is the investigation of olive oil by product (VW) inclusion in rainbow trout diet and its effect on productive traits and quality of product. **Two levels of VW inclusion were used and one control group**. Fish diets were isonitrogeonous (CP 40%) and isoenergetic (18 MJ kg-1 DM). 2400 rainbow trout were used. An in vivo digestibility experiment was performed in order to determine diets digestibility. All the fish diets and fillets were analyzed to determine the proximate and fatty acid composition. On final fish fillet, lipid oxidation was determined at 0, 24, 48, 72, 96, and 192 hours of storage using the 2-thiobarbituric acid method (TBARS). Aroma analyses on final cooked and raw fillet were performed with an electronic nose. The VW inclusion partially reduced protein digestibility. The fish growth varied between 1.08 and 1.1 % day. The supplemental level of VW led a better antioxidant status of fish fillet, in particular in the fillet sample after 72 hours of fillet conservation. **Principal component analysis (PCA) in raw and cooked fish fillet indicates that the VW inclusion in fish diet caused an aroma modification on fish fillet.**

Introduction

Fish feed affects the quality of product on aquaculture and antioxidant inclusion in farmed animal feed is a modern method largely used for improving the final product

(Chung et al., 2006). Several studies (Karpinska et al., 2001; Zia-ur-Rehman et al., 2004 – 2005; Surveswaran et al., 2007; Jayathilakan et al., 2007; Tung et al., 2007) have been conducted in order to identify natural sources of antioxidants and various compounds have been isolated, many of them being polyphenols (Moure et al., 2001). Antioxidants are a wide class of compounds utilised in animal nutrition for feed conservation and for improving of animal health. In particular, natural polyphenols from residual sources are of great interest because they represent a wide quantity of natural compounds (Moure et al., 2001). The increasing interest in natural antioxidants is due to the toxicity problems of synthetic antioxidants. In the last years, polyphenols in olive oil vegetation water mill (VW) have been studied for their antioxidant properties (De Lucia et al., 2006; Di Benedetto et al., 2006; Papadopoulos & Baskou, 1991; Soni et al., 2006) and their effects on human health (D'Angelo et al., 2005; O'Dowd et al., 2004; Singh et al., 2008; Visioli & Galli, 1998). Fish feed is stabilised with antioxidants to prevent the lipid oxidation. Polyphenols chemical structure is ideal for free radical-scavenging and have proved to be more effective in vitro than vitamin E and C (Rice-Evans et al., 1997; Dimitrios, 2006). Polyphenols exert, in vitro, powerful biological activities and they are useful for the pharmaceutical, cosmetic industry and food processing industry (Moure et al., 2001). The Mediterranean countries are the biggest olive oil producers in the world (Baskou, 1996). In modern oil mills, the main by-products are olive leaves, olive press cake and an aqueous by-product that makes up to 50% of the total yield of the olive paste and is named "olive mill waste water" (VW). The greatest part of antioxidants in the olive oil is lost with the wastewater (approximately 53%) and the pomace (approximately 45%) depending on the extraction system (Visioli et al., 1999). The

management of this liquid residue that is a mixture of polyphenols has been extensively investigated (Azbar et al., 2004; Niaounakis & Halvadakis, 2004;Visioli et al., 1999), in this context the potential VW use in fish nutrition could represent an interesting application. Spices and herbs are largely used as natural antioxidant in fish feed but there is still some criticism on them, as they give their typical flavour to the final product. For this reason, this study analyses fish fillet aroma. Some polyphenols affect the nutritional values of proteins because they reduce the nutritional values of foodstuffs and have a negative effect on the digestive enzymes (Gatlin et al., 2007), for this reason a digestibility trial and growth trial were planned before the antioxidant analyses on fish fillet. The aim of this work is the investigation on VW inclusion in the rainbow trout diet and on its effect on productive traits and quality product parameters.

Materials and Methods

2.1. Olive mill waste water analysis

Olive mill waste water (VW) utilized in this study was bought in a local oil mill and obtained by mechanical extraction from olive oil. VW was chemical characterized: sugars, proteins, lipids, polyphenols and ortho-diphenols, total suspended solids, natural organic material and pH were determined and are reported in table 1. VW total sugar content was determined by the spectrophotometric method at 550 nm (Bailey et al., 1992). The total protein content was evaluated by determination of total Nitrogen with the Kjeldahl Method. The total lipid content was measured by using gravimetric method (Delsal, 1944). The dry matter of VW is about 14% and it is chemically characterized

for the presence of polyphenols which could by considered interesting as potential natural antioxidant. Total polyphenols (TP) were measured using the Folin–Ciocalteau phenol reagent (Box, 1981). Total polyphenols were expressed as phenol (C_6H_5OH) in g L-1. TP were measured in VW after filtration through a 0.45 μm Millipore filter by a vacuum pump operating at 1 atm. Ortho-diphenol content was determined by using a colorimetric method (Gutfinger, 1981). Total suspended solids (TSS) measurements were performed according to a slightly modified technique (method 2540D) presented in APHA (1990). In particular, an accurately measured volume of sample (usually between 2 and 3 ml) was filtered through a 0.45 μm Millipore filter using a vacuum pump. The filter had been previously accurately weighted after drying at 105°C. After filtration, the filter was similarly dried at the same temperature and TSS were determined through the weight difference and expressed in g L-1. Natural Organic Material was analysed as the chemical oxygen demand (oxidisability) with the standardised permanganate Kubel method (Hofmann, 1965).

2.2. Diets

Two different diets were formulated with increasing level of olive mill wastewater (VW1 and VW5, respectively). These diets were tested against a control diet without olive oil by-product. Fish feeds were prepared as dry pellets with a diameter of 3.5-mm by a private feed manufacturer. The liver-protector integrator utilized is a product bought and registered by a private company (INVE, Belgium) and the specific composition is not given, even if generally is defined as a vitamin mixture. Diets analyzed by proximate composition according to standard methods (AOAC, 1995) show

that all diets were isonitrogeonous (CP 40%) and isoenergetic (18 MJ kg-1 DM) (Table 2). Diets were also analyzed for fatty acid methyl ester (FAME) profile (Table 3).

2.3 Growth trial and somatic indexes

Before starting the growth trial, a preliminary study for feed palatability was conducted for 15 days. During this period, three groups of trout were fed with experimental diet containing VW in order to check the diet palatability. No fish feed rejection was noticed. The growth trial was conducted at the Experimental Station of Department of Animal Husbandry of the University of Turin. 2400 rainbow trout individually weighted and having a mean initial body weight of 41.46 ± 0.1 g were randomly allotted to 12 tanks with a volume of 500l, (n=200), and a water flow rate of 12 l min^{-1}. Trout were acclimatized to experimental tanks for one week prior to commencement of the growth trial. Growth trial started on March 15th 2007 and ended on June 27th 2007, during first 2 weeks acclimatation period, the fish were progressively fed with experimental diets. The fish were fed once a day by hand (feeding ratio 1.5% of BW) six days per week. The feeding trial lasted for 94 days. Fish were bulk–weighted fortnightly in order to adjust the feeding rate and individually weighted at the end of the experiment. At the end of the trial productive traits and morphometric indexes were measured.

2.4 In vivo digestibility trial

During of the growth trial, an *in vivo* digestibility experiment was performed in order to determine the apparent digestibility coefficients (ADC) of diets. 12 groups of 3 trout (initial mean body weight 48 ± 3 g) from the same batch of those used for growth trial

were reared in 60 l cylindroconical tanks. Three tanks were randomly allotted to each diet. Water quality and temperature were the same as the growth trial. After 15 days of acclimatization to with a commercial diet, fish were fed by hand to satiety twice a day with the four experimental diets. The experimental design adopted was monofactorial, balanced with three levels of treatment. The apparent digestibility of protein and energy of each diet was measured using the indirect method (acid insoluble ashes). Faeces of each tank were collected using a continuous automatic collector (Choubert et al., 1982) 24 h after feeding over a period of six days per week and pooled per each experimental group together for chemical analyses. Faeces were collected every morning for three consecutive weeks and frozen daily (-20°C). Apparent Digestibility Coefficient of dry matter (ADCDM), crude protein (ADCCP), and gross energy (ADCGE) were calculated using the equation of Maynard and Loosly (1969).

2.5. Sampling and chemical analysis

At the end of the feeding trial, the fish were starved for 1 day, and after that, the fish were weighted for the final mean body weight. Five fish per tank, having body weight close to the mean body weight, were sampled and killed by anesthesia overdose in order to determine the somatic indexes. The gut and liver were separated from the rest of the body and weighted. The dorsal muscle tissues from the same fish were sampled and frozen until the next analyses. Upon the arrival in the laboratory, half muscle samples were freeze-dried in order to preserve the fatty acid quality whereas the remaining samples were kept frozen for the lipid oxidation analysis. All the fish diets and fillets were analyzed to determine the proximate composition according to standard methods

(AOAC, 1995). The gross energy content was determined using an adiabatic calorimetric bomb (IKA C7000, Staufen, Germany). The total nitrogen content was determined using a nitrogen analyzer (Rapid N III, Elementar Analysen systeme GmbH, Germany) according to the Dumas method and the crude protein was calculated as total N×6.25.

2.6 Fatty acid analysis

Fatty acid composition was determined on the feedstuffs, VW and fillet dorsal muscle samples. Lipid extraction of the samples was performed according to Caputi Jambrenghi et al. (2007) and the trans-methylation of the FA according to Christie (1982), with the modifications described by Chouinard et al. (1999). The fatty acid methyl esters (FAME) in isopropanol were then injected into a gas chromatograph (Hewlett Packard HP 6890 Series) equipped with a flame ionization detector. The helium carrier gas was used at a flow rate of 1 ml min-1. FAME were separated on a 30 m x 0.32 mm x 0.25 μm film thickness HP5 (Hewlett Packard) capillary column. The injector and detector temperature was maintained at 250 °C. The column was operated isothermally at 150 °C for 4 min, then programmed to 250 °C at 4 °C min-1. Peak identification was performed by using pure FAMEs standards (Sigma-Aldrich Co., Milan, Italy) and relative quantification was automatically carried out by peak integration.

The quality of fatty acid profiles was evaluated using atherogenicity indes (AI) and trombogenicity index (TI) of the fish fillets according to Ulbricht and Southgate (1991) as follows:

$$IA = (C12:0 + 4*C14:0 + C16:0) / (\sum MUFA + \sum n6 + \sum n3)$$

$$IT = (C14:0 + C16:0 + C18:0) / (0.5*\sum MUFA + 0.5*n6 + 3*n3 + n3/n6)$$

where MUFA and PUFA are monounsaturated fatty acids and polyunsaturated fatty acids, respectively.

2.7. Antioxidant analysis on fish fillet

To determine the effects of experimental diets on lipid oxidation, representative cutlets taken from each fillet were placed on ceramic dishes, wrapped in an oxygen permeable PVC and kept at 4 °C in a cool chamber. Lipid oxidation was determined at 0, 24, 48, 72, 96, 192 and 240 hours of storage using the 2-thiobarbituric acid method of Huang & Miller (1993) with slight modifications, thiobarbituric acid reactive substances (TBARS) were expressed as nmol malondialdehyde (MDA)• g–1 wet tissue.

2.8 Colour and pH detection on fish fillet

The colour of fish fillet on final samples was measured with colorimeter Minolta Chromameter Reflectance II CR200/08 and the measured parameters were L*, a*,b* (C.I.E., 1976). Fillet pH was measured with a portable pHmeter Delta OHM 9505 with Crison electrode and thermometer probe for automatic temperature calibration.

2.9 Flesh quality analysis

Eight samples of fish flesh per treatment were used for final quality analysis. The internal sample temperature was measured before baking (0 min). Cooking was performed at 165°C to reach 70 °C internal temperature in an electric forced-air convection oven. By forcing air into the oven, after passing through an active charcoal filter, regular cooking odour out flow was guaranteed. PEN 2 (AIRSENSE Analytics GmbH, Hagenower, Germany) is a portable electronic nose (EN) with 10 metal oxide sensors (MOS) that change their resistance in the presence of oxidising and reducing gaseous compounds (Kohl, 1992). The ten PEN2 sensors analyses 10 classes of chemicals: 2 sensors for aromatic (W1C and W3C), broad range (W5S), hydrogen (W6S), aromatic-aliphatic (W5C), broad-methane (W1S), sulphur-organic (W1W), broad-alcohol (W2S), sulphur-chloride (W2W) and methane-aliphatic (W3S). This instrument was utilized in the non-stop monitoring of volatile compounds on raw and cooked fish flesh. The temperature probe was put into the sample at the beginning of cooking phase and the internal temperature was checked while the sample reached 70°C. The statistical elaboration considered the average final 30 sec of the nose detection. In order to correctly measure the aromas, the samples for electronic nose were analysed following an increasing VW inclusion, as follow: control, VW1 and finally, VW5.

2.10. Statistical analysis

The experimental design was balanced monofactorial with randomized blocks, three levels of treatment and four replicates (3x4) for both digestibility and growth trial. Fish diet was the experimental factor tested. At the beginning of the growth trial, homogeneity of variance for individual fish weight was tested with Bartlett test in order to assess similar intra group variability. All data were analysed by one-way ANOVA using R software (R version 2.5.0, 2007-04-23) . After the ANOVA, differences among means were determined by the Tukey test multiple comparisons of means, using the significant level of $P < 0.05$ (Venables W. N. and Ripley, B. D. ,2002). For quality parameters analysis, two set of data for raw and cooked fish were used, with 24 rows (8 replicates per 3 treatments) and 10 columns corresponding to the sensors used for electronic nose. PCA multivariate analysis was performed for statistical elaboration of electronic nose data.

Results

3.1 Productive results and fillet composition

The VW dry matter is about 14% and it is chemically characterised for the presence of polyphenols (Tab. 2). Phenols contents in fish feed increased proportionally with VW inclusion and similarly the antioxidant activity (Tab.1). **However, it is to be noted that the antioxidant properties of fish feed were not exactly proportional to VW inclusion. This is probably caused by feed manufacture, where we prepared 500 kg of each different fish feed. However, we determined an increase of antioxidant**

properties of fish feed and this was considered sufficient. The most important fatty acid in the VW is the oleic acid (73 % of total), typical of olive oil (Tab.3). During growth trial the water temperature was 13 ± 1. °C and dissolved oxygen of 7.8 ± 0.1 mg l-1. The exploratory experimentation on experimental diets revealed that fish accepted the experimental diet. At the beginning of the growth trial, fish were individually weighted, thus the fish were considered suitable for the experimental design. Fish feed fatty acid composition shows a small increase of oleic acid in the VW5 diet (Tab. 4) that is related with VW inclusion. The diet digestibility is high in all the experimental groups (Tab. 1a) and the VW inclusion in fish feed partially reduced protein digestibility. Only for organic matter digestibility there is a decrease in the control diet. High dietary VW inclusion decreased biomass gain ($P < 0.05$), protein utilisation ($P < 0.05$) and fish growth ($P < 0.05$) (tab. 4). The fish growth (Tab. 5) varied between 1.08 and 1.1 % day. The feed conversion rate was little affected by variation in the dietary parameters. Somatic indexes were not different in the experimental treatments, only Body Index values were higher for the 1% VW inclusion (Tab. 6). The fillets proximate composition at the end of the trial showed no differences between treatments (Tab. 7). Fillet fatty acid composition (Tab. 8) is comparable in the experimental treatments and there is only a statistical significant ($P> 0,05$) decrease of C22:5 n6 between control group and fish feed with VW diets.

3.2 Antioxidants

The effect of experimental diets on susceptibility of fish fillet to oxidation as assessed by TBARS values was also measured for a period of 2 days (Tab. 9). The supplemental level of VW, bettered antioxidant status of fish fillets and there was a statistical significant difference (P> 0,05) in the fillet sample after 72 hours of conservation (Tab. 9). The antioxidant effect on fish fillet is much clearer if the VW fillet groups are considered together. In this case, after 24h, 48h and 192h of fish conservation there was a statistical significant difference (P> 0,05) indicating an ameliorative status in the fish fed with VW regardless the level of inclusion. It is also evident a progressive antioxidant effect in consideration of increasing of inclusion level from VW1 to VW5.

3.3 Fish quality

Analyses on colour and pH did not reveal any alteration on fillet, except a decrease in bL in the fillet of fish fed with 5% VW inclusion (Tab. 10). Electronic nose detected separated experimental groups on raw and cooked fish. More clear difference exist between control and VW groups, regardless of the inclusion level (Figg. 1 and 2), however the differences between experimental groups tend to decrease after cooking. The experimental groups are more confused in the cooked fish (fig 2) and the differences are unclear even considering second and third PCA axes. Considering the relation between PCA variables, i.e. electronic nose sensors, it is clear that there are 3 groups of correlate variables, which correspond to the sensor typologies. The variable groups are clearer with cooked fish than with raw ones (fig. 2). PCA scores in raw and

cooked fish indicate that aromatic compounds, (detected from W1C, W3C and W5C, on the right side of the graphs) are positively correlated with first axis, which explains more than 70% of variance in the two considered situations.

Discussion

4.1 Productive traits and fillet composition

The antinutritional effects of vegetal feedstuffs is the main obstacle for their inclusion in fish feed (Gatlin et al., 2007). In this study the partial decrease of feed digestibility induced by VW is better visible on gross energy and dry matter, that could indicate an interaction of VW polyphenols with dietary lipids and carbohydrates. The productive parameters are influenced by low digestibility, there is a negative effect of VW on protein utilisation, while other parameters are little affected. In general productive traits, digestibility, somatic indexes and fillet proximate composition are similar to other researches in experimental conditions (Sanchez-Muros et al., 2003; Sadek et al., 2004). Fillet fatty acid composition shows only an increase of docosopentenoic acid which is an elongation products of arachidonic acid that is sometimes associated with inflammation process. From histology of digestive tracts in these fishes there was not evident an inflammatory process (unpublished data).

4.2 Antioxidants

The attention of public opinion to modern aspect of zootechnics as organic production, led several researchers to explore substitution of artificial antioxidant with natural ones in animal feeding (Sebranek et al., 2005). The positive effect of antioxidant in farmed fish health has been largely studied in other sectors of animal production (Parpinello et al., 2006; Realini et al., 2004). Bandyopadhyay et al. 2007 used a mixture of natural antioxidants in dairy product and obtained similar results as BHT and BHA. Lycopene, occurring in red fruit and vegetable, has been extensively evaluated because of its property to scavenge free radicals in cell culture and in animal models (Srinivasan et al. 2007). There is large literature on the antioxidant effects of some plants (Yuan et al., 2006), as caffeic acid on rainbow trout (Chung et al. 2006), mushroom (Lee et al., 2007; Ktzberger et al. 2007), oil cakes (Ramachandran S. et al., 2007); Tamarindus indica (Siddhuraju P., 2007); blueberry in Artic charr semen quality (Mansour et al., 2006); mint, ginger and beet (Bandyopadhyay et al. 2007). The antioxidant properties of spices and herbs are attributed to their phenolic contents. Some of these compounds possess antioxidant activity similar or greater than BHA (Akthar et al., 1998). Vitamins are also extensively studied as antioxidant in fish feeds (Chaiyapechara et al. 2003; Guerriero et al. 2002; Hamre et al., 2004). The carry-over effect of dietary artificial antioxidant has been tested in a recent study on European salmon (Petri et al., 2008) and the fillet content of antioxidant has been shown to reflect that of the diet (Sigurgisladottir et al., 1994; Akhtar et al., 1998). In this study, the antioxidant effect of olive oil by product has been measured after 24 hours of fish conservation. This is interesting, if one considers that the fish is normally consumed within 24 hours. TBARS has been already used in researches on fish flesh quality (Mansour et al., 2006; Menoyo et al. 2002) and

our results, in terms of kinetic, are comparable with those found by Menoyo et al. 2002 in Atlantic salmon. In an our previous study on VW dietary antioxidant on gilthead sea bream similar effect had been reported indicating a more effective antioxidant deposition on trout fillet than sea bream fillet. In that study the same fish feed was used on gilthead sea bream and the reported values for TBARS in an 120 days long fish feeding trial were much higher. After 192 h of conservation VW1 TBARS was 2,1 (nmol malonaldehyde g-1 tissue). This high value, even considering higher control value for sea bream, clearly indicate that this phenolic antioxidant is better stored in trout than in sea bream. It is not clear for us what is the physiological explanation of this phenomenon, but it is likely due to physiology of absorption and transport of non-fatty acid materials in fish (Sigurgisladottir et al. 1994).

4.3 Fish quality

Considering that VW is very common in all the countries producing olive oil and it is very well known for its strong off-smell, caused also by the presence of oleuropein that contributes to the bitterness of olives, quality and aroma analyses were conducted on fish fillet, in order to investigate on possible alterations on final product. Fish odour is particularly considered in Italy were a recent research (Donati et al. 2000) reported that fish consumption is based mainly on its appearance, odour, point of sale hygiene and geographic origin. Moreover, several studies have been conducted on differences between farmed and wild fish (Farmer et al., 2000; Grigorakis et al., 2003). The quality

of farmed fish is clearly negatively affected by farming conditions, mainly on organoleptic characteristics and on other parameters as flesh colour, texture and firmness. The quality analyses conducted in this study show that electronic nose detected some differences in the samples and even if it is preliminary observation, it is useful for further consideration of VW inclusion in fish feed. Electronic nose is a useful and economic device really effective in exploratory studies in order to assess evident differences (Parisi et al., 2002). Aromatic compounds affect the flesh quality and discriminate between three experimental groups. In this study there is a correlation between the fish group with VW and the PCA 1st axis. This fact is a positive result, considering that aromatic compounds are correlated with the freshness aroma (Gregorakis et al., 2003). In other word, it seems that these VW diets could confer in the flesh fish a "freshness" odour. The darker colour is an other typical feature of wild fish and VW inclusion slightly increases darkness in this study. These b values indicates that the colour of raw fish in VW5 group tends to be darker than in the others groups. The quality of final product should be investigated in future utilisations of VW, because the few differences revealed by electronic nose should be compared with a panel test. In conclusion the utilisation of natural antioxidant in fish farming could be a promising direction for future aquaculture. This study investigated several aspects of fish nutrition and quality of product and the results obtained indicated that this by product can be used in the trout feed, no negative effects have been observed on fish growth, fish feed digestibility, somatic indexes, fillet quality and aroma analyses. The VW inclusion in rainbow trout feed increases the antioxidant activity in fish flesh and its carry-over effect as already demonstrated for other dietary phenols. In particular it was observed

the decrease of the TBARS values after 72 hours of conservation. In a similar study conducted on gilthead sea bream the VW resulted slightly different thought still positive. The inclusion of this by-product contributes to the valorisation of agriculture by-product since it opens new perspectives in the use of VW in other sectors of animal nutrition.

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Figure legends

Fig. 1 legend - WV1: vegetation water 1% group; WV5: vegetation water 5% group; ctrl: vegetation water control group. VARIABLES - W1C and W3C: aromatic sensors, W5S: broad range sensor; W6S: hydrogen sensor, W5C: aromatic-aliphatic sensor, W1S: broad-methane sensor; W1W: sulphur-organic sensor; W2S: broad-alcohol sensor; W2W: sulphur-chloride sensor; W3S: methane-aliphatic sensor

Fig. 2 legend - WV1: vegetation water 1% group; WV5: vegetation water 5% group; ctrl: vegetation water control group. VARIABLES - W1C and W3C: aromatic sensors, W5S: broad range sensor; W6S: hydrogen sensor, W5C: aromatic-aliphatic sensor, W1S: broad-methane sensor; W1W: sulphur-organic sensor; W2S: broad-alcohol sensor; W2W: sulphur-chloride sensor; W3S: methane-aliphatic sensor

Tables

Table 1 fish feed composition (%) . Ingredient and proximate composition of the experimental diets

Ingredients (%)	Control	VW1	VW5
Herring fish meal	47	47	47
Wheat meal	20.4	20.4	20.4
Soybean extraction meal	18.6	18.6	18.6
Fish oil	10	10	10
Mineral mixture [1]	1.5	1.5	1.5
Vitamin mixture[2]	1.5	1.5	1.5
Liver-protector integrator[3]	1.0	1.0	1.0
Vegetation water[4]	0	1	5
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-1)[5]	0.67±0.02	0.92±0.03	1.01±0.05
Ortho-diphenols (g kg-1)[5]	0.37±0.025	0.07±0.035	1±0.045
Antioxidant activity DPPH %	3.1	5.8	6.2

Fish feed digestibility **(%)**[5,6]			
ADCDM	79 ± 2.1 a	67.2 ± 4 c	73.9 ± 3.8 b
ADCCP	94.4 ± 0.4 a	91.9 ± 0.9 b	92.9 ± 1.9 b
ADCGE	83.5 ± 1.5 a	73.8 ± 3.6 b	78.9 ± 3.2 b

[1] Mineral mixture (g or mg kg-1 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-1 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).

[3] INVE TECHNOLOGIES, DENDERMONDE, BELGIUM

[4] Vegetation water is liquid by – product of olive oil extraction, its inclusion did not alter the percentage of other ingredient in fish feed

[5] (Mean ± Standard Deviation; n=4)

[6] In the rows, different letters mean statistical difference at $p<0.05$

Table 2. Vegetation water composition [a]

Compound	
Sugars (g L-1)	3.4 ± 0.9
Proteins (g L-1)	3.2 ± 1.3
Lipids (g L-1)	5.6 ± 1.6
Polyphenols (g L-1)	4.99 ± 0.02
Ortho-diphenols (g L-1)	0.46 ± 0.01
Total Suspended Solids (g L-1)	44.26 ± 6.66
Natural Organic Material (g L-1)	142.2 ± 21.3
pH	4.32 ± 0.39

[a] Mean ± standard deviation (n = 3)

Table 3. Vegetation water (VW) fatty acid composition (% of total fatty acids)

FA	%
14:0	0.03
16:0	16.90
16:1	1.60
17:0	0.18
17:1	0.06
18:0	0.40
18:1 n9	73.10
18:2 n6	7.70
18:3 n6	0.16
N6	7.86
Satured	17.51
Unsatured	82.62
MUFA	74.76
PUFA	7.86

Table 4. Fish feed fatty acid composition (% of total fatty acids)

FA	**Control**	**VW 1**	**VW 5**
C14:0	6.82	6.88	7.05
C14:1	0.11	0.11	0.10
C15:0	0.61	0.63	0.64
C16:0	22.10	22.50	23.30
C16:1	7.52	4.27	4.63
C17:0	0.56	0.57	0.56
C17:1	0.44	0.46	0.46
C18:0	2.83	1.61	1.66
C18:1n9	26.63	29.20	31.29
C18:2n6	13.11	15.30	10.33
C18:3n6	0.12	0.21	0.31
C20:4n6	0.57	0.59	0.58
C20:5n3	7.35	7.58	7.84
C22:5n6	1.50	1.45	1.57
C22:6n3	9.65	8.62	9.66
N3	17.00	16.20	17.50
N6	15.30	17.55	12.79
N3/n6	1.11	0.92	1.37
Satured	32.92	32.19	33.21
Mufa	34.40	34.04	36.48

Pufa	32.30	33.75	30.29
Unsatured	66.6	67.79	66.77
Sat/unsat	0.49	0.47	0.50
EPA+DHA	19.07	18.24	19.65

Table 5. Zootecnichal parameters [a]

Diets	**SGR**	**FCR**	**PER**	NPU	**BG**
Control	1,1 ± 0,01 a	1,4 ± 0,24 ns	1,95 ± 0,05 b	0,39 ± 0,01 a	80,4 ± 1,3 a
1% VW	1,06 ± 0,02 b	1,4 ± 0,04ns	1,81 ± 0,05 a	0,36 ± 0,01 b	72,2 ± 2,46 b
5 % VW	1,08 ± 0,03 b	1,4 ± 0,05ns	1,84 ± 0,05 a	0,37 ± 0,01 b	74 ± 3,29 b

In the columns, different letters mean statistical difference at $p<0.05$.

[a] Mean ± standard deviation (n = 4)

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

Specific Growth Rate SGR (%) = (ln final weight – ln initial weight)*100/feeding days

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

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

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

Table 6 Somatic indexes [a]

	HSI	VSI	BI
Control	1 ± 0.1 ns	10.8 ± 1.4 ns	1.4 ± 0.1 a
VW1%	1.1 ± 0.2 ns	11.3 ± 0.9 ns	1.8 ± 0.7 b
VW5%	1 ± 0.1 ns	10.7 ± 2.7 ns	1.4 ± 0.1 a

In the columns, different letters mean statistical difference at $p<0.05$.

[a] Mean ± standard deviation (n = 12)

VSI = (viscera/body weight) x 100

HSI = (liver/body weight) x 100

BI = (fish weight (g)/(fish length (cm))^3)x 100

Table 7. Fillets composition (% DM) and gross energy (MJ kg-1 DM)[a]

	Control	VW1	VW5
DM	22.1 ±0.89 $_{ns}$	22.1 ± 0.41 $_{ns}$	22.0 ± 1.19 $_{ns}$
CP	88.93 ± 1.34 $_{ns}$	88.74 ± 1.59 $_{ns}$	89.47 ± 1.39 $_{ns}$
EE	6.61 ± 0.85 $_{ns}$	6.48 ± 1.0 $_{ns}$	5.93 ± 0.83 $_{ns}$
Ash	6.69 ± 0.35 $_{ns}$	6.68 ± 0.25 $_{ns}$	6.53 ± 0.16 $_{ns}$
GE	22.62 ± 0.67 $_{ns}$	22.37 ± 0.8 $_{ns}$	22.5 ± 0.78 $_{ns}$

In the rows, different letters mean statistical difference at $p<0.05$.

[a] Mean ± standard deviation (n = 4)

Table 8. Fish fillet fatty acids composition (% of total fatty acids)[a]

FA	Control	VW1	VW5
14:0	3.94 ± 0.2 n.s	3.88 ± 0.4 n.s	4.14 ± 0.6 n.s
14:1	0.07 ± 0 n.s	0.1 ± 0 n.s	0.07 ± 0 n.s
15:0	0.47 ± 0 n.s	0.48 ± 0 n.s	0.5 ± 0.1 n.s
15:1	0.08 ± 0 n.s	0.09 ± 0 n.s	0.08 ± 0 n.s
16:0	22.75 ± 1.4 n.s	23.76 ± 1 n.s	23.94 ± 1.2 n.s
16:1	5.52 ± 0.2 n.s	5.46 ± 0.5 n.s	5.81 ± 0.7 n.s
17:0	0.39 ± 0 n.s	0.4 ± 0 n.s	0.41 ± 0 n.s
17:1	0.39 ± 0 n.s	0.39 ± 0.1 n.s	0.4 ± 0.1 n.s
18:0	2.85 ± 0.4 n.s	2.45 ± 0.1 n.s	2.37 ± 0.5 n.s
18:1 n9	30.21 ± 1.6 n.s	30.03 ± 1.5 n.s	29.85 ± 1.6 n.s
18:2 n6	9.52 ± 1.1 n.s	9.43 ± 1.1 n.s	9.68 ± 1.7 n.s
18:3 n3	0.47 ± 0.1 n.s	0.5 ± 0.2 n.s	0.33 ± 0.1 n.s
20:4 n6	0.74 ± 0.1 n.s	0.75 ± 0.1 n.s	0.74 ± 0.1 n.s
20:5 n3	3.66 ± 0.1 n.s	3.55 ± 0.4 n.s	3.61 ± 0.2 n.s
22:5 n6	0.81 ± 0.2 a	0.4 ± 0.1 b	0.4 ± 0.1 b
22:6 n3	18.21 ± 1.7 n.s	18.21 ± 2.4 n.s	17.78 ± 2.6 n.s
n3	22.34 ± 1.8 n.s	22.25 ± 2.5 n.s	21.72 ± 2.7 n.s
n6	11.07 ± 1.3 n.s	10.58 ± 1 n.s	10.81 ± 1.5 n.s
n3/n6	2.04 ± 0.3 n.s	2.12 ± 0.3 n.s	2.05 ± 0.4 n.s
satured	30.4 ± 1.8 n.s	30.98 ± 1.1 n.s	31.35 ± 1.3 n.s

MUFA	36.26 ± 1.9 n.s	36.07 ± 2 n.s	36.21 ± 2 n.s
PUFA	33.41 ± 2.5 n.s	32.83 ± 2.7 n.s	32.53 ± 2.6 n.s
unsatured	69.67 ± 1.8 n.s	68.9 ± 1.1 n.s	68.74 ± 1.4 n.s
Sat/Unsat	0.44 ± 0 n.s	0.45 ± 0 n.s	0.46 ± 0 n.s
EPA+DHA	23.42 ± 1.8 n.s	22.91 ± 2.6 n.s	22.52 ± 2.7 n.s
UI	191.28 ± 10.1 n.s	188.39 ± 13.4 n.s	186.2 ± 13.3 n.s
AI	0.55 ± 0 n.s	0.57 ± 0 n.s	0.59 ± 0.1 n.s
TI	0.32 ± 0 n.s	0.33 ± 0 n.s	0.34 ± 0 n.s

In the rows, different letters mean statistical difference at $p<0.05$.[a] Mean ± standard deviation (n = 4)

Table 9 . TBARS values on fish fillet (nmol malondialdehyde / g–1 tissue) (means, n=4)

Diets	0 h	24 h	48 h	72 h	96 h	192 h	240 h
Control	0.615 n.s.	0.680 n.s.	0.681 n.s.	0.708 a	0.644 n.s.	1.112 n.s.	1.027 n.s.
VW1%	0.523 n.s.	0.528 n.s.	0.528 n.s.	0.522 b	0.544 n.s.	0.807 n.s.	0.964 n.s.
VW5%	0.621 n.s.	0.483 n.s.	0.500 n.s.	0.462 b	0.435 n.s.	0.735 n.s.	0.720 n.s.

In the columns, different letters mean statistical difference at $p<0.05$.

Table 10. Colour measurement and pH in the fish fillet [a]

	L	a	b	CrL	HL	pH
Control	49.5 ±1.4 n.s.	4.4 ±0.5 n.s.	5.2 ±0.4a	6.8 ±0.5 n.s.	0.9 ±0.1 n.s.	6.5±0.04 n.s.
VW1%	50.0 ±1.5 n.s.	4.5 ±1.4 n.s.	5.1 ±0.5a	6.9 ±0.9 n.s.	0.9±0.2 n.s.	6.5±0.05 n.s.
VW5%	50.0 ±0.7 n.s.	4.5 ±0.8 n.s.	4.6 ±0.4b	6.5 ±0.6 n.s.	0.8 ±0.1 n.s.	6.5±0.07 n.s.

In the columns, different letters mean statistical difference at $p<0.05$. L (lightness), a (redness), b (yellowness), CrL (Chrome = radq (a^2+ b^2)), HL (Hue = arctg (b a-1))

[a] Mean ± standard deviation (n = 8)