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Effects of α-tocopheryl acetate supplementation in preslaughter diet on antioxidant enzyme activities and fillet quality of commercial-size *Sparus macrocephalus**

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Abstract: This study examined the effects of dietary α-tocopheryl acetate supplementation on antioxidant enzyme activities and fillet quality in commercial-size *Sparus macrocephalus*. Three hundred fish [main initial weight (350±12) g] were divided into three groups (E250, E500 and E1000) and reared in 9 cages. The fish were fed for 8 weeks with three diets containing different levels of dietary α-tocopheryl acetate (289, 553, 1 069 mg/kg). Over the experimental period, fish were fed to satiation and reached a final mean weight of (465±28) g without significant body weight difference and proximate composition difference. Fillet α-tocopherol was significantly (P<0.05) different between groups, reaching levels of 14.2, 22.1, 30.9 μg/mg fillet for groups E250, E500 and E1000, respectively. Total serum superoxide dismutase (SOD) activity increased significantly (P<0.05) in fish fed the diets high in α-tocopheryl acetate, but serum glutathione peroxidase (GPX) activity was unaffected. In storage on ice, fillets of fish fed the diets high in α-tocopheryl acetate exhibited significantly lower (P<0.05) levels of oxidation. These results suggested that increased dietary α-tocopheryl acetate could increase its flesh deposition, increase the activity of SOD and prevent lipid peroxidation of *Sparus macrocephalus* fillets in retail storage on ice.

Key words: Fillet quality, Lipid peroxidation, *Sparus macrocephalus*, α-Tocopherol
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INTRODUCTION

Lipid peroxidation is a serious problem for biological materials containing unsaturated fatty acids. This is particularly important for aquatic animals since they normally contain greater amount of polyunsaturated fatty acids (PUFA) than other species. Frequently, high value seafood products are stored for retail purposes on ice under refrigerated conditions, often without any further protection from oxidation. As a consequence, lipid peroxidation phenomena, which have a large influence on flesh quality along with autolytic and bacterial spoilage (Watanabe *et al*., 1996), may easily occur.

Among natural antioxidants, α-tocopherol is a potent biological antioxidant that can protect biomembranes and lipid components containing unsaturated fatty acids against attack from oxygen free radicals. Decreased lipid peroxidation has been found in fish when supplemented with α-tocopheryl acetate (a stable ester of α-tocopherol) at elevated levels in the diet (Huang and Huang, 2004; Kiron *et al*., 2004; Kolkovski *et al*., 2000; Tocher *et al*., 2002). To date, the influence of α-tocopherol on seafood quality has been studied for several species such as Atlantic salmon (*Salmo salar*) (Onibi *et al*., 1996; Scaife *et al*.,

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2000), rainbow trout (*Oncorhynchus mykiss*) (Jensen *et al*., 1998a), sea bass (*Dicentrarchus labrax*) (Pirini *et al*., 2000; Stéphan *et al*., 1995) and turbot (*Scophthalmus maximus*) (Frigg *et al*., 1990; Ruff *et al*., 2003), but many of these studies have been carried out on sub commercial-size fish and few studies exist for *Sparus macrocephalus*.

In fish, the relationship between dietary α-tocopheryl acetate and antioxidant enzyme activities has been reported in several studies. However, the reported effects of α-tocopherol on the levels of antioxidant enzymes in fish are not uniform (Lygren *et al*., 2000; Mourente *et al*., 2002).

The purpose of the present study was to evaluate the effect of α-tocopheryl acetate levels in pres-laughter diet on tissue α-tocopherol levels, serum antioxidant enzymes activity and the susceptibility of flesh fillets (stored on ice) to lipid oxidation.

MATERIALS AND METHODS

Diets

The components of a basal diets presented in Table 1 should provide adequate nutrient supply to *Sparus macrocephalus*. White fishmeal, soybean meal and protein powder of blood corpuscles were used for sources of protein; fish oil and α-starch for non-protein energy. α-Tocopherol content in basal diet (E250) was 289 mg/kg. Additional α-tocopheryl acetate (Mingo Tech-Bank Co., Ltd., China) was added in fish oil and data recorded after analysis 553 and 1069 mg/kg for diets E500 and E1000, respectively.

Table 1 Formulation and proximate composition of the experimental diet (on dry matter basis)

	Basal composition
Ingredients (%)	
White fishmeal	65.8
Soybean meal	5
α-Starch	14
Protein power of blood powder	2
Vitamin pre-mixture*	1
Mineral pre-mixture*	2
Cellulose	0.2
Fish oil	10
α-Tocopherol content (mg/kg)	
Diet E250 (basal diet)	289
Diet E500	553
Diet E1000	1069

*Vitamin and mineral pre-mixtures were provided by the Mingo Tech-Bank Co., Ltd., China; Total energy is 15.59 kJ/kg

To avoid lipid peroxidation, diets were stored at −20 °C until used. Every morning, before feeding fish, the quantities needed for the day were taken out from the freezer and left to reach room temperature.

Fish and rearing conditions

Sparus macrocephalus weighting (350±12) g were purchased from net-cage culture in a private fish farm (Fenghuangjiao Port, Zhejiang Province, China). Three hundred fish collected from a single net-cage, containing a homogeneous lot, were placed in 9 cages of 3 m^3 (1.5 m×1 m×2 m) size and divided into 3 groups. After two weeks of adaptation (basal diet fed), the fish were fed twice daily until satiety for 8 weeks. During the experimental period, water temperature and salinity were 22~29 °C and 38 g/L respectively.

Sampling and storage conditions

Ten *Sparus macrocephalus* per diet were sampled at the end of the experiment. Blood was drawn from the caudal vein of the individual fish. The whole blood was collected in a syringe, allowed to clot for 1 h in microtubes at room temperature, followed by 5 h at 4 °C and then serum was harvested by centrifuging at 1500×g for 5 min at 4 °C. All serum samples were preserved at −20 °C prior to analysis.

The fish were filleted in a box containing marine water and ice after blood extraction. Four fillet pieces per fish were sampled. One fillet was stored at liquid nitrogen for proximate composition, α-tocopherol content and induced thiobarbituric acid reactive substances (TBARs). Further fillets were stored on and covered with ice in boxes for up to 9 d after slaughter. On days 0, 3, 6 and 9, fillet samples for the determination of induced TBARs were taken from each fillet. Each sample was stored in liquid nitrogen until analysis.

Proximate composition analysis

The proximate composition analysis of fillets was carried out according to the AOAC International (1999) and reported on a wet weight basis.

Detection of superoxide dismutase

The activities of serum antioxidant enzymes

were determined with commercial kits purchased from Jiancheng Institute of Biotechnology (Nanjing, China). The assay for total superoxide dismutase (SOD) was based on its ability to inhibit the oxidation of oxymine by the xanthine-xanthineoxidase system. The red product (nitrite) produced by the oxidation of oxymine had absorbance at 550 nm. One unit of SOD activity was defined as the amount that reduced the absorbance at 550 nm by 50%.

Detection of glutathione peroxidase

The activity of glutathione peroxidase (GPX) was determined by quantifying the rate of H_2O_2-induced oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSH). A yellow product which had absorbance at 412 nm could be formed as GSH reacted with dithiobisnitrobenzoic acid. One unit of GPX was defined as the amount that reduced the level of GSH by 1 μmol/L in 1 min per ml.

Detection of α-tocopherol

The samples of fillet tissue were removed from liquid nitrogen for weighing, and a 1:10 (w:v) tissue homogenate was made with normal saline. After centrifugation, the supernatant was collected. α-Tocopherol content was measured with phenanthroline colorimetry (Jiancheng Institute of Biotechnology, Nanjing, China). α-Tocopherol has the ability to deoxidize ferric iron to ferrous iron, which chelates phenanthroline to form an orange red complex. The orange red complex (ferrous-phenanthroline) had absorbance at 533 nm. α-Tocopherol content was calculated in μg/g by calibration curve of standard addition method. The standards were prepared from kits.

Detection of lipid oxidation

Fillet samples were treated in the same way as for the detection of α-tocopherol content. Levels of TBARs were expressed as equivalents malondialdehyde (MDA). MDA content was determined by the thiobarbituric acid method (Jiancheng Institute of Biotechnology, Nanjing, China). MDA forms a red adduct with thiobarbituric acid, which had absorbance at 532 nm. MDA content was calculated in nmol/mg by calibration curve of standard addition method. The standards were prepared from kits.

Statistical analysis

All data were analyzed using SPSS 10.0 (SPSS Inc., Chicago, IL, USA). Regression analysis was used to determine the relationship between dietary α-tocopheryl acetate and its fillet deposition. One-way analysis of variance (ANOVA) was used to determine dietary difference on wet weight, proximate composition, enzyme activities and TBARs levels followed by a multiple comparison test (Student-Neuwman-Keuls). The relationship between fillets TBARs levels and conservation time was analyzed by the same way.

RESULTS

Growth performance and proximate composition

No difference in growth was observed among groups. After two months of feeding, the fish had reached the mean weight of (465±28) g. The proximate composition of the fish (Table 2) was not affected by dietary α-tocopheryl acetate over the experimental period.

Table 2 Fillet proximate composition (g/kg) (mean±*SD*)

Groups	Moisture	Crude lipid	Crude protein	Ash
E250	788.4±6.0	28.4±2.4	177.6±3.4	12.8±0.5
E500	782.5±6.1	29.3±2.2	180.5±2.6	12.8±0.4
E1000	781.2±7.3	28.1±1.7	181.1±2.7	12.4±0.6

Activities of SOD and GPX in serum of *Sparus macrocephalus*

The serum antioxidant enzyme activities showed some notable differences among the dietary treatments. The serum SOD activities were significantly higher in *Sparus macrocephalus* with diets E500 and E1000 compared to E250 (Table 3). No difference was found between E500 and E1000. The serum GPX activities (Table 3) did not change significantly among groups.

Table 3 Effect of treatment on SOD and GPX activities in serum (mean±*SD*)

Groups	Activity	
	SOD (U/ml)	GPX (μmol/L)
E250	123.1±8.3[a]	394.5±14.5
E500	188.4±11.4[b]	399.0±19.1
E1000	192.5±10.9[b]	400.9±16.6

Values with different superscript letters are significantly different ($P<0.05$) within the same column; $n=10$

Tissue α-tocopherol levels in *Sparus macrocephalus*

Tissue α-tocopherol levels are presented in Table 4. Diet significantly (P<0.05) affected α-tocopherol incorporation in fillet in a dose-dependent manner, with fish fed the diet high in α-tocopheryl acetate incorporating (E500 and E1000) more than those receiving low level of α-tocopheryl acetate (E250). Regression analysis (Fig.1) indicated a linear relationship between dietary and fillet concentration of α-tocopherol:

$$y=9.19+0.0223x,\ r^2=0.81,$$

where y is the fillet α-tocopherol content (μg/g), and x is the dietary α-tocopheryl acetate content (mg/kg).

Table 4 Effect of treatment on α-tocopherol (mean±*SD*) fillet content

Groups	α-Tocopherol content (μg/g)
E250	14.2±1.8[a]
E500	22.6±2.6[b]
E1000	30.9±2.3[c]

Values with different superscript letters are significantly different (P<0.05) within the same column; n=10

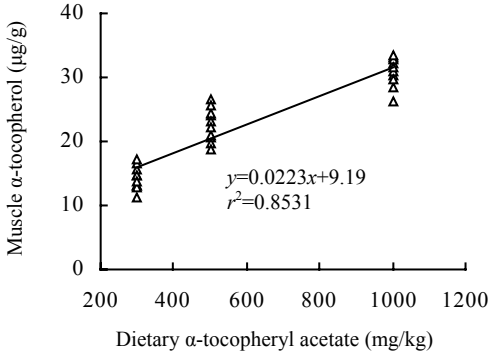

Fig.1 Regression analysis between dietary α-tocopheryl acetate and fillet α-tocopherol content

Tissue TBARs levels in *Sparus macrocephalus*

Tissue TBARs levels increased significantly (P<0.05) in *Sparus macrocephalus* during refrigeration and dietary treatment significantly affected flesh oxidation (Fig.2). The level of TBARs was significantly (P<0.05) lower in fish fed high α-tocopheryl acetate supplemented diets (E500 and E1000) than those receiving low level of α-tocopheryl acetate (E250) on days 0, 3, 6 and 9. Tissue TBARs levels did not change significantly between groups E500 and E1000.

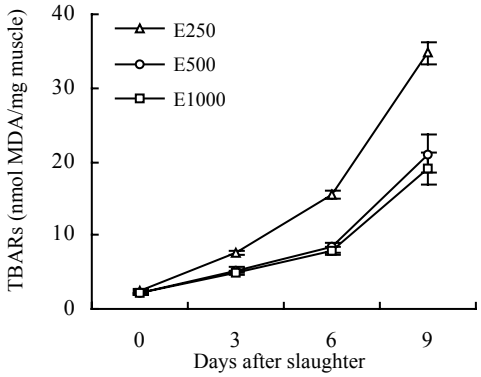

Fig.2 Effect of dietary α-tocopheryl acetate supplementation on TBARs (nmol MDA/mg muscle) fillet content of *Sparus macrocephalus* displayed on ice for 9 d (mean±*SD*)

DISCUSSION AND CONCLUSION

In the present study, dietary supplementation with α-tocopheryl acetate had no effect on *Sparus macrocephalus* growth, which is in agreement with studies by Gatta *et al*.(2000) on sea bass and Ruff *et al*.(2002) on Atlantic halibut, respectively. Previous studies showed growth differences in Atlantic salmon (Hamre *et al*., 1997; Tocher *et al*., 2002), but these results were based on α-tocopherol deficiency. Furthermore, Waagbø *et al*.(1993) and Gatta *et al*.(2000) found that α-tocopherol did not affect the proximate composition of Atlantic salmon and sea bass, respectively, which is in agreement with the findings in *Sparus macrocephalus* fillets of the present study.

Fillet α-tocopherol concentration is most likely affected by various factors, such as fish species and fat content, size and age as well as rearing conditions, while diet α-tocopheryl acetate levels may be the major factor (Ruff *et al*., 2002). In this study, the average concentrations of α-tocopherol incorporated corresponded very well with the dietary supplementation levels of α-tocopheryl acetate in *Sparus macrocephalus*. Fig.1 clearly showed an increase in α-tocopherol fillet content, with minimum and maximum values of 14.2 and 30.9 μg/g fillet for groups E250 and E1000, respectively. These findings suggest that *Sparus macrocephalus* might have the ability to store large amounts of α-tocopherol, when fed high α-tocopheryl acetate supplemented diets. Other aquaculture species such as Atlantic salmon, sea bass, turbot and Atlantic halibut show comparable

results (Bjerkeng *et al*., 1999; Pirini *et al*., 2000; Ruff *et al*., 2002; Stéphan *et al*., 1995).

The level of dietary α-tocopheryl acetate also showed some significant effects on the activities of the serum enzymes of the antioxidant defense system. These effects have to be interpreted within the knowledge of the commonly perceived biochemical mechanisms of these enzyme systems. For instance, SOD is scavenger of active oxygen species, acting on hydrogen peroxide (H_2O_2) and superoxide (O_2^-), respectively (Halliwell and Gutteridge, 1996; Winston and Di Giulio, 1991). Thus, the lower activity of SOD in *Sparus macrocephalus* fed with the E250 diet and the higher levels of SOD fed with the E500 and E1000 diets are all consistent with the expected pattern. Similar results were also found in other aquatic species, such as turbot (*Scophthalmus maximus* L.), halibut (*Hippoglossus hippoglossus* L.), sea bream (*Sparus aurata* L.) and *Litopenaeus vannamei* (Tocher *et al*., 2002; Liu *et al*., 2007). However, the activity of serum GPX was not related to dietary or tissue α-tocopherol levels in *Sparus macrocephalus*, which may indicate that elevated dietary α-tocopheryl acetate levels do not have the same effect on different serum antioxidant enzyme activities.

Since α-tocopherol protects against the development of rancidity, α-tocopherol level and TBARs values can be used as indices in fish quality evaluation (Frigg *et al*., 1990), and high levels of α-tocopherol may have the potential to improve sea food products produced in aquaculture. In the present study, the level of TBARs was significantly (P<0.05) lower in fish fed the high α-tocopheryl acetate supplemented diets (E500 and E1000) than those of E250 treatment on day 0. This is in agreement with the findings of Frigg *et al*.(1990), Stéphan *et al*.(1995), Onibi *et al*.(1996) and Gatta *et al*.(2000) who demonstrated that high levels of dietary α-tocopherol significantly reduced tissue lipid peroxidation in rainbow trout, juvenile turbot (after induced oxidation), Atlantic salmon and sea bass, respectively. Dietary α-tocopherol levels also had a significant (P<0.05) influence on levels of TBARs on ice storage days 3~9, which was shown in Fig.2. In contrast, Gatta *et al*.(2000) found lipid oxidation in sea bass fillets stored under refrigeration for up to 12 d was not induced. This may be caused by different storage condition; in our case, all fillets were stored under the same conditions (i.e. low temperature and without handling until day of preparation) with the aim of avoiding oxygen contact and other factors that may initiate lipid oxidation. Moreover, the results also showed that E1000 diet did not show better effect on reducing lipid preoxidation in *Sparus macrocephalus* than E500 diet. These may indicate that excessive dietary α-tocopheryl acetate supplementation does not have further contribution to shelf-life of fish. These findings above suggested that high levels of α-tocopherol can significantly reduce tissue lipid peroxidation and enhance shelf-life stability, but excessive dose of dietary α-tocopheryl acetate is not necessary. The TBARs value (expressed as MDA) of 0.5 μg/g in stored cooked pork tissue was suggested as a borderline level for the detection of off-flavours by taste panels (Jensen *et al*., 1998b). Weather this value applies to fish still remains to be investigated. If so, then fish fed the high α-tocopheryl acetate diets would have a distinct flavour advantage over the fillets of fish fed the low α-tocopheryl acetate levels.

The present study has demonstrated relationships between dietary α-tocopheryl acetate levels, the activities of the serum antioxidant enzymes and the levels of fillet lipid peroxidation products. Our findings allow us to conclude that α-tocopherol incorporation was directly related to dietary levels of α-tocopheryl acetate, and that elevated doses of dietary α-tocopheryl acetate significantly reduced lipid peroxidation. Therefore, it is suggested that preslaughter (8 weeks) feeds with about 500 mg/kg of α-tocopheryl acetate for commercial-size *Sparus macrocephalus* will significantly improve the quality of the final product.

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