Oral administration of Gum Arabic: effects on haematological parameters and oxidative stress markers in *Mugil cephalus*

Faggio C.[1]; Fazio F.[2]*; Marafioti S.[2]; Arfuso F.[2]; Piccione G.[2]

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Abstract

The aim of this research was to investigate the effects of the oral administration of Gum Arabic on haematological and some oxidative stress markers in *Mugil cephalus*. For this purpose 60 mullet caught in Faro Lake (Italy), were acclimatized and fed with commercial diet for 20 days prior to the start of experiment, which lasted 15 days. After acclimatization, fish were randomly divided into two equal groups: control group was fed with commercial pellets. Haematological profile on whole blood and sera was assessed, reactive oxygen metabolites (d-ROMs), antioxidant barrier (OXY-ads) and thiol-antioxidant barrier (SHp) were immediately assessed by means of a UV spectrophotometer. Two-way repeated data analysis (ANOVA) showed significant changes only on Thrombocyte Count (TC). In the experimental group an increase in OXY-ads and SHp levels and a significant decrease of d-ROMs were observed after administration of GA.

Keywords: Gum Arabic, Diet supplement, Haematological profile, Immuno-stimulant capacity, Oxidative stress, Mullet

1-Department of Biological and Environmental Science. University of Messina. Viale Ferdinando Stagno d'Alcontres 31, 98166 S. Agata-Messina Italy

2-Department of Veterinary Sciences. Polo Universitario Annunziata, University of Messina. 98168, Messina, Italy.

*Corresponding author's email: ffazio@unime.it

Introduction

Biomarkers are defined as a response to biological changes, which can be related to the toxic exposure or to the toxic effects of environmental chemicals (Depledge *et al*., 1995). Fish are largely used for the assessment of aquatic environment quality and are accepted as bio-indicators of environmental pollution (Borkovic *et al*., 2008). Among the different fish species, *M. cephalus* has several characters required in a "sentinel" such as wide geographic distribution, great abundance, salinity and temperature tolerance; it is common in coastal waters and enters lagoons, estuaries, harbour and rivers that are frequently subjected to pollution. Moreover this specie has high economic and appreciated food value and is also demonstrated to be suitable for biomarkers studies (Ferreira *et al*., 2004; Gorbi *et al*., 2005; Pacheco *et al*., 2005). In addition to haematological and biochemical parameters that are already used to provide information about the health status of fish and water quality in which they live (Coz-Rakovac *et al*., 2008; Fazio *et al*., 2012a), changes in the levels of stress oxidative parameters have been proposed as biomarkers in this fish (Mieiro *et al*., 2011; Ben Ameur *et al*., 2012; Yonar *et al*., 2012). A stressful condition leads to excessive production of free radicals, which results in oxidative stress (Khadadija *et al*., 2009). This is commonly defined as a disturbance in the balance between the pro-oxidants and antioxidants leading to detrimental biochemical and physiological effects. Oxidative stress might occur when the antioxidant defence system is overwhelmed by an increased oxidant burden or a reduced antioxidant supply (Kirschvink *et al*., 2008). Reactive oxygen species (ROS) are produced continuously in cells by products of aerobic cellular metabolism, largely via leakage from the electron transport chain in mitochondrial respiration. Due to their high reactivity, these species may show damage in lipids, proteins, carbohydrates and nucleic acids (Hermes Lima, 2004). In farm animals, oxidative stress is involved in a number of pathological conditions, including those associated with animal production, reproduction and welfare (Lykkesfeldt and Svendsen, 2007).

Sources of ROS include irradiation, UV light, production of H_2O_2, NO and O_2•- by activated macrophages and phagocytes, metal catalyzed oxidation systems, air and water pollutants, and auto oxidation of electron transport carriers (Stadtman and Levine, 2000).

Cultured fish usually faces stressful conditions such as hypoxia/re-oxygenation and hydrogen peroxide exposure, all of which generate oxidative stress. Low oxygen pressure is common in intensive fish farming and during fish handling, and the subsequent re-oxygenation after hypoxia generates an increase in free radical formation. On the other hand, H_2O_2 is used in aquaculture as a treatment to control ectopic diseases of cultured fish. When toxic agents against the natural protective systems overrun, exogenous antioxidative and protective compounds must be administered (Devillers *et al*., 2001). Nowadays, many efforts are being performed to search new antioxidants as potential therapeutic agents. In recent years, several organic forms of antioxidant

molecules have been studied as preventive agents and natural therapeutics. To bring about diseases without exposure to pro-oxidant agent and to avoid oxidative stress phenomena, fish should use food supplement as possible natural immumostimulant, antioxidant and protective agent. The usefulness of immunostimulants has been demonstrated in modern aquaculture (Sakai, 1999), and fish farmers use a wide range of immunostimulants which may need to be purified (vitamins, chitin, glucans, etc.) or not (microorganisms, animal and plant extracts, sub-products of other industries, etc.). These categories of immunostimulants has recently received increasing attention since they combine lower costs with the advantage that they can be easily incorporated into the diet and have a low impact on the environment. Furthermore, they have many additional effects on fish physiology because they act as a "cocktail" containing many macronutrients, micronutrients as well as immunostimulant substances. Some natural substances such as aloe and roots (e.g. from Astragalus and Chinese Angelica) and propolis have also been demonstrated to be good immunostimulants in fish (Kim *et al*., 1999; Jian and Wu, 2003; Cuesta *et al*., 2005; Talas and Gulhan, 2009). Gum Arabic (GA) is one of these substances, due to its immunostimulatory and anti-inflammatory effects in mammals (Cuesta *et al*., 2005) and also antioxidant properties, so it has attracted aquaculture researchers' interest. There are no reports about the possible use of GA administration on haematological and oxidative markers in fish, so, this study was designed to assess the effects of orally administered GA on mullet (*M. cephalus*) through analyzing of several blood parameters and oxidative stress markers.

Materials and methods

Experimental design

A number of 60 *M. cephalus* (fork length: 20.36±0.79cm; body weight: 293.50±15.88g) were caught in Faro Lake (Sicily), acclimatized and fed with commercial diet (Aller Aqua, DK-6070 Christiansfeld, Table 1) for 20 days prior to the experiment. After acclimatization, fish were randomly divided into two equal groups (control and experimental). Each group was equally subdivided to form their triplicate (10 fish for each subgroup) and transferred into 100 L tanks respectively. The control group was fed with the same commercial pellets used during acclimatization period, while the experimental group was fed with 12% GA-pellets for 15 experimental days. Both groups were fed with 2% of body weight once daily at 10:30. At each feeding stage, all food was consumed and no leftover was observed. Temperature, salinity, pH and total ammonia were measured three times weekly and did not exceed values recommended for fish (Table 2).

Table 1: Composition of the commercial diet with which the fish were fed.

Commercial diet composition	Percentage
Crude Protein	46%
Crude Fat	20%
Ash	10%
Fiber	1.5%

Table 2: Mean values ± SEM of water quality parameters measured in three different subgroup tanks.

Parameters	Control group	Experimental group
Temperature (°C)	22.04 ± 0.30	22.20 ± 0.10
Salinity (ppt)	32.80 ± 0.15	32.50 ± 0.18
pH	8.10 ± 0.02	8.20 ± 0.01
NH_3/NH_4 (mg/L)	0.31 ± 0.03	0.32 ± 0.01

GA composition

Gum arabic (GA), is a natural composite polysaccharide derived from exudates of Acacia senegal and Acacia seyal trees. GA consists of mainly three fractions (Randall *et al*., 1988; Idris *et al*., 1998): the major one is a highly branched polysaccharide (MW=3x105) consisting of b-(1-3) galactose backbone with linked branches of arabinose and rhamnose, which terminate in glucuronic acid (found in nature as magnesium, potassium, and calcium salt) (Dror *et al*., 2006). A smaller fraction (~10 wt % of the total) has a higher molecular weight (~1x106 g/mol) called arabinogalactan–protein complex (GAGP – Gum arabic glycoprotein) in which arabinogalactan chains are covalently linked to a protein chain through serine and hydroxyproline groups. The attached arabinogalactan in the complex contains ~13% (by mole) glucoronic acid (Goodrum *et al*., 2000). The smallest fraction (~1% of the total) having the highest protein content (~50 wt %) is a glycoprotein which differs in its amino acids composition from that of the GAGP complex. The major amino acids present in the protein of AG and AGP are hydroxyproline, serine and proline, whereas in GP, aspartic acid is the most abundant (Islam *et al*., 1997).

Food supplement preparation

A net weight of 25 g of commercial basal pellets (Table 1) was crushed and mixed with water and 3 g of Gum arabic (Sigma-Aldrich, St. Louis, MO, USA) to obtain 12 % gum supplemented diet. The obtained mash was reformed into pellets, and these allowed drying at ambient temperature until use.

Analytical procedures

Before starting of experimental protocol (T0) and at the end of trial (T15), fish blood samples were collected. Both control and experimental fishes were anesthetized with 2-phenoxyethanol at the concentration of 400 mg/l. Blood samples were transferred into two different tubes: one (Miniplast 0.5

ml, LP Italiana Spa, Milano) containing EDTA (1.26 mg/0.6mL) as an anticoagulant agent and the other without EDTA. The time lapse from capture to blood withdrawal was less than 3 minutes. After blood sampling, the fish were individually weighted to the nearest 0.1g (KERN 440-49N) and their fork length (L) were recorded. Condition factor (CF) was calculated as: W x 100 L-3, where W is the wet weight of animal and L is length. After the dietary intake of GA pellets, the specific growth rate (SGR, %body weight/day) for each group was determined using the equation:

Specific growth rate (SGR) = [(LnW2-LnW1)/t]×100

Where: Ln = the natural log, W1= initial fish weight; W2 = the final fish weight in "grams" and t = period in days.

Haematological profile was measured, on blood samples collected in EDTA tubes, within 1 hour after blood samples were taken. Blood haematological profile was measured in a blood cell counter HeCo Vet C (SEAC, Florence, Italy) as described before by Fazio *et al*. 2012b, 2012c. The parameters evaluated were: Red Blood Count (RBC), Haematocrit (Hct), Haemoglobin concentration (Hgb), White Blood Cell Count (WBC), Thrombocyte Count (TC), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH), and Mean Corpuscular Haemoglobin Concentration (MCHC).

On sera obtained after centrifugation (7 min at 1500 g), from blood samples without EDTA, reactive oxygen metabolites (d-ROMs), antioxidant barrier (OXY-ads) and thiol-antioxydant barrier (SHp) were immediately determined by means of commercial kits (DIACRON International s. a. s., Grosseto, Italy) using UV spectrophotometer (Slim, SEAC, Florence, Italy). All analyses were performed in duplicate by the same technician according to the procedures described by the manufacturers. Protocols of fish and experimentation were reviewed and approved in accordance with the standards recommended by the Guide for the Care and Use of Laboratory Animals and Directive 63/2010 EU.

Statistical analysis

Data obtained after fish biometry and different blood parameters were tested for normality using Kolmogorov-Smirnov test. *P*-value for statistical significance was 0.05. Two-way analysis of variance (ANOVA) was used to determine significance of the effects of time and GA administration on biometric, haematological and serum parameters recorded in two different groups.

Results

During the test, no fish mortality in any groups and the water parameters were monitored and kept constant. As reported in Table 3, two-way repeated measure ANOVA showed that dietary supplemented GA significantly influenced fork length (p=0.03) and weight gain (p= 0.01).

Table 3: Mean values ± SEM of biometric parameters, condition factor (CF) and specific growth rate (SGR) recorded in mullet at T0 and after GA administration (T15).

Parameters	Control group		Experimental group	
	T0	**T15**	**T0**	**T15**
Weight (g)	293.00 ± 7.42	294.80 ± 7.08	294.00 ± 6.78	301.60 ± 4.88*
Fork length (cm)	20.06 ± 0.43	20.20 ± 0.58	20.66 ± 0.28	22.90 ± 0.48 *
CF	3.66 ± 0.23	3.64 ± 0.31	3.35 ± 0.15	2.54 ± 0.17
SGR (%)	0.14		0.17	

* *Significant Vs* Control group (T0 and T15) and Experimental group (T15).

The specific growth rate was not significantly affected by the dietary intake of GA.

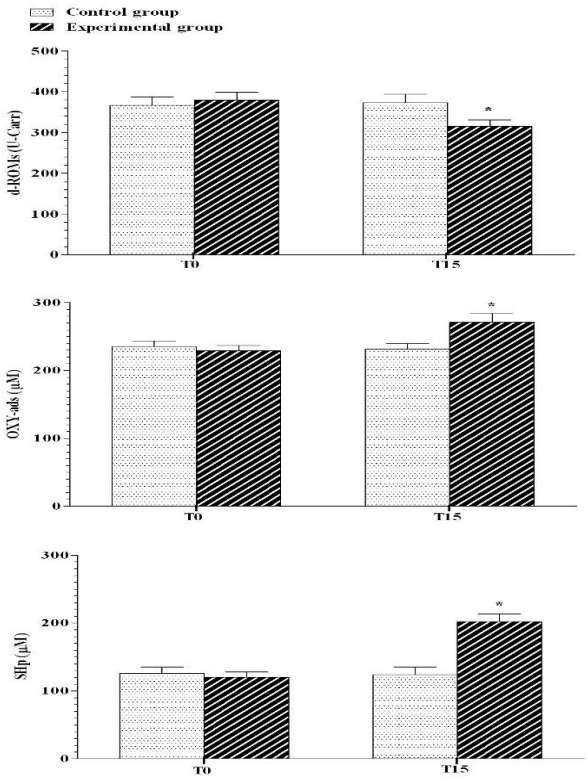
Among haematological parameters (Table 4), GA administration had a significant effect only on TC (P=0.0015).

Table 4: Mean values ± SEM of haematological parameters recorded in two groups before (T0) and after GA administration (T15).

Parameters	Control group		Experimental group	
	T0	**T15**	**T0**	**T15**
RBC ($x10^6/\mu L$)	2.24 ± 0.20	2.24 ± 0.11	2.23 ± 0.17	2.27 ± 0.11
Hct (%)	24.60 ± 1.60	24.98 ±1.61	24.76 ± 1.44	26.46 ± 3.70
Hgb (g/dL)	6.94 ± 0.49	6.42 ± 0.43	7.00 ± 0.57	6.92 ± 0.51
WBC ($x10^3/\mu L$)	13.18 ± 1.20	14.98 ± 0.58	13.42 ± 0.99	14.48 ± 1.13
TC ($x10^3/\mu L$)	21.80 ± 1.58	22.40 ± 1.21	22.00 ± 1.14	33.40 ± 1.21*
MCV (fL)	110.00 ± 5.52	113.00 ± 7.01	112.60 ± 7.47	116.8 ± 12.70
MCH (pg/cell)	31.16 ± 2.46	32.19 ± 1.32	31.42 ± 0.94	34.17 ± 1.76
MCHC (g/dL)	28.34 ± 1.60	25.81 ± 1.26	28.23 ± 1.41	25.18 ± 0.38

* *Significant Vs* Control group (T0 and T15) and Experimental group (T15).

Figure 1 shows the average value of oxidative markers expressed in their conventional units, together with the relative standard deviations and statistical significance obtained by statistical analysis. Two way ANOVA showed a highly significant effect of GA administration on all three oxidative stress markers studied. In particular, an increase of OXY-ads (p=0.016) and SHp (p=0.010) levels was seen and a significant decrease of d-ROMs (p=0.038) in experimental group was detected compared to the control group.



** Significance Vs Control group (T0, T15) and Experimental group (T0)*

Figure 1: Mean values ± SE of oxidative stress markers recorde in two group before (T0) and after GA administration (T15).

Discussion

This is the first study carried out to evaluate GA immunostimulant and antioxidant activity in fish. In recent years, there have been different studies carried out on GA effects in rats but no one on GA and its effects on biochemical parameters of fish. The use of compounds with immunostimulant and/or antioxidant effects as dietary supplement can improve the innate defence on animals providing resistance to pathogens during periods of high stress. Therefore, the use of immunostimulant is being introduced into fish farming routine procedures as a prophylactic measure (Kumari and Sahoo, 2006).

Natural products have been used lately as an alternative for different purposes. For example, studies conducted on propolis showed that this substance has a significant effect on haematological and biochemical parameters in fish and so its use may contribute to the health of fish (Cuesta *et al*., 2005; Talas and Gulhan, 2009; Yonar *et al*., 2012). Many polysaccharides, such as β-glucans, chitosan and raffinose are available in the fish aquaculture industry and are extensively used as immunostimulants to induce and build up protection against a wide range of disease (Lin *et al*., 2011). In our study, diet containing GA showed no adverse effects on fish, rather a positive effect was seen on biometric parameters. CF, is a measure of

fish condition in which CF<1.0 is poor and CF>1.4 shows a fish is in good to excellent condition. Our results showed that, this factor was always >1. This confirms that GA administration did not have negative effects on the welfare status of mullet. Over the duration of the study, specific growth rates ranged between 0.00 and 0.07% in the control group and between 0.00 and 0.38% in the experimental group. Some recent studies have shown that feeding natural supplementary food for fish aquaculture, resulted in increased disease resistance and in improved survival and growth rate, which may be attributed to an improvement of immune functions (Christybapita *et al*., 2007; Divyagnaneswari *et al*., 2007; Ardó *et al*., 2008; Cheng *et al*., 2008;). It is known that exogenous agents can change heamatological parameters and that these provided valuable information for fishery biologists in the assessment of fish health (Banaee *et al*., 2008). Haematological values found in this study are similar to those found by Satheeshkumar *et al*. (2010) and Fazio *et al*. (2012c) on *M. cephalus*. Regarding these parameters our study showed that oral administration of GA at concentrations of 12% for 15 days have significant effects only on TC values. There are reports suggesting that fish thrombocytes have phagocytic ability and participate in defence mechanisms (Stosik *et al*., 2001). Fish thrombocytes represent a link between innate and adaptive immunity (Passantino *et al*., 2005) and express surface and intracellular molecules that are involved in the immune function (Kollner, 2004). It is already agreed that the fish thrombocytes are blood phagocytes that form one of the protective barriers (Tavares-Dias and Moraes, 2004; Prasad and Charles, 2010; Prasad and Priyanka, 2011). On WBC levels, GA administration did not show any effect, however the higher values found in TC level in the experimental group highlights the possible immunostimulant power of this natural gum.

The absence of changes in WBC levels can be due to dosage or short-term administration of 12% GA-pellets. Dose-dependent effects of GA on fish blood can be favourable, opening new perspectives of investigation on their biological properties and utilization. So, further studies are necessary to understand the right concentration and time of administration of GA to obtain immunostimulatory effects in fish. Changes in the levels of stress oxidative parameters have been proposed as biomarkers in fish and some research have been carried out to understand the role of some substance as antioxidant in aquatic animals. The feeding of probiotics, for example, increased the antioxidant status in shrimps (Castex *et al*., 2009) and melatonin administrations provoked slight antioxidant effect in goldfish (López-Olmeda *et al*., 2006). The purported action of GA as an antioxidant has suggested new researches that have evidenced protective effects of GA against experimental gentamicin and cisplatin nephrotoxicity (Al Majed *et al*., 2002), doxorubicin cardiotoxicity (Abd-allah *et al*., 2002) in rats, and acetaminophen hepatotoxicity (Gamal el-din *et al*., 2003) in mice. All these studies were based on the assumption that GA has strong anti-oxidant properties, and a major mechanism for the induction of these toxicities is the generation of free radicals

(Hinson *et al*., 2004; Ali and Al Moundhri 2006). In our study, the anti-oxidant effect was also found in fish, and GA administration resulted in an increase of OXY-ads and SHp levels and in a depletion of d-ROMs. These results confirm the high antioxidant power of this natural substance, suggesting a promising potential use of GA as food supplement.

GA administration has a protective effect against oxidative damage and a slight immunostimulantory effect that is valued by the increase of TC in fish, as well as in mammals. Probably, these findings underlines that the immunostimulatory effect of GA is related to the dosage and time of administration. So there is still a need for further studies involving a larger number of fish, various concentrations and times of administration of GA and the assessment of additional fish blood parameters to confirm the utility of this substance for fish health.

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