Enhancement of growth in tilapia larvae (*Oreochromis niloticus)* by sulfated D-galactans extracted from the red marine alga *Botryocladia occidentalis*[1]

Incremento do crescimento de larvas de tilápias (*Oreochromis niloticus*) por D-galactanas sulfatadas extraídas da alga marinha vermelha *Botryocladia occidentalis*

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ABSTRACT

Sex reversal with synthetic androgens is nowadays one of the most frequently applied techniques to produce monosex male populations in tilapia. Moreover, intensive tilapia larviculture exposes the larvae to a considerable level of stress. These conditions are harmful to the larvae by the fact that administration of sex hormones and stress, are potent suppressors of the immune system in fish. The present study aimed to investigate the effect of sulfated D-galactans (SDG), extracted from the red marine alga *Botryocladia occidentalis,* during the sex reversal of fry from the Nile tilapia, *Oreochromis niloticus,* with the synthetic androgen 17-alpha-methyl-testosterone. SDG was incorporated to the dried ration before the addition of the synthetic androgen and administered to the fingerlings during 28 days. Four experimental treatments were carried out using 0.05, 0.1 e 0.2 mg SDG g^{-1} body weight and the control without incorporation of SDG. Maximal final weight and weight gain was observed in fingerlings feed with 0.1 mg SDG g^{-1} after 3 and 4 weeks. Furthermore, oral administration at a higher dose (0.2 mg SDG g^{-1} body weight) did not increase weight gain. Survival rate was low in all treatments probably due to a low content of dissolved oxygen, high stock density and fish escape. The results obtained in the present study revealed that administration of low amount of SDG from *B. occidentalis*, during the sex-reversal treatment, might be a valuable tool to increase growth in fingerlings from the Nile tilapia, *O. niloticus*.

***Index terms**:* Tilapia, sulfated polysaccharides, immune system.

RESUMO

A reversão sexual com andrógenos sintéticos é uma das técnicas mais utilizadas para a produção somente de machos de tilápias. Por outro lado, a larvicultura intensiva de tilápias expõe as larvas a um nível considerável de estresse. Essas condições são prejudiciais, pois a administração de hormônios sexuais e o estresse são potentes inibidores do sistema imunológico dos peixes. Esse estudo teve como objetivo investigar o efeito de D-galactanas sulfatadas (DGS), extraídas da alga marinha vermelha *Botryocladia occidentalis*, durante a reversão sexual de larvas de tilápia do Nilo, *Oreochromis niloticus*, com o hormônio 17-alfa-metil-testosterona. As DGS foram incorporadas à ração seca antes da adição do hormônio, sendo administradas aos alevinos durante 28 dias. Foram realizados quatro procedimentos experimentais utilizando 0,05, 0,1 e 0,2 mg DGS g^{-1} de peso vivo e um controle sem DGS. Após a 3ª e 4ª semanas do início do experimento, foram observados os maiores valores de peso final e ganho de peso nos peixes que receberam a dose de 0,1 mg DGS g^{-1}. A administração de uma dose mais elevada (0,2 mg DGS g^{-1}) não aumentou o ganho de peso. A taxa de sobrevivência foi baixa em todos os tratamentos, provavelmente devido ao baixo teor de oxigênio dissolvido, uma elevada densidade de estocagem e o escape de larvas. Os resultados obtidos nesse estudo revelaram que a administração de uma baixa quantidade de DGS de *B. occidentalis*, durante a reversão sexual, poderá ser uma ferramenta valiosa para incrementar o crescimento em alevinos de tilápia do Nilo, *O. niloticus*.

Termos para indexação: Tilápia, polissacarídeos sulfatados, sistema imunológico.

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Introduction

Marine algae are rich in carbohydrates, mainly polysaccharides and it is well known that they produce sulfated polysaccharides possessing anticoagulant and antithrombotic activities. Fucans extracted from marine brown (Church et al., 1989; Nishino et al., 1991; Pereira et al., 1999) and red alga (Carlucci et al., 1997; Yamada et al., 2000) have been described as anticoagulant. Furthermore, some of these sulfated polysaccharides have been shown to exhibit many other biological properties such as antiinflamatory, antiviral and antitumoral activities. It has been reported that these effects are probably related to their chemical structure that promotes binding to a large number of proteins (Boisson-Vidal et al., 1995). For instance, Baba et al. (1988) described the antiviral effect of fucoidan, a sulfated polysaccharide extracted from marine brown algae. This polysaccharide inhibits cell invasion by various virus including Herpes virus (HSV-I) and HIV-1 human virus. The same effect was also observed with a sulfated polysaccharide isolated from the blue-green algae *Spirulina platensis* (Hayashi et al., 1996). Moreover, fucoidan has been reported to exhibits an antiinflamatory activity on an experimental model of induced acute peritonitis in rats (Semenov et al., 1998).

In a recent review, Sakai (1999) described the immunostimulant effects in fish and shrimp of some polysaccharides preparations including peptido-glucans, yeast glucans, protein-bound polysac-charides, lentinan, schizophyllan and others. A protein-bound polysaccharide preparation, PS-K, isolated from *Coriolus versicolor* enhanced the resistance of tilapia to bacterial infection through the activation of the non-specific immune system (Park e Jeong, 1996). Miles et al. (2001) showed that an alginate extracted from the marine brown algae *Laminaria digitata* has an immunostimulant activity in striped snakehead *Channa striata* against epizootic ulcerative syndrome. Recently, Campa-Córdova et al. (2002) reported that a sulfated polysaccharide extracted from the blue-green algae *Cyanothece* sp enhances the non-specific immune response in American white shrimp (*Litopenaeus vannamei*). Interestingly, after the administration of a hot water extract of *Spirulina platensis* to human volunteers, Hirahashi et al. (2002) observed an activation of the human innate immune system.

The intensive fish larval rearing process exposes larvae to considerable stresses of a chemical, physical and biological nature. These conditions can be detrimental to the larvae, and this situation is amplified by the fact that stress is a potent suppressor of the immune system of fish (Valdstein, 1997). Another kind of stress is the exposure to sex hormones that occurs in mature fish and in the sex reversal phase in tilapia culture. Therefore, immunostimulants should be used in these situations to overcome immune suppression caused by sex hormones (Sakai, 1999).

Several authors have reported the relationships between immunostimulation and growth-promoting activity. Black tiger shrimp fed with peptidoglycan-supplemented feed or immersed in a glucan solution showed better growth and feed conversion rates than control treatments (Sakai, 1999). Nandeesha et al. (2001) showed that the replacement of fishmeal by more than 25 % with blue-green algae *Spirulina platensis* resulted in significantly superior growth of Indian major carp *Labeo rohita*.

Farias (2000) reported the extraction of sulfated polysaccharides from 54 species of marine algae and tested their anticoagulant activity using clotting assays. The sulfated polysaccharide from *Botryocladia occidentalis* showed the highest anticoagulant activity among the species tested. This sulfated polysaccharides occurs as -4-α-D-Gal*p*-1→3-β- D-Gal*p*-1→, but with a variable sulfation pattern. Clearly, approximately one third of the total α-units is 2,3-di-*O*-sulfated and another one third is 2-*O*-sulfated. This algal sulfated D-galactan has a potent anticoagulant activity similar as unfractionated heparin (Farias et al., 2000) and also exhibits antithrombotic and platelet-aggregation effects (Farias et al., 2001).

In our continuous work on isolation and biological activity of sulfated polysaccharides from marine algae, we have carried out studies to evaluate the effect of the addition of the sulfated D-galactans from the marine red alga *Botryocladia occidentalis* in the ration of Nile tilapia, *Oreochromis niloticus*, during the sex reversal phase with the synthetic androgen 17-alpha-methyl-testosterone.

Material and Methods

Fish

Tilapias, *Oreochromis niloticus,* weighting 300-350 g were used as broodstock. The fish were acclimated to reproduction conditions for 15 days in resting cages and fed twice daily at 0.8% body weight with a 28% crude protein commercial ration. After reproduction, larvae (12 days live) were collected by a 1.5 mm net and placed in a plastic tray water recirculation system.

Sulfated polysaccharides

Sulfated D-galactans (SDG) were extracted from the dried tissue of the marine red alga *Botryocladia occidentalis* as published elsewhere (Farias et al., 2000). Briefly, the dried algae tissue (5 g) was cut in small pieces, suspended in 0.1 M sodium acetate buffer (pH 6.0) containing 510 mg of papain (E. Merck, Darmstadt, Germany), 5 mM EDTA, and 5 mM cysteine, and then incubated at 60°C for 24 h. The incubation mixture was filtrated and sulfated polysaccharides in solution were precipitated with a 10% cetylpyridinium chloride solution. After standing at room temperature for 24 h, the mixture was centrifuged at 2,560 x *g*, for 20 min, at 5°C. The sulfated polysaccharides in the pellet were washed with 0.05% cetylpyridinium chloride solution dissolved with 2 M NaCl, ethanol (100:15, v/v) solution, and precipitated with absolute ethanol. After 24 h at 4°C, the precipitate was collected by centrifugation (2,560 x *g*, for 20 min, at 5°C), washed twice with 80% ethanol, and once with the same volume of absolute ethanol. The final precipitate was dried at 60°C overnight and ~200 mg (dry weight) of crude polysaccharide was obtained after these procedures.

Incorporation of SDG in ration

SDG was dissolved in distilled water and soaked in dried ration to give the final desirable concentrations based on fish weight gain estimated by the Popma e Green equation (Popma and Green, 1990). After dried at 60°C the ration was reduced to a fine powder at the same size of the commercial food.

Incorporation of the androgen 17-alpha-methyl-testosterone

The synthetic androgen 17-alpha-methyl-testosterone was incorporated in the ration after the addition of the sulfated D-galactans at a dosage of 60 mg kg^{-1} dried ration. After diluted in commercial ethanol, the hormone was mixed with the ration and then dried in a shaded place (Popma and Green, 1990).

Experimental and sampling protocols

Fry (23.03 ± 0.13 mg) used for sex reversal were divided into four groups of 500 larvae each and randomly held in 6.3 L plastic trays with three replicates under constant water exchange (2 L h^{-1}). Groups of fry were weighted prior to experiment and the initial weight of fish in each group was not significantly different ($P < 0.05$). The SDG concentrations in the food were 0 (control), 0.05 mg g^{-1}, 0.1 mg g^{-1} and 0.2 mg g^{-1}. Fish were fed each test diet at a rate of 20% body weight per day in the first week, 17% in the second, 15 % in the third and 10 % in the last week of the experiment. In order to ensure the complete ingestion of ration fish were fed four times daily. Larvae were sampled every week to assess weight gain and survival.

Physical-chemical parameters

Water temperature, pH and dissolved oxygen were measured weekly. Conductivity, salinity, ammonia, nitrite, nitrate, phosphate and total iron were measured as described by Boyd (1997), only one time just before the experiment.

Statistical Analysis

All data were subjected to one-way ANOVA and *t*-test (independently). For all tests, a significance level of $p < 0.05$ was regarded as significant.

Results and Discussion

All fish fed with SDG supplemented ration exhibited a higher final weight than those observed in the control. As shown in Figure 1, after 3 weeks of feeding, the mean weight of larvae fed at a dose rate of 0.1 mg SDG g^{-1} body weight (Treatment C) differ significantly ($P < 0.05$) from control without SDG (Treatment A) and from treatments B and D (0.05 and 0.2 mg SDG g^{-1} body weight, respectively). This effect was also observed in the mean weight gain of fish treated with the same dose (Figure 2). However, a higher dose (0.2 mg SDG g^{-1} body weight) did not increase fish growth. Means daily weight gain were 9.23, 10.94, 14.90 and 10.79 mg d^{-1} for treatments A, B, C and D, respectively. As shown in Figure 3, daily weight gain in treatment C was significantly higher then those observed in the other treatments ($P < 0.05$). Therefore, the results indicate that the addition of a sulfated polysaccharide extracted from the red marine alga *B. occidentalis* in tilapia ration may enhance the fish growth during the sex reversal phase.

Growth-promoting activity is considered to be an indirect effect of non-specific immunostimulation by polysaccharides. Furthermore, overdoses of several immunostimulants induce immunosuppression in fish (Sakai, 1999). Boonyaratpalin et al.,

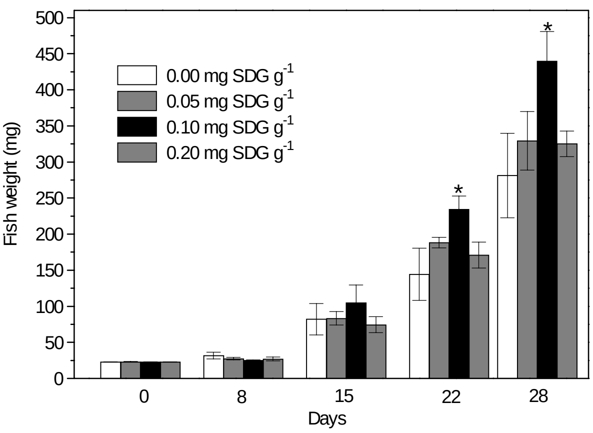

Figure 1 - Weight growth of tilapia, *Oreochromis niloticus*, larvae fed with different doses of sulfated D-galactans (SDG) during the sex reversal phase with 17-alpha-methyl-testosterone. Bars with * are significantly different ($P < 0.05$).

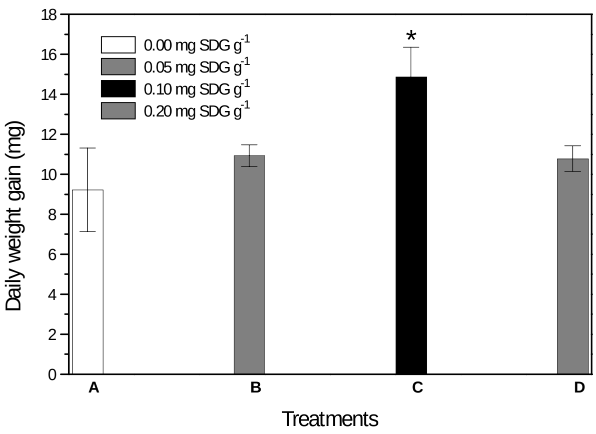

Figure 3 - Daily weight gain of tilapia, *Oreochromis niloticus*, larvae fed with different doses of sulfated D-galactans, SDG, (0.00, 0.05, 0.10, and 0.20 mg SDG g^{-1} in treatments A, B, C, and D, respectively) during the sex reversal phase with 17-alpha-methyl-testosterone. Bar with * is significantly different ($P < 0.05$).

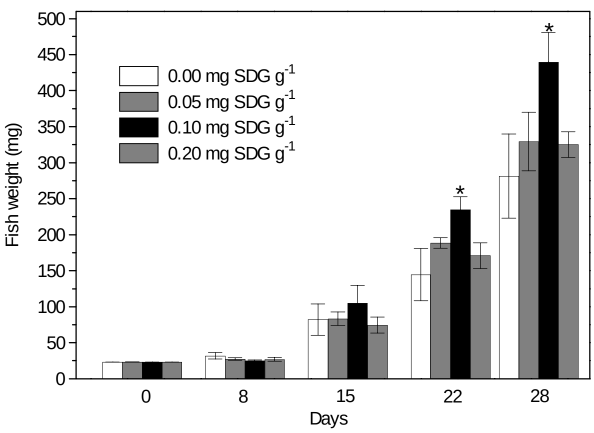

Figure 2 - Weight gain of tilapia, *Oreochromis niloticus*, larvae fed with different doses of sulfated D-galactans (SDG) during the sex reversal phase with 17-alpha-methyl-testosterone. Bars with * are significantly different ($P < 0.05$).

(1995) reported that the shrimp *Penaeus monodon* fed with a peptidoglycan-supplemented feed showed better growth and feed conversion rates than those fed a normal diet. This effect was observed with 0.01% peptidoglycan supplementation, but not with the highest level administered (0.1%). Glucan immersion also increased the growth of the shrimp *Penaeus monodon* (Sung et al., 1994). Park e Jeong (1996) showed the enhanced resistance against *Edwardsiella tarda* infection in tilapia (*Oreochromis niloticus*) by administration of protein-bound polysaccharide. This effect was higher at a dose rate of 0.1 mg PS-K g^{-1} body weight than at 0.9 mg PS-K g^{-1} body weight. Tinman et al. (2000) reported the immunostimulant effect of peptidoglucan in tilapia hybrids (*Oreochromis aureus* x *Oreochromis niloticus*), infected by *Streptococcus difficile*. They observed that fish treated with peptidoglucan exhibited an increase in growth 50% faster than controls groups, and only fish treated with a low dose showed better survival and immunostimulation. All these findings indicate that the administration of these substances is dose-response related.

In the present study a negative effect of SDG treatment was observed when high doses were administered to tilapia larvae. The better growth-promoting activity was only achieved with a determined dose (0.1 mg SDG g^{-1} body weight). In a previous paper, we described the antithrombotic activity of the sulfated D-galactans from *B. occidentalis* in rats. Surprisingly, they showed unexpected dose-response curves when compared with standard heparin. At low dose they prevent thrombus formation but as we increased the dose administered to the animal the antithrombotic activity disappeared (Farias et al., 2001). Then, optimal biological activity of polysaccharides should be achieved with a unique dose. Generally, the better effect is obtained with low doses. Recently, Campa-Córdova et al. (2002) showed that although the dose of sulfated polysaccharide administered in American white shrimp was 500 times lower than that of glucan (not sulfated polysaccharide), the immune responses were similar in both treatments. According to Farias et al. (2000) the addition of two sulfate esters to a single α-galactose residue in *B. occidentalis* sulfated polysaccharides has an «amplifying effect» on its anticoagulant action. Probably, the sulfate groups and/

or the sulfation pattern make these polysaccharides also more effective in the stimulation of the non-specific immune system.

The route of administration seams to be very important when considering larviculture. Although many authors reported that the administration by injection is the most effective method of immunostimulation, this is in fact, an intensive work, relatively time-consuming and becomes impractical when fish weight less than 15 g. Thus, oral administration or immersion should be used and exploited (Sakai, 1999). On one hand, oral administration is an easy method to use in fish larvae because the feed administration is precisely based on fish weight gain. On the other hand, the immersion method should be selected in shrimp larviculture, since it is difficult (and often inexact) to quantify the feed intake in shrimp culture.

In tilapia larviculture, survival is always over than 60% (Popma and Green, 1990; Marengoni, 1999; Kubitza, 2000). Immunostimulation of fish and shrimp also enhances survival, but in our studies we observed a very low survival rate (Table 1). This low survival was indeed expected due to the intentional physical stress created in our experiment in order to suppress fish immune system. However, this high mortality was in fact observed in all treatments, including control (without SDG). In addition, increasing the dose of SDG, the survival rate was not affected. Moreover, survival did not differ among all the treatments ($P < 0.05$). The high mortality observed was probably due to the dissolved oxygen tenor (Table 2), which was not suitable to the high stocking density (80 fry/L) forcing larvae escape. In fact, dissolved oxygen values for aquaculture organisms depends on the stocking density. In our study the average of dissolved oxygen was 3.4 mg/L, which was probably low to support the high initial stocking density. Furthermore, survival rapidly decreases in the first 15 days and then stabilized up to the end of the experiment in 12.6% as average survival in all treatments, during the third and the last week of the experiment (Figure 4). Therefore, this low survival should not be taken as an adverse or a toxic effect of SDG administration.

Conclusion

The results of this present study demonstrated that the orally administration of sulfated D-galactans from the marine red algae *B. occidentalis* to tilapia fry might be a very useful natural substance to improve fish growth during the sex reversal phase.

Table 1 - Average survival (%) of tilapia, *Oreochromis niloticus*, larvae in all treatments (A, B, C, and D) during the sex reversal phase.

Treatments*	Days				
	0	8	15	21	28
A	100.0	86.13	22.80	13.80	10.27
B	100.0	62.33	12.60	10.73	9.33
C	100.0	66.80	10.73	9.13	7.93
D	100.0	82.73	17.80	14.13	11.67

*After 15 days of culture survival was not significantly different among all treatments ($P < 0.05$).

Table 2 - Physical-chemical parameters measured in the culture water of tilapia, *Oreochromis niloticus*, during the sex reversal phase.

Parameters*	Days				
	0	8	15	22	28
Temperature (°C)	28.10	28.60	29.80	28.10	29.30
Dissolved oxygen (mg/mL)	3.68	3.52	3.22	3.56	3.10
pH	7.38	7.84	7.49	7.67	7.69

* Parameters are valid for all treatments.

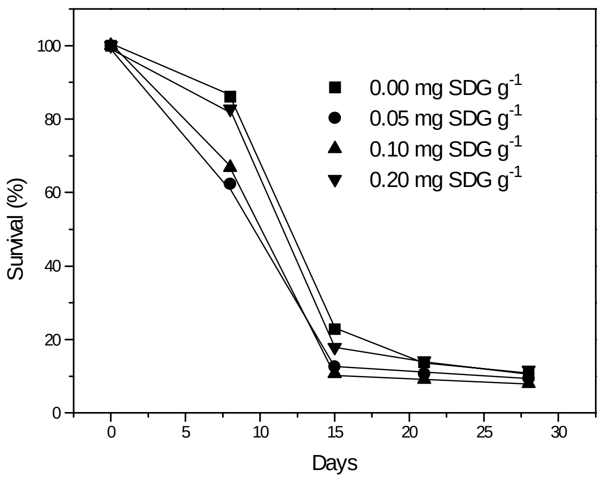

Figure 4 - Average survival of tilapia, *Oreochromis niloticus*, larvae during the sex reversal phase fed with different doses of sulfated D-galactans (SDG). The means are not significantly different ($P < 0.05$).

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