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Inflammatory responses of Nile tilapia *Oreochromis niloticus* to *Streptococcus agalactiae*: effects of vaccination and yeast diet supplement

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ABSTRACT: This study evaluated the effects of dietary supplementation with 0.3% *Saccharomyces cerevisiae* yeast cell wall and of vaccination against *Streptococcus agalactiae* on the cellular component of acute inflammation induced in the coelomic cavity of Nile tilapia *Oreochromis niloticus* and on survival of the fish after challenge. A total of 84 tilapia of mean (±SD) weight 125.0 ± 1.5 g were distributed among twelve 310 l fiberglass tanks according to a 2 × 2 × 3 factorial design in the following manner: with and without supplementation; 2 stimulations (oily solution without *S. agalactiae* vaccine and vaccination); 15 d later all fish were intracoelomically challenged with 10^8 CFU ml^{-1} of a homologous strain of *S. agalactiae*, and evaluated after 6, 24 and 48 h, with 7 replicates. The fish received the non-supplemented or supplemented diet for a total of 77 d. The vaccination was performed on the 60th day, intracoelomically, as a single injection of 0.5 ml of the vaccine containing 10^8 CFU ml^{-1}. Fifteen days later, all the fish were challenged with *S. agalactiae* by means of an intracoelomic inoculation of 10^8 CFU ml^{-1}. No mortality was observed among the supplemented fish. The fish that were fed the non-supplemented diet and immunized with the bacterium presented a mortality rate of 28.5%. Among the non-supplemented and non-immunized fish, the mortality rate was 38.09%. Supplementation, in both vaccinated and non-vaccinated fish, induced larger accumulations of thrombocytes, lymphocytes and macrophages at the inflammatory focus. The results suggest that supplementation with 0.3% yeast cell wall, in both vaccinated and non-vaccinated fish, improved the inflammatory response of the fish and protected against the challenge. Vaccination increased the defense response, but the effect was stronger when associated with supplementation with *S. cerevisiae*.

KEY WORDS: Inflammation · *Saccharomyces cerevisiae* · Vaccination · *Streptococcus agalactiae* · Challenge

INTRODUCTION

Streptococcus spp. are responsible for high morbidity and mortality among various species of fish in different parts of the world (Kitao et al. 1981, Ming-Chen et al. 1985, Al-Harb 1994, Pasnik et al. 2006). In the state of Paraná, Brazil, *Streptococcus agalactiae* is the predominant agent (Salvador et al. 2005), but no data on the extent of the losses attributable to this species have been published.

Food supplementation with the yeast *Saccharomyces cerevisiae* improves the efficiency of the

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defense system of fish, while vaccination is a good alternative to the indiscriminate use of antibiotics and other products for preventing infections in Nile tilapia *Oreochromis niloticus* and pacu *Piaractus mesopotamicus* (Klesius et al. 2000, Freimund et al. 2003, Reque et al. 2010).

In an evaluation of the cellular component in the inflammation induced by thioglycolate, lipopolysaccharide (LPS) and inactivated *Aeromonas hydrophila* in *Piaractus mesopotamicus*, Bozzo et al. (2007) demonstrated that thioglycolate induced the greatest accumulation of total cells 6 h after application and *A. hydrophila* caused the greatest accumulation of total cells after 24 h. The differential count demonstrated that thrombocytes predominated and the numbers of lymphocytes and macrophages were smaller in all of the stimulations used (Bozzo et al. 2007). In Nile tilapia supplemented with *Saccharomyces cerevisiae* and stimulated with inactivated *A. hydrophila*, greater accumulations of thrombocytes were predominantly observed, and smaller numbers of neutrophils, macrophages and lymphocytes were observed at the inflamed focus (Reque et al. 2010).

The present trial evaluated acute inflammation among Nile tilapia that were fed a diet supplemented with 0.3% *Saccharomyces cerevisiae* cell wall, vaccinated against *Streptococcus agalactiae*, and then challenged.

MATERIALS AND METHODS

Storage, experimental design and feed

Eighty-four clinically healthy Nile tilapias (125.0 ± 1.5 g [mean ± SD]), obtained from the Aquaculture Center, State University of São Paulo, were distributed among twelve 310 l fiberglass tanks, each supplied with 300 l of free-flowing water from an artesian well at an output of 1 l min^{-1} with continuous aeration. The water temperature (28.0 ± 1.7°C), the pH (7.3 ± 0.3) and the dissolved oxygen concentration (5.54 ± 0.82 mg l^{-1}) were measured every day. These water quality variables remained within the suitable culture range for the fish (Boyd 1990).

Fish were divided into 4 treatment groups with 3 tanks per treatment and 7 fish per tank. Each of the 3 tanks in each treatment represented a different evaluation time (6, 24 and 48 h). In treatment Group 1 (G1), the diet was not supplemented but the fish were vaccinated against *Streptococcus agalactiae*; in Group 2 (G2), the diet was not supplemented and the fish were not vaccinated; in Group 3 (G3), the diet was supplemented with 0.3% *Saccharomyces cerevisiae* cell wall (Reque et al. 2010) and the fish were not vaccinated; and in Group 4 (G4), the diet was supplemented and the fish were vaccinated. The groups were completely randomized with a 2 × 2 × 3 factorial design: with and without supplementation, 2 stimulations (oily solution without *S. agalactiae* vaccine and vaccination) and 3 evaluation times (6, 24 and 48 h; n = 7 at each evaluation time). The feed (NRC 1993) contained isoprotein (32.0% DP), isoenergy (3200 kcal $kg^{-1}$of DE) and isophosphorus (3.0% P available phosphorus) with the same level of gross fiber (5.0%) (Baccarin & Pezzato 2001) (Table 1).

Table 1. *Oreochromis niloticus*. Percentage chemical–bromatological composition of the experimental diets

Ingredients	Diet composition (%)
Soybean meal	45.00
Corn gluten	9.50
Fish meal	5.50
Corn meal	15.00
Wheat bran	8.00
Broken rice	7.60
Alginate	0.30
Cellulose	1.84
DL-methionine	0.45
Threonine	0.40
Soybean oil	0.50
Dicalcium phosphate	3.00
Limestone	2.00
Vitamin C[a]	0.08
Common salt	0.10
Vitamin/mineral supplement[b]	0.41
β-hydroxytoluene (BHT) as antioxidant	0.02
Autolyzed yeast[c]	0.30
Digestible energy (kcal kg^{-1})	3240.00
Digestible protein (%)	32.00
Gross fiber (%)	5.00
Ether extract (%)	3.00
Ca:P ratio	2.95

[a]Vitamin C, 35.0% active;

[b]Vitamin and mineral supplement (Supremais) kg^{-1} of feed: vitamin A, 1 200 000 IU; vitamin D3, 200 000 IU; vitamin E, 1000 mg; vitamin K3, 2400 mg; vitamin B1, 4800 mg; vitamin B2, 4800 mg; vitamin B6, 48 000 mg; vitamin B12, 4800 mg; folic acid, 1200 mg; pantothenic acid, 12 000 mg; vitamin C, 56 mg; biotin, 48 mg; choline, 65 mg; niacin, 24 000 mg; Fe, 10 000 mg; Cu, 600 mg; Mn, 4000 mg; Zn, 6000 mg; I, 20 mg; Co, 2 mg; Se, 20 mg;

[c]Absent in the control feed

The ingredients were finely ground (0.7 mm), homogenized, pelleted in a pellet mill (model 20A, California Pellet Mill) at 40°C and dried in a heated chamber with forced air circulation (55.0°C, 24 h). The pellets were fractionated into compatible diameters (6 mm) and stored at a temperature of −20.0°C. The fish were fed (3.0% of biomass) ad libitum, 3 times a day for 77 d.

Standardization of the vaccine inoculation and challenge

Streptococcus agalactiae was isolated from the encephalon of tilapias that had been naturally infected, and was identified from its culturing, morphological, tincture and biochemical characteristics (Vandamme et al. 1997, Salvador et al. 2005). The lethal concentration (i.e. test concentration that causes 50% mortality, LC_{50}) and the challenge inoculation dose were previously determined. The mortality rate with a LC_{50} of 10^8 CFU ml^{-1} was analyzed by using the Spearman-Karber software (Hamilton et al. 1977).

For the vaccine, the microorganisms were cultured in 500 ml of brain-heart infusion (BHI) at 29.0°C for 5 d, and were washed 3 times in phosphate-buffered saline (PBS). The bacterial mass was diluted in 100 ml of PBS to contain 10^8 CFU ml^{-1}, i.e. MacFarland grade 8, and was then inactivated in a water bath (30 min, 40.0°C). After confirmation that there had been no activity in BHI over a 7 d period, incomplete Freund's adjuvant was incorporated at proportions of 1:1 followed by emulsification of the solution. The mass was then subjected to the emulation test, and the doses were prepared before the time of immunization.

Vaccination (groups G1 and G4) was carried out after 60 d of feeding, by means of intracoelomic inoculation of 10^8 CFU of *Streptococcus agalactiae* in 0.5 ml of oily solution, as a single injection. In the non-vaccinated groups (G2 and G3), 0.5 ml of oily solution without *S. agalactiae* was injected into the intracoelomic cavity. Fifteen days later (Neumann & Tripp 1986), all the fish were challenged by means of an intracoelomic inoculation of 10^8 CFU of a homologous strain of *S. agalactiae*.

Each treatment was evaluated at 6, 24 or 48 h after the challenge. Each time was represented by 1 tank, each of which contained 7 fish. The efficacy of the vaccination was calculated as the relative percentage survival (RPS) (Amend 1981) as follows:

$$RPS = [1 - (\%\ \text{mortality among the vaccinated fish} / \%\ \text{mortality among the control fish})] \times 100$$

Evaluation of the inflammatory cellular component

After killing the fish in a solution of benzocaine, the coelomic cavity of each fish was washed with 0.5 ml of PBS with 0.01 ml of EDTA at 5%. The same volume was collected and one aliquot was placed in a Neubauer chamber to count the total number of inflammatory cells. For the differential counts of thrombocytes, lymphocytes, macrophages and granulocytes, the exudate was centrifuged at 1000 rpm (3000 × *g*) for 5 min in a clinical centrifuge and the sediment was placed on histological slides, homogenized and a smear prepared. After drying, the smears were fixed in methyl alcohol for 1 min and underwent panchromatic staining with May-Grunwald-Giemsa-Wright stain, and up to 100 cells of the different cell types were counted by means of microscopy (Tavares-Dias & Moraes 2003).

Statistical analysis

The data were analyzed by means of the *F*-test with significance at $p < 0.05$ (Snedecor & Cochran 1974). The mortality data were analyzed with the Kruskal-Wallis non-parametric test. Significant results were analyzed with Tukey's test ($p < 0.05$).

RESULTS

After the challenge, there was no mortality among the fish that had been fed the diets supplemented with *Saccharomyces cerevisiae*, regardless of whether or not they had been vaccinated with *Streptococcus agalactiae* (RPS 100%); relative to mortality among the other fish the difference was significant ($p < 0.05$). Among the non-supplemented fish, the mortality rate of the vaccinated fish (G1) was 28.57% and that of the non-vaccinated fish (G2) was 38.09%, which corresponded to an RPS of 25%, but these rates were not significantly different ($p > 0.05$). The mortality rate was significantly higher ($p < 0.05$) among the non-supplemented fish compared with those fed supplemented diets, regardless of whether they had been vaccinated (Table 2). It was possible to re-isolate *S. agalactiae* (Vandamme et al. 1997, Salvador et al. 2005) from kidneys and brains of all the fish that died.

The exudate cell count demonstrated that there was no significant interference from the triple interaction of the effects of diet, vaccination and time (Table 3). The total number of cells in the

Table 2. *Oreochromis niloticus*. Mortality in Nile tilapia with and without yeast diet supplement (Yeast), and with and without vaccination (Vacc.). Asterisk (*) indicates significant difference according to Tukey's test ($p < 0.05$) between fish on supplemented and non-supplemented diets

Treatment (n = 21)	Yeast	Vacc.	No. (and %) of dead fish
G1	–	+	8 (28.5)*
G2	–	–	6 (38.0)*
G3	+	–	0
G4	+	+	0

interaction of diet and vaccination was greater in the supplemented and vaccinated fish and smaller in the fish that were not supplemented or vaccinated. This number was significantly greater in the fish received the supplemented diet, regardless of whether they were vaccinated. In the non-supplemented fish the greatest number of cells occurred in the vaccinated fish, and there was a significant difference relative to those that were not vaccinated (Table 4).

The fish fed supplemented diet presented greater total numbers of inflammatory cells than fish fed non-supplemented diet in all treatments (Table 4). The total number of cells decreased over time in the vaccinated and non-vaccinated fish. However, regardless of the time of analysis, this value was significantly greater in the vaccinated fish (Table 5).

A greater percentage of thrombocytes occurred in the vaccinated fish. In the fish that were not vaccinated there were no differences ($p > 0.05$) in the cell

Table 3. *Oreochromis niloticus*. Mean total number of cells and mean percentage of cells present in the inflamed focus, in Nile tilapia fed diets supplemented with 0.3% *Saccharomyces cerevisiae* yeast cell wall and vaccinated against *Streptococcus agalactiae*. For each measured variable, within each variation factor, means followed by different superscript letters differ at the 5% probability level. *Differ at 5% with CV. EGC: eosinophil granular cells

Treatments	Variables					
	Mean total cells (n)	Thrombocytes (%)	Neutrophils (%)	Macrophages (%)	Lymphocytes (%)	EGC (%)
With yeast (G3, G4)	1775.47[a]	24.13	1.05	0.45	56.76	17.58
Without yeast (G1, G2)	778.03[b]	21.01	2.53	1.27	64.08	11.08
With vaccine (G1, G4)	1517.64[a]	22.88	1.25	1.04	57.73	17.07
Without vaccine (G2, G3)	1243.19[b]	22.89	2.00	0.53	61.54	13.01
6 h	1648.34[a]	22.94	2.49	0.86	61.48	12.21
24 h	1389.26[b]	23.59	1.16	0.14	66.89	11.18
48 h	1103.75[c]	25.03	1.29	1.32	51.07	21.28
***F*-value for:**						
Yeast (L)	407.37*	0.44	2.01	2.01	1.95	1.82
Vaccine (V)	32.10*	0.00	0.54	0.80	0.55	0.74
Time (T)	42.52*	0.31	0.67	1.46	3.31	1.87
L vs. V	5.45*	4.04*	6.09*	2.12*	1.84*	0.60
L vs. T	39.14*	1.26	3.10*	0.17	4.02*	1.33
V vs. T	5.71*	2.06	1.54	2.46*	5.69*	0.68
L vs. V vs. T	23.34	0.33	0.17	0.55	0.34	0.88
Coefficient of variation, CV (%)	14.7	74.36	61.2	85.77	35.95	131.9

Table 4. *Oreochromis niloticus*. Effect of the interaction between yeast-supplemented diet and vaccination on the values for total cells, thrombocytes, neutrophils, macrophages and lymphocytes. Means followed by different superscript letters (lower case in the same yeast treatment, i.e. with vaccine vs. without vaccine, and upper case between different yeast treatments, i.e. with yeast vs. without yeast) differ at the 5% level

Treatments	Mean total cells (n)	Thrombocytes (%)	Neutrophils (%)	Macrophages (%)	Lymphocytes (%)
With yeast					
With vaccine (G4)	1824.76[aA]	27.84[aA]	4.31[aA]	2.25[aA]	66.84[aA]
Without vaccine (G3)	1726.19[aA]	20.43[aA]	3.65[aA]	0.51[bA]	61.69[bA]
Without yeast					
With vaccine (G1)	1021.53[aB]	26.33[aA]	0.48[aB]	2.09[aA]	61.44[aA]
Without vaccine (G2)	567.00[bB]	14.87[aA]	1.75[aB]	0.56[bA]	52.09[aB]

Table 5. *Oreochromis niloticus.* Effect of the interaction between time and yeast or vaccination on the values for total inflammatory cells and percentages of neutrophils, macrophages and lymphocytes. For each factor, means followed by different superscript letters (lower case in the rows and upper case in the columns) differ at the 5% level

Treatment	Interaction time		
	6 h	24 h	48 h
Mean total cells (n)			
With yeast	2292.85^{Aa}	1655.00^{Ab}	1378.57^{Ac}
Without yeast	645.77^{Ba}	975.88^{Bb}	719.00^{Bc}
With vaccine	1900.00^{Aa}	1520.00^{Ab}	1165.00^{c}
Without vaccine	1417.66^{Ba}	1269.41^{Bb}	1042.50^{c}
Neutrophils (%)			
With yeast	4.80^{Aa}	2.97^{Ab}	0.16^{Ac}
Without yeast	1.00^{B}	0.12^{B}	0.14^{B}
Macrophages (%)			
With vaccine	1.21^{a}	0.18^{b}	2.42^{Ac}
Without vaccine	0.22	0.11	0.47^{B}
Lymphocytes (%)			
With yeast	64.75^{a}	65.61^{a}	39.93^{Ab}
Without yeast	56.41	68.85	66.66^{B}
With vaccine	69.35^{a}	68.58^{a}	37.85^{Ab}
Without vaccine	54.27	66.10	64.26^{B}

counts of those that received different diets. The percentage was greater in the supplemented fish than in the non-supplemented fish (Table 4).

The percentage of neutrophils was higher ($p < 0.05$) in the supplemented fish, and greater values were observed in the vaccinated fish. In the non-supplemented fish, the percentage was greater in the non-vaccinated fish. Regardless of the diet, vaccination did not cause differences ($p > 0.05$) in the percentages of these cells (Table 4). The supplemented fish presented a higher percentage of neutrophils ($p < 0.05$) than that found in the non-supplemented fish, 6 and 24 h after the challenge. In the supplemented fish there was a significant decrease over time, while in the non-supplemented fish there was no difference ($p < 0.05$) (Table 5).

The percentage of macrophages was greater ($p < 0.05$) in the vaccinated fish, regardless of the diet. In the non-vaccinated fish the percentage was greater in the non-supplemented fish (Table 4). This percentage decreased ($p < 0.05$) in the vaccinated fish between 6 and 24 h and increased ($p < 0.05$) during the 24 to 48 h period. In the fish that were not vaccinated there was no difference over time ($p > 0.05$). The percentage of macrophages was greater in the vaccinated fish than in the non-vaccinated fish only after 48 h ($p < 0.05$) (Table 5).

The highest percentage of lymphocytes occurred in the vaccinated and supplemented fish. The smallest percentage occurred in the non-supplemented and non-vaccinated fish. In the non-vaccinated fish those that were supplemented had a significantly ($p < 0.05$) higher percentage of lymphocytes (Table 4). In the supplemented fish there was no difference between 6 and 24 h, but there was a decrease ($p < 0.05$) after 48 h. In the vaccinated fish, the decrease ($p < 0.05$) was greater after 48 h (Table 5).

DISCUSSION

There was no mortality among the supplemented fish, which suggests that the defense mechanisms in these fish were more efficient. Lower percentage mortality among immunosuppressed fish caused by supplementation with cell wall material from *Saccharomyces cerevisiae* has been described in rohu *Labeo rohita* (Sahoo & Mukherjee 2001) and salmonids infected with *Vibrio anguillarum* (Burrells et al. 2001) and other forms of infection or inflammation (Sakai et al. 2001, Ortuño et al. 2002, Li & Gatlin 2004, Li et al. 2004, Reque et al. 2010).

In this trial, the lower mortality in non-supplemented and vaccinated fish, in comparison to the non-supplemented and non-vaccinated fish, suggests that the vaccine improves the resistance to the challenge. This corroborates previous observations (Klesius et al. 2000, Pasnik et al. 2006). Nevertheless, the low RPS observed in the fish fed with a non-supplemented diet and vacccinated with *Streptococcus agalactiae* (25%), relative to the fish fed diets supplemented with *Saccharomyces cerevisiae* and vaccinated (100%), indicates that supplementation with *S. cerevisiae* alone was capable of protecting the fish against the challenge.

In this study, inoculation of *Streptococcus agalactiae* into the coelomic cavity caused progressive total cell accumulation. This was greater in the groups of supplemented fish, and a favorable effect was observed in relation to inflammation and resistance, which corroborates results of others (Esteban et al. 2000, Ortuño et al. 2002, Reque et al. 2010).

Thrombocytes accumulated at the inflamed focus in all of the groups because in fish, these cells have defensive functions. *Staphylococcus aureus* can be phagocytized *in vitro* by thrombocytes in the exudate of carp *Cyprinus carpio* (Suzuki 1986), as can *Edwardsiella tarda* in Japanese eel *Anguilla japonica* (Kusuda & Ikeda 1987). Tavares-Dias et al. (2007) showed endocytosis and digestion of blood

cellular debris in *Colossoma macropomum* thrombocytes. Matushima & Mariano (1996) and Martins et al. (2006) confirmed the defense function of thrombocytes.

In this trial, the fish receiving the supplemented diet presented a greater number of neutrophils than did the non-supplemented fish, regardless of vaccination. These yeast cells have nonspecific cytotoxic activity (Sasaki et al. 2002) and it is possible that the increased resistance through the action of the yeast cell wall is due to nonspecific inflammation. This is attributable to the neutrophils, thrombocytes and macrophages.

There was a decrease in the accumulation of neutrophils after 24 h, which may be due to pathogen virulence factors (Fearon & Locksley 1996). At this time there was an increase in the number of macrophages in the vaccinated fish, regardless of the diet. Thus, it is possible that vaccination contributes to this increase in the later phases.

The greatest number of lymphocytes occurred among the cell-wall supplemented and vaccinated fish, which suggests that there was a positive synergistic effect. Klesius et al. (2000) and Shelby et al. (2002) demonstrated an increase in the number of lymphocytes among tilapias vaccinated against *Streptococcus iniae*. In the present study, the number of lymphocytes increased in the fish that received vaccination alone, but not as much as in the fish that received both *Saccharomyces cerevisiae* supplementation and vaccination.

When fish diets are supplemented with vitamin C (Gill 1991, Moraes et al. 2003, Petric et al. 2003), vitamin E (Ortuño et al. 2003, Belo et al. 2005) or yeast cells (Jeney et al. 1997, Reque et al. 2010), it is possible that the fish can reach a better homeostatic level, thereby reducing the release of cortisol and thus allowing inflammation and resistance to occur.

The present results suggest that diet supplementation with 0.3% *Saccharomyces cerevisiae* cell wall can, on its own, increase the inflammatory response and the resistance of the fish to the challenge, thus giving 100% protection. Vaccination also has a positive effect and, when used on its own, it protected 75% of the fish against the same challenge. Diet supplementation and vaccination together also protected 100% of the fish through improvement of the inflammatory cell response.

Nevertheless, other elements of the response that were not evaluated in this trial, such as the effect of lysozymes, the complement system and the acute-phase proteins, should be taken into consideration. In any case, accumulation of leukocytes and thrombocytes in the inflamed focus is fundamental in the process. This represents the resultant effect from a highly complex system that involves multiple actions by several pharmacological mediators of cellular and plasmatic origin, as well as hormonal and non-hormonal modulators. Detailed investigation on this system remains a topic for future research.

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