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Probiotic expressing heterologous phytase improves the immune system and attenuates inflammatory response in zebrafish fed with a diet rich in soybean meal

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ABSTRACT

Although aquaculture is among the fastest growing food production sectors in the world, one of the bottlenecks for the continuity of its expansion is the dependence of animal protein on commercial feed formulations. Vegetable proteins are an alternative due to the low cost and high availability. However, this protein source is accompanied by a series of antinutritional and pro-inflammatory compounds, including phytate. Phytases can be added in feed for phytate degradation and increase nutrient availability. However, the use of purified phytases significantly increases the production costs. An interesting alternative is to use probiotics genetically modified as bioreactors for phytase production. In the present study, a strain of *Bacillus subtilis* secreting a fungal phytase was used to evaluate the effect of a feed with high content of soybean meal on zebrafish (*Danio rerio*). We analysed the condition factor (K) of fish, and the expression of genes related to the immune system, inflammatory response and oxidative.

stress. The results obtained demonstrate that the transgenic probiotic was efficient in improving the fish condition factor, stimulating the immune system, reducing the inflammatory response and oxidative stress. Thus, probiotics acting as phytase bioreactors can be considered an interesting tool for the adaptation of commercial species to feed of lower cost.

1. Introduction

Fishmeal is the main source of protein in aquaculture feeds and the increase in demand from the rapid expansion of this sector of food production has made this input expensive and limited. Thus, reducing the use of fishmeal has become a crucial issue for the development of aquaculture production [1,2]. Soy protein is widely investigated as an alternative substitute for fishmeal, and this is favoured mainly by a wide range of soybeans in recent years, tied to its reasonable cost, the constant supply and its attractive nutritional characteristics such as high protein content, digestibility and balanced amino acid levels [3]. However, the inclusion and substitution levels of soy-based ingredients is limited for the various species of fish because soy is composed of several antinutrients such as phenolic compounds, saponins, glucosinolates, oligosaccharides, antigenic proteins (for example, glycine and β-conglycinin) and phytates [3,4].

Studies indicate that the inclusion of soybean meal in feed adversely influences fish health, triggering disturbances in immune function, inflammatory process and decreased growth [4–7]. In Japanese sea bass (*Lateolabrax japonicus*), for example, the replacement of fishmeal with soybean meal in the diet resulted in high expression of intestinal pro-inflammatory genes [6]. In zebrafish larvae (*Danio rerio*), Hedrera et al. [8] indicated that antinutrients present in soybean meal supplemented the diet developed intestinal inflammation. In some cases, supplementation of microorganisms such as microalgae or substances such as lactoferrin, butyrate and taurine in feed can minimize the inflammatory effects in fish fed with soy-based ingredients [5,9,10].

Phytate is a known antinutrient in diets with soy-derived ingredients. It is resistant to heat and even after application of various processing methods in soybean, it is still possible to observe phytate levels ranging from 2 to 18 g/kg [3]. This antinutrient is able to chelate minerals and also affect the availability of proteins, amino acids and

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digestive enzymes in the animal organism [11]. One way to minimize the phytate negative effects is to add the phytase enzyme in the diets, which catalyzes the hydrolysis of phytic acid and its salts (phytates), generally producing inositol, monophosphate and inorganic phosphate [12,13]. Due to these characteristics, phytase represents a product widely used in animal feed [14].

Fungal phytases are widely used commercially because of their excellent biochemical and catalytic characteristics. In addition to the phytate hydrolyzing function, phytase appears to have indirect effects on important physiological systems in fish. Lazado et al. [15] showed, through an *in vitro* assay, that phytase was able to boost the immune response in renal leukocytes of *Gadus morhua*. Hu et al. [16] observed that phytase induced anti-inflammatory responses in tilapia. These evidences indicate that phytase also has a potential action on the immune system and anti-inflammatory response in fish. However, the relationship between these immune responses and feeding with high soybean diet is to must be analysed.

The production and purification of phytases is an expensive and laborious process. An alternative is the production of recombinant phytases using an expression system in bioreactors such as *Escherichia coli*, for example. However, as the enzyme's destination is food, gram-negative bacteria such as *E. coli* are not the best models of heterologous protein expression since they can produce toxins that can contaminate the feed and render commercial production unfeasible. The use of gram-positive bacteria with probiotic characteristics such as *Bacillus subtilis* seems to be the best option because this type of bioreactor can be incorporated into the feed and produce the enzyme directly in the lumen of the host digestive tract, saving the production and purification steps that represent a very high cost. *Bacillus subtilis* compose a group of probiotics commonly used in aquaculture as a source of protein or in the fermentation of plant ingredients [17–19]. On the other hand, genetic engineering of *B. subtilis* has advanced considerably and made this probiotic a potential bioreactor for heterologous expression of proteins [20].

Zebrafish (*Danio rerio*) is an interesting model for physiological studies, which may encompass several molecular and cellular mechanisms, including immunological and anti-inflammatory responses [21–23]. Zebrafish demands high protein content in the composition of their diets, with fishmeal as the main source of protein [24,25]. In this context, the objective of this study was to evaluate the effect of a diet rich in soybean meal supplemented with the probiotic *Bacillus subtilis* KM0 expressing a fungal phytase in the zebrafish immune, anti-inflammatory and antioxidant responses.

2. Material and methods

2.1. Preparation of probiotic strains

The strain of *B. subtilis* KM0/Phy-Af from the collection of the Laboratory of Molecular Biology of the Federal University of Rio Grande (FURG, Brazil) was used as a probiotic phytase producer. This strain was previously transformed with a replicative plasmid for gram-positive bacteria kindly provided by Dr. Josef Altenbuchner (University of Stuttgart, Germany). The plasmid has an origin of replication for *Bacillus* and expresses the phytase gene from *Aspergillus fumigatus* fused to the signal peptide of the SacB gene (levansucrase) from *B. subtilis*. *Bacillus subtilis* stationary phase promoter (P_{ylb}) was used for the expression of the target gene [26]. For the selection of the transformants, the plasmid contains the spectinomycin resistance gene.

A single colony of strain KM0/Phy-Af was inoculated into 10 mL of Luria Bertani medium (LB) under constant stirring at 250 rpm at 37 °C for 16 h. After, the culture was centrifuged at 8000 × *g* for 10 min at 4 °C. The supernatant was discarded and the bacterial pellet washed with sterile saline (0.85% NaCl), and re-centrifuged under the above conditions. The pellet was resuspended with sterile water and sprinkled on the feed. Then, the feed was oven dried at 37 °C for 1 h. Bacterial counts were performed to determine the colony forming units (CFU) per gram of feed. The same procedure was performed for the non-transgenic KM0 strain, which served as the experimental control.

2.2. Phytase activity

B. subtilis KM0 and the transgenic KM0/Phy-Af were screened for their ability to produce phytase on LB agar plates supplemented with 2 mM phytic acid using the two-step counter-staining treatment as described by Bae et al. [27]. Phytase activity was observed by the formation of light zones around the colonies, indicating phytate hydrolysis. The observation was performed daily and followed for a period of 8 days.

Phytase activity was also measured in the culture medium as the fungal phytase used was fused to the SacB signal peptide. For this, the cultures were grown to the stationary phase (approximately 16 h), and centrifuged at 8000 × *g* for 30 min at 4 °C. The supernatant was collected and analysed for phytase activity by the method proposed by Heinonen and Lahti [28], in which 1 M sodium phytate ($C_6H_6O_{24}P_6Na_{12}$; Sigma-Aldrich) buffered with 2 M sodium acetate (pH 5.0) is used as substrate. The reaction occurred for 30 min at 40 °C. The released inorganic phosphate was analysed by the addition of acetone, 5 M sulfuric acid and 10 mM ammonium molybdate (2: 1: 1, v/v). After 30 s, the reaction was stopped by the addition of 1 M citric acid and the released inorganic orthophosphate was measured in spectrophotometer (Absorbance = 405 nm). Five repetitions were used for the assay. A unit of phytase activity was defined as the amount of enzyme required to release 1 μmol phosphate per minute under the assay conditions.

2.3. Feed formulation

A basal diet was formulated to contain soybean meal as the main protein source. The diet consisted of 40.84% of protein, of this total 73.5% was derived from vegetable ingredients, soybean meal being the most abundant vegetable ingredient (Table 1). All dry ingredients were a mixture. The oil and water were added further. The diet was forced

Table 1
Basal diet used to fed zebrafish (*Danio rerio*).

Ingredients	%
Fish meal	2.0
Casein[a]	10.0
Soybean meal	50.0
Maize starch	5.0
Wheat bran	8.0
Fish oil	6.0
Mineral and vitamin blend[b]	2.0
Meat and bone meal	4.0
Corn gluten (prothenosis)[c]	13.0
Total	100.0
Proximate composition	
Crude protein	40.8
Crude fat	8.7
Non-nitrogenous extract (NNE)d	44.8
Crude ash	5.7

[d]Calculated. NNE = 100 – (crude protein + crude fat + crude ash).

[a] LabSynth, SP, Brazil.

[b] Premix M. Cassab, SP, Brazil. Vitamin A (500.000 Ul/kg), Vit. D3 (250.000 Ul/kg), Vit. E (5000 mg/kg), Vit. K3 (500 mg/kg), Vit. B1 (1000 mg/kg), Vit. B2 (1000 mg/kg), Vit. B6 (1000 mg/kg), Vit. B12 (2000 mcg/kg), Niacin (2500 mg/kg), Calcium pantothenate (4000 mg/kg), Folic acid (500 mg/kg), Biotin (10 mg/kg), Vit. C (10000 mg/kg), Choline (100.000 mg/kg), Inositol (1000 mg/kg), Selenium (30 mg/kg), Iron (5000 mg/kg), Copper (1000 mg/kg), Manganese (5000 mg/kg), Zinc (9000 mg/kg), Cobalt (50 mg/kg), Iodine (200 mg/kg).

[c] Ingredion, SP, Brazil.

through a meat grinder and dried at 60 °C. The diet was crushed into the desired size to feed the fish. The diet proximal composition (protein, lipid and ashes) was determined in accordance with the AOAC [29].

2.4. Zebrafish assay

We used 120 fish with a final weight of 246.91 ± 7.60 mg and a final length of 2.56 ± 0.04 cm (data are presented as mean values ± SEM), distributed in 12 glass aquaria (5 L) at the density of 2 fish/L, as recommended by Schneider et al. [30]. The fish were acclimated in the experimental environment for 15 days, the fish were fed the commercial Tetra Color Bits (47.5% protein) and were fasted for 24 h before the start with the experimental diet. The experimental design was completely randomized, consisting of 2 groups. The groups were designated control (basal diet supplemented with the non-transgenic strain *B. subtilis* KM0) and Phy-Af group (basal diet supplemented with *B. subtilis* KM0/Phy-Af). Following the recommendations of Xu et al. [31], the dose of *B. subtilis* was 10^{10} CFU/g of feed. According to the authors, this dosage administered orally is sufficient to colonize the intestinal tract of the zebrafish. The daily portion of the feed was supplemented with the probiotic and administered to the fish twice daily.

The temperature of the aquaria was maintained at 28 °C, and the aquariums were equipped with a biofilter system. About 50% of the aquaria water was changed every two days. The experiment lasted 30 days. To calculate the condition factor (K), we used the Fulton equation $K = 100\ (W/L^3)$, where W = weight and L = standard length. At the end of the experiment, the weight (mg) and length (mm) of all fish were determined. All fish were photographed for length determination by image analysis [32,33] using ImageJ software (Image processing and analysis in Java, http://rsb.info.nih.gov/ij).

2.5. Tissue collection, RNA extraction and cDNA synthesis

At the end of the experimental period, the fish were fasted for 24 h. For dissection of the tissues (kidney and intestine), ten fish per treatment were randomly selected from each replica and euthanized with tricaine methanesulfonate lethal dose (500 mg/L). Total RNA was isolated from tissues shortly after dissection using the TRIZOL reagent (Life Technologies, Brazil), following the manufacturer's recommendations. Total RNA was treated with the RNAse free DNAse I kit (Invitrogen, Brazil) and spectrophotometrically quantified (BioDrop, Isogen Life Science, B·V, Veldzigt, Netherlands). The quality and integrity of total RNA was analysed by 1% agarose gel electrophoresis. The cDNA was synthesized using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Brazil), following the manufacturer's instructions.

2.6. Gene expression

The analysed genes were previously selected in GenBank (https://www.ncbi.nlm.nih.gov), with the specific primers designed by GenBank Primer-Blast tool (Table 2). Quantitative analysis of the genes was performed by quantitative real-time PCR (qRT- PCR). Each sample ($n = 10$) was analysed in duplicate. The 7500 Real-time PCR system (Applied Biosystems, Brazil) was used for qRT-PCR reactions. The cDNA was diluted 5x and used according to the recommendations of the PowerUP SYBR Green Master Mix kit (Applied Biosystems, Brazil). The efficiency of the primers was measured by qRT- PCRs with serial dilutions of the cDNA. The conditions of the reactions used in the qRT- PCR assays were: 50 °C/2 min, 95 °C/2 min, followed by 40 cycles at 95 °C/15 s and 60 °C/15 min. The dissociation curve was performed at 95 °C/15 s, 60 °C/1 min and 95 °C/30 s. The genes analysed in the kidney were those related to the immune response: *cd4* and *ikzf1*. Genes related to the inflammatory response (*ifnphi1*, *ifng*, *tnf-α* and *il1b*) and the antioxidant response (*sod1*) were analysed in the intestine. For the normalization of the expression data, the *ef1α* (elongation factor 1 alpha), *actb1* (β-actin) and *rpl13a* (ribosomal protein L13 alpha) (Table 2) genes were used. All normalizing genes were evaluated by the geNorm VBA applet for Microsoft Excel software [34].

2.7. Ethics statement

Zebrafish cultivation and experimental procedures were approved by the Ethics Commission on Animal Use (CEUA) of the Federal University of Rio Grande (FURG), under approval number P002/2019.

2.8. Statistical analyzes

The normality of the data was previously tested. The condition factor (K) and phytase activity were statistically analysed by the Student *t*-test for independent samples. The gene expression was analysed by the $2^{-\Delta\Delta CT}$ method, and the data were transformed into $\log_{10}$ and expressed as mean (± SEM) of $-1/\log_{10}$ of $2^{-\Delta\Delta CT}$ [35]. $P < 0.05$ was considered statistically significant.

3. Results

Fig. 1 shows the higher activity of phytase from KM0/Phy-Af when compared with the KM0 strain. The enzymatic assay in liquid medium detected significantly higher phytase activity ($P < 0.05$) in transgenic *B. subtilis* (69.37 ± 5.06 U mL^{-1}) when compared to non-transgenic control (25.74 ± 4.29 U mL^{-1}).

The changes in the condition factor (K) related to the different foods used and the treatments with the two types of probiotics supplemented (transgenic and non-transgenic) are presented in Fig. 2. The fish in the control group showed a significant decrease in K when comparing the beginning (1.93 ± 0.07) with the end of the experiment (1.43 ± 0.13). The K of the fish of the Phy-Af group showed no significant difference when comparing the initial condition (1.73 ± 0.07) to the final period (1.55 ± 0.14).

The expression of *cd4* and *ikzf1* in controls was 0.17 ± 0.04 (Fig. 3A) and 0.16 ± 0.04 (Fig. 3B), respectively. In the Phy-Af group the expression of *cd4* was 0.71 ± 0.27 (Fig. 3A) and *ikzf1* 1.02 ± 0.37 (Fig. 3B), significantly higher than in the controls.

Expression of genes related to viral response is not a zebrafish problem based on the soybean diet but was significantly decreased ($P < 0.05$) in the Phy-Af group, where the diet was supplemented with fungal phytase expressing probiotic transgenic (Fig. 4). More specifically, the expression of *ifnphi1* in the controls was 0.64 ± 0.03, while in the Phy-Af group it was 0.03 ± 0.00 (Fig. 4A). For the *ifng* gene, control = 0.64 ± 0.23; Phy-Af = 0.03 ± 0.00 (Fig. 4B). For *tnf-α*, control = 0.30 ± 0.10; Phy-Af = 0.03 ± 0.01 (Fig. 4C). For *il1b*, control = 0.68 ± 0.06; Phy-Af = 0.05 ± 0.01 (Fig. 4D).

Regarding the antioxidant defenses, the gene coding for superoxide dismutase (*sod1*) was analysed in the zebrafish intestine. The results of the expression analysis are presented in Fig. 5, where *sod1* presented values of 0.23 ± 0.02 for the control group and 0.16 ± 0.02 for the Phy-Af group.

4. Discussion

In the present study, *B. subtilis* genetically modified to express a fungal phytase significantly influenced the overall health status of zebrafish fed with a soybean meal rich diet. The detection of high phytase activity in the culture medium showed that the signal peptide fused to the gene of interest increased the secretion capacity of the recombinant enzyme. Fig. 2 shows the evolution of the condition factor (K) of the fish fed with the commercial diet and the subsequent drop in this parameter from the substitution by the diet rich in soybean meal. The diet change impacted the zebrafish differently, where the control group presented a decrease in K of 26%, which was statistically

Table 2
Genes and primers used in qRT- PCR analyses.

Gene symbol	Sequence (5′-3′)	Efficiency (%)	GenBank accession
Normalizing genes			
ef1α	F: 5′-CAAAATTGGAGGTATTGGAACTGTAC-3′ R: 5′-TCAACAGACTTGACCTCAGTGGTT-3′	89	NM131263
rpl13a	F: 5′-TCTGGAGGACTGTAAGAGGTATGC-3′ R: 5′-AGACGCACAATCTTGAGAGCAG-3′	89	NM212784
actb1	F: 5′GCTGTTTTCCCCTCCATTGTT-3′ R: 5′-TCCCATGCCAACCATCACT -3′	99	NM131031
Target genes			
ikzf1	F: 5′-GCCGACATGGTGGTCAGCCC-3′ R: 5′-GTGCTCTGCGGCGCTGTCTT-3′	84	NM130986
cd4	F: 5′-GGTTCTGGTGCCACTGATCATTGG-3′ R: 5′- AGAGGCTGCCGCATGGATCTCA-3′	84	NM001366061
il1b	F: 5′-CCACGTATGCGTCGCCCAGT-3′ R: 5′-GGGCAACAGGCCAGGTACAGG-3′	93	NM212844
tnf-α	F: 5′-TGAGCCATGCAGTGATGCGCT-3′ R: 5′-TCCTGCGTGCAGATTGAGCGG -3′	97	NM212859
ifnphi1	F: 5′- AGCATGGGAGCAGATCCGGA-3′ R: 5′- TGACCCTTGCGTTGCTTGCGA-3′	98	NM207640
ifng	F: 5′- ACGCTTGCAAAGGATTGGGTTGG-3′ R: 5′-ACACAGCCTGGCAAGTGCAGG -3′	91	NM001020793
sod1	F: 5′-CACCGTCTATTTCAATCAAGAGG-3′ F: 5′-AGAATGTTGGCCTGACAAAGTTA-3′	82	NM131294

significant ($P < 0.05$). On the other hand, the group treated with the transgenic probiotic had a K loss of approximately 10%, which was not statistically significant. These results demonstrate that the phytase produced by the probiotic mitigates the negative impact of a diet with high vegetable content.

The K result is related, in part, to the immune responses observed in fish treated with *B. subtilis* expressing heterologous phytase. Among the analysed genes *cd4*, is expressed in the T-helper lymphocytes ($CD4^+$) performing the important function in activating and stimulating other leukocytes to multiply and attack antigens, and coordinating the immune response by the release of cytokines [36]. Fig. 3A shows that the probiotic expressing phytase induced a 4.4 fold increase in *cd4* expression relative to the control group. Another gene related to the immune system was *ikzf1*. This gene encodes for a zinc finger protein, which is involved not only in the regulation of lymphopoiesis [37–40] but also in a wide range of processes such as apoptosis, cell cycle arrest, proliferation and differentiation [41]. Fig. 3B shows that this gene was strongly upregulated (6.4 times) in the zebrafish group treated with the transgenic probiotic. The increase in expression of genes like *cd4* and *ikzf1* is a strong indication that the phytase produced by *B. subtilis* is causing a major effect on the immune system of zebrafish, through the production of lymphocytes. The results observed here are corroborated by Lazado et al. [15], which showed that phytase was able to boost the immune response in renal leukocytes of Atlantic cod *Gadus morhua*. Likewise, Liu et al. [42] showed that the percentages of $CD4^+$ and $CD8^+$ T cells were increased by the addition of phytase in the broiler feed.

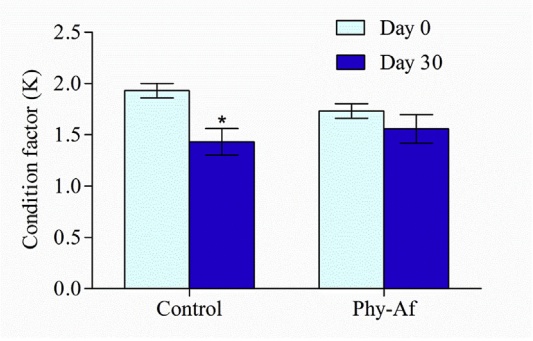

Fig. 2. Condition factor (K) of the zebrafish (*Danio rerio*) fed a diet rich in soybean meal. In the control group, the feed was supplemented with *B. subtilis*, and in the Phy-Af group, the feed was supplemented with *B. subtilis* expressing *A. fumigatus* phytase (Phy-Af). Day 0 refers to the analysis of biometrics data (weight and length) shortly after the end of the acclimation period with commercial feed, and day 30 refers to the analysis of the biometrics data (weight and length) shortly after 30 days of feeding with the experimental diet. Data are expressed as means ± SEM. Significant differences are indicated by an asterisk (*) (Student's t-test, $p < 0.05$).

The second group of genes analysed here involves the inflammatory response. These genes were selected by the fact that soybean meal has a number of antinutrients that can cause inflammation in the intestine.

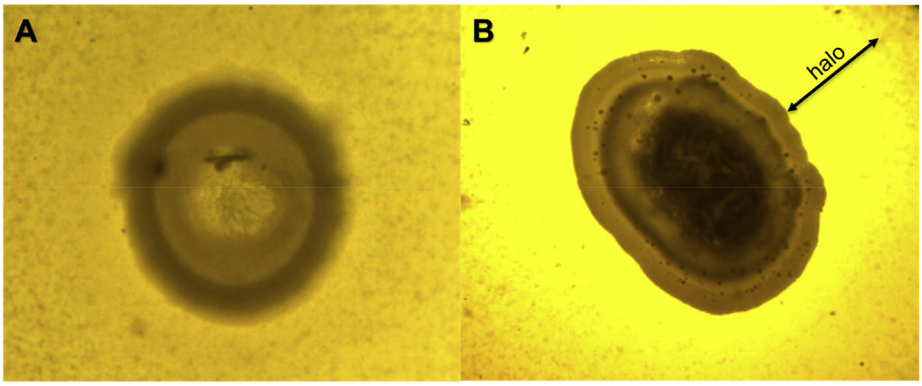

Fig. 1. Identification of phytase activity by halo formation around the colonies of *B. subtilis* (A) KM0 and (B) KM0/Phy-Af. 10X magnification.

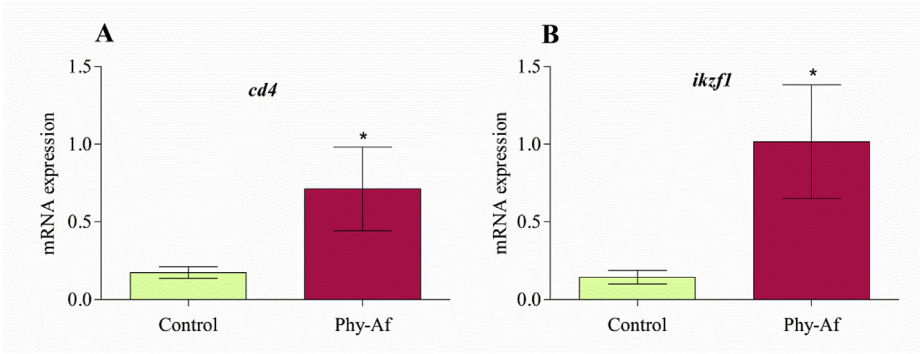

Fig. 3. Expression of genes related to the immune system (A: *cd4*; B: *ikzf1*) in the zebrafish kidney (*Danio rerio*). Control: fish fed with a diet supplemented with non-transgenic *Bacillus subtilis*. Phy-Af: fish fed a diet supplemented with *B. subtilis* transgenic expressing *Aspergillus fumigatus* phytase. Values are expressed as means ± SEM (n = 10 individuals/treatment). Significant differences are indicated by an asterisk (*) (Student's *t*-test, $p < 0.05$).

Fuentes-Appelgren et al. [43] have shown that the inclusion of soy-derived components at high levels in the diet of zebrafish larvae not only stimulates the immune system but also produce inflammatory signals that lead to the development of intestinal enteritis. The authors attributed this inflammatory process not to phytate, but to the presence of saponins. These compounds are amphipathic glycosidic molecules, which contain a hydrophobic aglycone to which one or more hydrophilic sugar chains are attached. The presence of saponins in commercial diets has also been linked to intestinal inflammation in cultured fish such as salmon, carp and flounder [44–46].

The genes related to the inflammatory response analysed here were inf*1*, *infg*, *tnf-α* and *il1b*. Interferons (inf*1* and *infg*), interleukin (*il1b*), and tumour necrosis factor (*tnf-α*) are proinflammatory cytokines that play a critical role in host resistance predisposed to infection, acting as immunomodulators and mediators of inflammatory responses [47,48]. Fig. 4 (A, B, C and D) shows that the expression of these four genes had a drastic decrease in the group of zebrafish treated with the diet supplemented with the transgenic probiotic. A 95% reduction for inf*1*, 95% for *infg*, 90% for *tnf-α* and 93% for *il1b* was observed. These results indicate that the phytase produced by the probiotic drastically reduced the inflammatory process induced by the diet with high soybean meal content. Taking into account the findings of Fuentes-Appelgren et al. [43], the question that arises is: how would phytase be reducing the pro-inflammatory effect of saponins? Although phytases are not enzymes with high substrate specificity, it is virtually impossible to hypothesize that phytase would be promoting the direct degradation of saponins. The most reasonable explanation may come from the study of Liu et al. [42], which analysed the effects of phytate and phytase on chicken immune functions. These authors suggest that phytate complexed with amino acids and proteins are refractory to digestion and lead to increased secretion of mucins by the gastrointestinal epithelium. These mucins are high molecular weight proteins that serve to protect

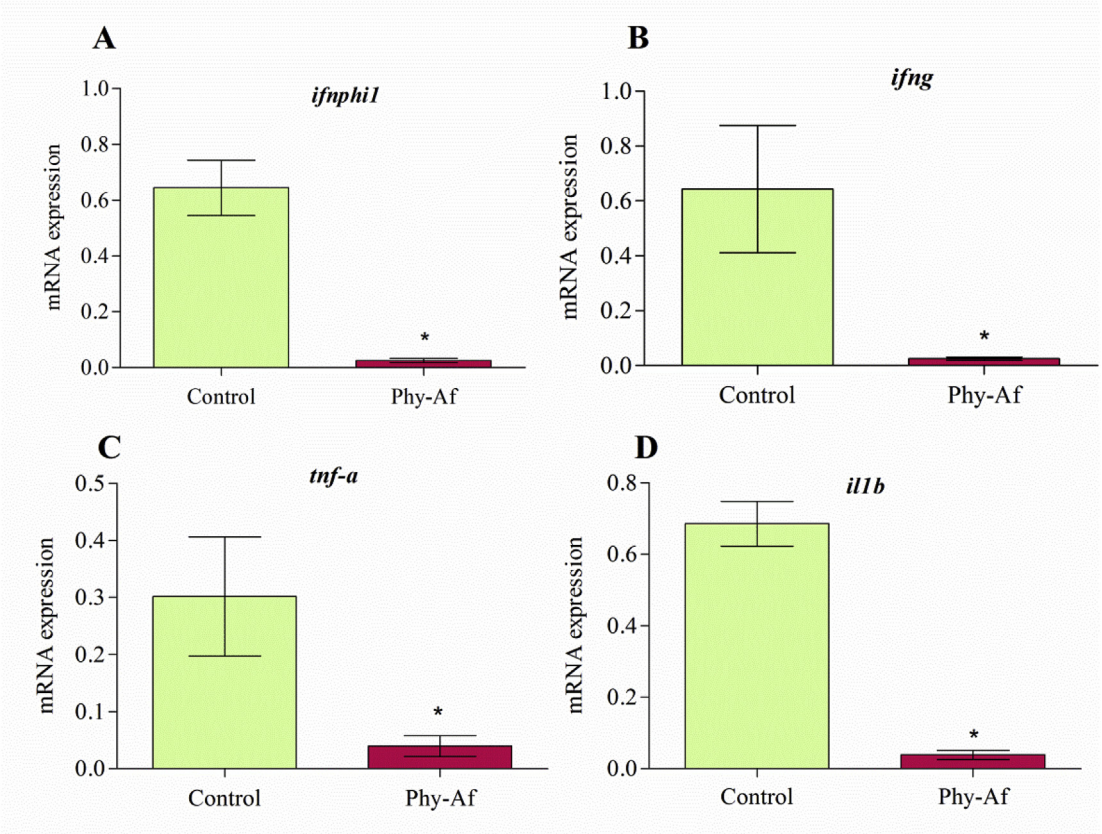

Fig. 4. Expression of the genes related to the inflammatory response (A: *ifnphi1*; B: *infg*; C: *tnf-α*; D: *il1b*) in the zebrafish intestine (*Danio rerio*). Control: fish fed with a diet supplemented with non-transgenic *B. subtilis*. Phy-Af: fish fed a diet supplemented with *B. subtilis* transgenic expressing *A. fumigatus* phytase. Values are expressed as means ± SEM (n = 10 individuals/treatment). Significant differences are indicated by an asterisk (*) (Student's *t*-test, $p < 0.05$).

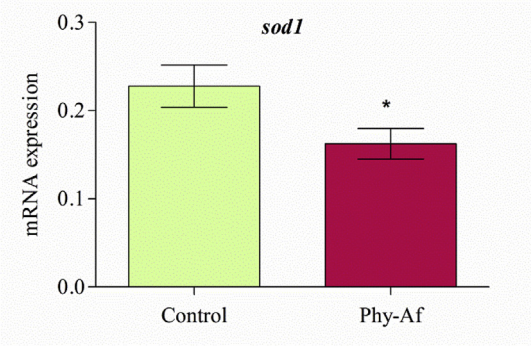

Fig. 5. Expression of the gene related to the antioxidant defenses (*sod1*) in the zebrafish intestine (*Danio rerio*). Control: fish fed with a diet supplemented with non-transgenic *B. subtilis*. Phy-Af: fish fed a diet supplemented with *B. subtilis* transgenic expressing *A. fumigatus* phytase. Values are expressed as means ± SEM (n = 10 individuals/treatment). Significant differences are indicated by an asterisk (*) (Student's *t*-test, $p < 0.05$).

the epithelium against injury and are strongly related to the onset of cancer and inflammatory diseases [49,50]. Thus, phytase hydrolyzes phytate, releases the complexed nutrients to it and decreases the production of mucins. After, the epithelium returns to its natural state, enhancing host immunity and perhaps reducing the pro-inflammatory effects of soybean meal compounds such as saponins. Ramos et al. [51] observed that the addition of an enzymatic complex consisting of phytase and carbohydrases prevented enteritis in *Mugil liza* fed a diet rich in soybean meal.

It is known that inflammatory processes are related to the production of reactive oxygen species (ROS). In fact, according to Lugrin et al. [52], inflammation and oxidative stress are two processes that feed one another, establishing a vicious cycle that perpetuates and propagates the inflammatory response. Thus, in relation to the experiment that was carried out in the present study, the genes related to zebrafish's antioxidant defense system should respond in the same way as the genes related to the anti-inflammatory responses. The gene coding for superoxide dismutase (*sod1*) was analysed and the result is shown in Fig. 5. Expression of this gene had a significant reduction of 30% in the zebrafish group that had the feed supplemented with the transgenic probiotic. This result corroborates with the results observed for genes related to the inflammatory response, showing that both systems are directly related and also that phytase plays an important role not only in the reduction of inflammation but also in the reduction of oxidative stress in zebrafish intestine.

In conclusion, the results obtained in the present study prove the efficiency of phytase in the activation of the zebrafish immune system fed with soybean meal rich diet. This source of vegetable protein is made up of a number of antinutritional and pro-inflammatory factors such as phytate and saponins. The use of a genetically modified probiotic to express a fungal phytase has been shown to be an interesting strategy to overcome the presence of antinutritional factors in vegetal feed. In addition, this probiotic produced an active enzyme in sufficient quantity to influence the immune system of zebrafish and attenuate the inflammatory responses from the diet rich in vegetable matter. Thus, genetically modified probiotics seem to be an interesting tool for adapting commercially important species to aquaculture at lower cost diets, which proteins can come from cheaper and more abundant sources.

Conflicts of interest

The authors declare no conflict of interest.

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