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Effect of dietary supplements in American bullfrogs reared in low and high stocking densities

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ABSTRACT

The aim of this study was to evaluate the effect of the probiotic *Bacillus subtillis* and beta-glucan from the fungus *Agaricus blazei* on survival, growth and immunological capacity in bullfrogs (*Lithobates catesbeianus)* cultured in low and high stocking densities. Animals weighing 24.3 ± 2.38 g were randomly distributed into four treatments with four simultaneous replicates: D100: 100 frogs/m^2 (control); D236: 236 frogs/m^2; D236 + Prob.: 236 frogs/m^2 supplemented with probiotic; and D236 + BG: 236 frogs/m^2 supplemented with beta-glucan. The parameters evaluated were weight gain, survival, plasma corticosterone (CORT), phagocytic capacity (PC) and phagocytic index (PI), at 24 h and 15 and 30 days. There is significant interaction between treatments and time for CORT levels. At 30 days, these values were very close for the D100 (control) and D236 + BG groups. Meanwhile, no statistical differences were observed between treatments for PC and PI. These results indicate that beta-glucan reduced the effects of stress caused by high density in bullfrogs, but the probiotic did not reduce these effects. Both compounds are not efficient at increasing survival rates, weight gain and neither immune response of animals. Thus, the use of commercial food additives may not have the favorable impact desired by the farmer. Their use in aquaculture should be further studied in experiments involving a longer trial period and taking into account the cost of their use.

1. Introduction

Stress is an expression of metabolic or physiological changes when faced with challenging situations, whether they be environmental, organic, acute or chronic. Stress agents induce compensatory or adaptive physiological responses in an organism to make it possible to overcome the condition (Wendelaar Bonga, 1997). During stress, various endocrine responses are activated to improve the performance of the organism, including the release of glucocorticoids (GCs), which enhance the mobilization of energy and the performance of the organism. Chronic stress can lead to immunosuppression, tissue atrophy and a decrease in reproductive performance, resulting in consequences such as increased incidence of disease and mortality (Mostl and Palme, 2002).

In vertebrates, responses to stress are regulated by GCs, and corticosterone (CORT) is the main hormone linked to this process in amphibians (Belden et al., 2005; Denver, 2009). In amphibians, GCs are released by the anterior pituitary gland and interrenal gland in response to activation of the HPI (hypothalamus-pituitary-interrenal) axis, and mediate the physiological and behavioral responses to adverse stimuli (Glennemeier and Denver, 2002b; Wada, 2008; Denver, 2009; Belden et al., 2010).

The majority of commercial frog farms observe animal mortality due to stress agents, such as inadequate installations or management, poor water quality, dietary deficiency or incidence of disease (Rocha et al., 2010; Teixeira et al., 2012). In frogculture, it is suggested that the ideal density is 100 frogs per square meter in the pre-fattening stage (up to 30 g) in semi-dry systems and up to 200 frogs per square meter in so-called flooded systems. In the fattening stage, 50 frogs per square meter are recommended in semi-dry systems and 100 frogs per square meter

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in flooded systems (Cribb et al., 2013; FAO, 2017; Moreira et al., 2013). However, many breeders use higher densities predisposing animals to chronic stress that results in mortality and economic losses. Therefore, there is special interest in reducing the effects of stress and increasing immune response, which can be induced by the use of immunostimulants. Probiotics and beta-glucans have been used in aquaculture as immunostimulants. Probiotics, are growth promotors and regulators of the gut microbiota, and has a positive immunomodulator effect. Most are prepared with *Lactobacillus acidophillus, Streptococcus faecium, Bacillus subtilis* and, in some cases, yeasts (Robertsen et al., 1990; França et al., 2008). Beta-glucans of yeasts and fungi are also able to increase the function of immune cells (Volman et al., 2008).

The aim of this study was to evaluate the effect of the probiotic *Bacillus subtillis* and beta-glucan from the fungus *Agaricus blazei* (Cogumelo do Sol™) on survival, growth and immunological capacity in bullfrogs (*Lithobates catesbeianus)* cultured in low and high stocking densities.

2. Materials and methods

The study included 440 animals after metamorphosis at 45 days of age, and mean weight of 24.3 ± 2.4 g, acquired from a commercial frog farm (23° 31′S and 47° 08′W). The organisms were transported to the Interinstitutional Laboratory of Health in Aquaculture, in São Paulo/APTA/SAA. At this location, they were acclimated for five days in a room with ambient temperature (measured every day) and controlled photoperiod (12:12 h L:D) into polypropylene boxes filled with tap water up to a level of 0.03 m and density of 50 frogs per square meter with daily renewal water. Afterwards, the animals were weighed and distributed into 16 polypropylene boxes (0.47 × 0.30 × 0.17 m), also filled up to a water level of 0.03 m. Tap water was first dechlorinated by aeration and allowed to set overnight. It was used both for maintenance of the animals and for daily cleaning of boxes. The physical and chemical parameters of this water were 23.7 ± 2.0 °C, pH 7.2 ± 0.1, electric conductivity 150.0 ± 0.5 µS/cm and dissolved oxygen 6.5 ± 0.4 mg/L.

The experimental design was entirely randomized with complete block composed by four treatments and four replicates: Treatment 1 (control)–D100: stocking density of 100 frogs/m^2, without dietary supplementation; Treatment 2–D236: stocking density of 236 frogs/m^2, without dietary supplementation; Treatment 3–D236 + Prob: stocking density of 236 frogs/m^2, supplemented with commercial probiotic with *Bacillus subtillis* (Strain C-3102, 10^9 CFU/g); Treatment 4–D236 + BG: stocking density of 236 frogs/m^2, supplemented with beta-glucan from the fungus *Agaricus blazei* (Cogumelo do Sol ™, Cogumelos Valemar, 167 mg/g beta-glucan, 40 mg free beta-glucan, 2.4 mg/g protein, 0.2 mg/g phenol). Both supplements were added in a proportion of 10 g/kg feed. Densities of 100 animals/m^2 and 236 animals/m^2 were equivalent to 14 and 32 animals per box, respectively. The experimental period was 30 days. The probiotic and beta-glucan were emulsified in 2.0% soybean oil (per kg of diet) and sprayed on the extruded feed. The animals were fed (3% of biomass) with commercial extruded (pellets with 6 mm) fish feed (45% crude protein, 14% ether extract, 6% crude fiber, 2.5% calcium, 1% phosphorus, 14% ash, 21% carbohydrate, 300 mg vitamin C, 4180 kcal/kg crude energy), two times a day.

The parameters evaluated were weight gain, survival, corticosterone value and nonspecific immunity by immunologic challenge. Blood from two animals per replicate was collected for the determination of CORT (eight animals per treatment) at times of 24 h and 15 and 30 days, totaling 96 animals. Samples were taken by puncture of the sciatic artery using disposable and heparinized syringes and needles. The collection of blood occurred in the morning by wrapping the animals in gauze and applying a topical anesthetic (Lidocaina™—40 mg/g). The procedure of capture and blood collection took a mean time of 3 min. After sampling, aliquots of blood were placed in microtubes and centrifuged at 2000 × *g* for five minutes to obtain plasma, which was frozen for later analysis. Plasma CORT was measured by radioimmunoassay (RIA) in the liquid phase, using a commercial diagnostic kit (ImmunoChem™ Double Antibody Corticosterone I^{125} RIA kit, MP Biomedicals, LLC, Orangeburg, NY, USA), according to the manufacturer's directions as previously validated for this species (Teixeira et al., 2012). The sampled animals were anaesthetized with a lethal concentration of benzocaine (3 g/L) and killed by cervical dissection after blood collection.

Immunologic challenge was performed in eight animals at the beginning of the study (TZ—time zero) and in 32 other animals at the end (30 days) (two from each replicate). The frogs were inoculated with 2 mL of yeast solution (*Saccharomyces cerevisiae*) into visceral cavity, using a concentration of approximately 11,000 cells/mm^3 (Dias et al., 2010). After 2 h, the animals were euthanized and the abdominal cavity was washed with Ringer solution for amphibians, via a lateral cut of the abdomen. The material was centrifuged at 251 × *g* for five minutes. The precipitate was separated from the supernatant, resuspended and placed on a glass slide. Active phagocytes and phagocytized cells were counted using a phase contrast microscope. Active phagocytes and phagocytized cells were counted using a phase contrast microscope. The phagocytic capacity (PC) was calculated by multiplying the number of active phagocytes by 100, and the phagocytic index (PI) was calculated by dividing the total number of phagocytized yeast by the number of active phagocytes (Silva et al., 2005; Dias et al., 2010).

Tests were performed to verify the normality of the data (Lilliefors-test) and homogeneity of variance (*F-test*). The corticosterone data were log (x + 1) transformed to meet the assumptions of normality. Comparison of means was done by analysis of variance (two-way ANOVA) followed by Tukey's test. Differences were considered significant when $P < 0.05$ (Zar, 1999).

3. Results

During the experimental period, the mean minimum temperature was 22.1 ± 1.1 °C, and the maximum, 24.0 ± 1.0 °C, showing no alterations that could interfere with the results obtained, once the temperatures were within the thermal comfort levels for these animals (Cribb et al., 2013).

There were no statistical differences between treatments for survival rates ($P > 0.05$), but a significant difference in weight gain between non-supplemented (D100 and D236) and supplemented (D236 + Prob and D236 + BG) treatments were observed (Table 1). Inter and intra-assay sensitivity and variation were tested to guarantee the laboratory quality of analyses (MP Biomedicals) of plasma corticosterone (CORT) levels. The sensitivity of the assay was 1.99 ng/mL, with a low and high inter-assay coefficient of variation of 6% and 3.66%, respectively.

The data showed significant interaction between the level of plasma corticosterone (CORT) and the time of culture (Table 2). In addition, there is a general increase in CORT levels until 30 days of

Table 1
Means and standard deviations of weight gain and survival rates in bullfrog (*Lithobates catesbeianus*) in the different treatments at 30 days of experimentation. Statistical significant differences are indicated by values with different letters in the same columns ($P < 0.05$).

Treatment	Weight gain (g)	Survival rates (%)
D100	37.5 ± 6.9 [a]	96.4 ± 4.1 [a]
D236	28.5 ± 5.2 [a]	98.4 ± 1.8 [a]
D236 + Prob	13.5 ± 7.5 [b]	96.9 ± 3.1 [a]
D236 + BG	16.8 ± 1.5 [b]	98.7 ± 1.4 [a]

Notes: D100 (control): 100 frogs/m^2, without dietary supplementation; D236: 236 frogs/m^2, without dietary supplementation; D236 + Prob: 236 frogs/m^2, supplemented with probiotic based on *Bacillus subtillis*; and D236 + BG: 236 frogs/m^2, supplemented with beta-glucan from *Agaricus blazei*.