Animal Feed Science and Technology 211 (2016) 137–144

Contents lists available at ScienceDirect

Animal Feed Science and Technology

journal homepage: www.elsevier.com/locate/anifeedsci

Probiotic feeding improves the immunity of pacus, *Piaractus mesopotamicus*, during *Aeromonas hydrophila* infection

Thaís Heloísa Vaz Farias[a,b], Nycolas Levy-Pereira[a], Lindomar de Oliveira Alves[a], Danielle de Carla Dias[c], Leonardo Tachibana[c], Fabiana Pilarski[a], Marco Antonio de Andrade Belo[b,d,*], Maria José Tavares Ranzani-Paiva[c]

[a] *Aquaculture Center of São Paulo State University (CAUNESP), Rodovia Paulo Donato Castellane, s/n, CEP 14884-900 Jaboticabal, SP, Brazil*

[b] *Department of Preventive Veterinary Medicine, São Paulo State University, Rodovia Paulo Donato Castellane, s/n, CEP 14884-900 Jaboticabal, SP, Brazil*

[c] *Fishery Institute (APTA-SAA), Av. Francisco Matarazzo, 455, CEP 05001-970, São Paulo, SP, Brazil*

[d] *College of Veterinary Medicine, Camilo Castelo Branco University, Av. Hilário da Silva Passos, 950, CEP 13690-000 Descalvado, SP, Brazil*

ARTICLE INFO

Article history:
Received 22 November 2014
Received in revised form 29 October 2015
Accepted 2 November 2015

Keywords:
Bacterial infection
Bacillus
Immunostimulation
Innate immune system
Piaractus mesopotamicus

ABSTRACT

The utilization of probiotic bacteria have been widely tested and applied in intestinal microflora modulation, through competitive exclusion of the pathogenic bacteria. *Aeromonas hydrophila* is a gram-negative bacterium, responsible for fish outbreaks in farms around the world, and is one of the major loss causes for neotropical fish farmers. 660 pacus (67 ± 7 g) were distributed in 20 tanks ($n = 33$), constituting five groups (four tanks for each treatment): four groups were fed with different levels (2, 4, 8 and 16 g kg^{-1}) of *Bacillus cereus* and *Bacillus subtilis* (1:1, 10^8 CFU g^{-1}), and the fifth group was fed with a control diet (without probiotic). Pacus fed with probiotic showed increment in the ROS production associated to elevated neutrophil and monocyte counts and increased phagocytic activity without affecting the growth parameters. Probiotic fed fish presented higher survival rates, subjected to an i.p. challenge with 10^8 CFU mL^{-1} of *A. hydrophila*. The results demonstrated a dose response effect and the ideal level of the probiotic (*Bacillus cereus* and *Bacillus subtilis*, 1:1, 10^8 CFU g^{-1}) in *P. mesopotamicus* diets was around 8 g kg^{-1}, as the highest survival rates and immunological responses were found in groups of fish fed with this diet. In addition, too much probiotic should be avoided, as pacus fed diet with 16 g kg^{-1} showed partial suppression of these responses.

1. Introduction

Aeromonas hydrophila is a gram negative, motile, rod-shaped bacterium, widely known as an opportunist pathogen of farmed fish, with worldwide distribution. In rearing systems, in which occurs stressful conditions, this bacterial agent is responsible for disease outbreaks, as described in east Asia (Xia et al., 2004; Crumlish et al., 2010), Europe (Boran et al.,

* Corresponding author at: Department of Preventive Veterinary Medicine, São Paulo State University, Rodovia Paulo Donato Castellane, s/n, CEP 14884-900 Jaboticabal, SP, Brazil. Tel.: +55 016 99961 7299.
E-mail address: maabelo@hotmail.com (M.A.d.A. Belo).

http://dx.doi.org/10.1016/j.anifeedsci.2015.11.004

2013), middle East (Ahmed and Shoreit, 2001; Sarkar et al., 2012), North America (Pridgeon et al., 2013; Li et al., 2013) and South America (Reque et al., 2010; Silva et al., 2012), being responsible for considerable economic losses in intensive rearing systems.

Also known as hemorrhagic septicemia, the motile *A. hydrophila* infection is characterized by ulcerative lesions on the skin surface, local hemorrhages in gills and opercula, exophthalmia, liquid accumulation in the coelomic cavity, anemia and liver and kidney lesions (Crumlish et al., 2010; Silva et al., 2012). Aeromoniosis is one of the main diseases in farmed pacu (*Piaractus mesopotamicus*), a neotropical freshwater species of great importance in Brazilian finfish aquaculture, and this teleost fish has demonstrated to be a good bioindicator of water quality and has been used as model for ecotoxicity studies during the process of chemical registrations in Brazil (Castro et al., 2014a). Among the chemicals available for bacterial disease treatment, oxytetracycline and florfenicol are most frequently used in aquaculture (Rigos and Troisi, 2005), and, in some countries, they are the only drugs allowed for fish treatment. The lack of control in antibiotic use, especially for prevention, can trigger undesirable effects in fish, terrestrial animals and environment, which could threaten human health (Cabello, 2006). The use of immunostimulants, as dietary supplements, can improve the innate defense of fish which provide resistance to pathogens, such as vitamins (Belo et al., 2012, 2014), minerals (Castro et al., 2014b, 2014c), essential fatty acids (Sakabe et al., 2013) and yeast *Saccharomyces cerevisiae* (Reque et al., 2010; Castro et al., 2014b). In this context, many studies regarding probiotic-use in farmed animals have reported improvements not only on the productivity indexes in fish, but also in promoting the welfare and maintenance of host health, especially through gut microbiota modulation, inhibition of pathogen colonization in the intestinal epithelium and stimulation of the innate immune system (Yanbo and Zirong, 2006; Zhou et al., 2009; Ai et al., 2011), when used at the appropriate levels.

In aquaculture, the inclusion of exogenous *Bacillus* in fish diets have shown a positive overall effect, improving the productive parameters and immune responses and increasing the resistance to pathogens (Raida et al., 2003; Salinas et al., 2005; Merrifield et al., 2010; Nayak, 2010; Sun et al., 2010; He et al., 2011). In this context, the present study aimed to evaluate the effect of diets containing 2, 4, 8 and 16 g kg^{-1} of probiotic (*Bacillus cereus* and *Bacillus subtilis*, 1:1, 10^8 CFU g^{-1}) on productivity performance, innate immune parameters and resistance to experimental infection by *A. hydrophila* in pacus, *P. mesopotamicus*.

2. Materials and methods

2.1. Fish and feeding

P. mesopotamicus juveniles, from a commercial fish farm (Águas Claras, Mococa-SP), were maintained for 15 days in 500 L plastic tanks and fed with basal diet (commercial diet) in order to allow them to acclimate to experimental conditions. 660 fish (67 ± 7 g) were distributed in 20 tanks (n = 33; four tanks for each treatment) with continuous water flow (~16 L min^{-1}) and artificial aeration. No differences were observed among the water quality parameters throughout the trial period (temperature: 26.6 °C, pH: 7.9 ± 0.09, dissolved oxygen: 6.7 ± 0.59 mg L^{-1} and ammonia: 0.12 ± 0.11 mg L^{-1}).

Five experimental diets were prepared: one without probiotic (control diet) and four with different levels (2, 4, 8 and 16 g kg^{-1}) of the commercial probiotic PAS-TR™ (*Bacillus cereus* and *Bacillus subtilis* in a 1:1 proportion, lyophilized at a concentration of 10^8 CFU g^{-1}). Count of colony forming units of *Bacillus cereus* and *Bacillus subtilis* incorporated in *Piaractus mesopotamicus* diets was performed before the start of the experiment and the reisolation results showed 1.6×10^1, 1×10^5, 7×10^5, 4.2×10^6 and 1.2×10^7 CFU g^{-1} for diets supplemented with 0 (control), 2, 4, 8 and 16 g kg^{-1} of the probiotic, respectively. Every week during the feeding period, the probiotic was incorporated in the pellets via soybean oil (20 g kg^{-1} of diet), mixed manually and the diets were stored at 4 °C (refrigerated). The control diet was made with the same commercial feed (32% crude protein, 7% crude fiber and 6.5% fat, Pirá 32® – Guabi Company), also mixed with soybean oil, but without probiotic. The fish were fed a quantity equal to 3% of their body mass, twice a day, at 9:00 and 17:00, for 60 days.

The protocol for the animal experiment was approved by Ethics Committee on the Use of Animals of São Paulo State University under the protocol n°22.518/10.

2.2. Blood samples and NBT assay

During the feeding period (at days 7, 15, 30 and 60), ten fish per treatment were anesthetized in a benzocaine solution (100 mg L^{-1}) for blood collection from the caudal vein, afterwards the fish were euthanized by deepening anesthesia. White blood cell (WBC) counts were made using a hemocytometer with Natt and Herrick solution. Blood smears for differential leukocyte counts were stained with a combination of May-Grünwald Giemsa and Wright's Method (Belo et al., 2013). For nitro blue tetrazolium (NBT) assay, 0.5 mL of blood was transferred to 2 mL plastic tubes containing 15 μL of heparin (5.000 IU mL^{-1}). The respiratory burst of leukocytes was measured according to Biller-Takahashi et al. (2013). Briefly, 100 μL of heparinized blood was mixed with 100 μL of an NBT-buffered solution (Sigma, St. Louis, MO, USA). The solution was homogenized and incubated in a dark room for 30 min at 25 °C. After the incubation, 50 μL of the solution was added in 5 mL tubes containing 1 mL *n*,*n*-dimethyl-formamide, (DMF, Sigma, St. Louis, MO, USA) and centrifuged at 3000 g for 5 min. The optical density of the supernatant was measured using a spectrophotometer with a wave length of 540 nm.