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Efficacy of *Aeromonas hydrophila* S-layer bacterins with different protein profiles as a vaccine in Nile tilapia (*Oreochromis niloticus*)

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Farming of Nile tilapia is in full development throughout the world. However, an increase is still seen in the prevalence and severity of bacterial diseases. The aim of this study was to develop a vaccine against *Aeromonas hydrophila* in Nile tilapia (*Oreochromis niloticus*) based on protein patterns of S-layer proteins. The proteins were extracted using glycine and NaOH with subsequent visualization through SDS-PAGE. Based on the protein patterns observed, bacterins were produced that were tested in an experiment *in vivo* with the use of 144 fingerlings of Nile tilapia that were distributed in 24 aquariums in a completely randomized design. Eight distinct protein patterns were observed in SDS-PAGE, with apparent molecular mass from 52 to 72 kDa. All the unvaccinated fish inoculated with *A. hydrophila* died within 24 h after inoculation. The bacterins produced reduced the probability of death of the vaccinated fish when compared to unvaccinated ones. Hepatic histological analysis showed that the use of vaccines was able to revert changes in the liver of the fish.

Key words: S-layer protein, SDS-PAGE, *Aeromonas hydrophila,* vaccine, *Oreochromis niloticus.*

INTRODUCTION

In recent years, Brazil has stood out in the area of fishery production in the world, achieving greater prominence in the international market (MPA, 2011). Among the species most commonly farmed in the country are those of exotic origin, such as Nile tilapia (*Oreochromis niloticus*, Linnaeus, 1758). A tropical climate species that has adapted very well to conditions in Brazil (IBAMA, 2007; MPA, 2011).

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With the growth of intensive fish farming, disease outbreaks particularly, bacterial one are encountered in production systems, *Aeromonas hydrophila* are considered to be the most virulent species (Pavanelli et al., 2008). The *Aeromonas* have multifactorial pathogenesis and diverse virulence factors, including S-layer proteins (Pablos et al., 2009). The presence of the S layer at the surface of bacterial cells is strongly correlated with their virulence Proteins present in this layer are involved with different biological roles related to water reserve, nutrient functions, increase of surface adherence through formation of biofilms, invasive capacity of pathogenic bacteria, and microbial resistance to antimicrobial drugs (Kinns and Howorka, 2008).

Immunoprophylaxis of fish by vaccines is an alternative approach for preventing bacterial infections (Figueiredo and Leal, 2008). There are vaccines against some pathogens available on the market; however, they have not been shown to be effective in Brazilian samples. Because of variability, a vaccine produced abroad may be ineffective in Brazil (Figueiredo and Leal, 2008).

The aim of this study was to evaluate the effect of two bacterins produced as based on the profile of S-layer proteins from isolates of *A. hydrophila* obtained from aquatic organisms in protection of challenged Nile tilapia *(O. niloticus)*.

MATERIALS AND METHODS

Samples

S-layer proteins were extracted from 20 isolates of *A. hydrophila* originating from the bacterial collection of the Microbiology and Animal Immunology Laboratory of the Universidade Federal do Vale do São Francisco. These isolates were obtained from the kidney, integument, intestine, and lesions of Nile tilapia (*O. niloticus*) and Pac-man catfish (*Lophiosilurus alexandri,* Steindachner, 1876) showing clinical signs, collected from the Sobradinho Dam area (Sobradinho, Bahia, Brazil) and from the Centro Integrado de Recursos Pesqueiros e Aquicultura (CIRPA) (Integrated Center of Fishery and Aquaculture Resources) located in the Bebedouro district (Petrolina, Pernambuco, Brazil) in 2009 and 2010. The *A. hydrophila* isolates were previously identified through their morphological, tinctorial, and biochemical characteristics, according to Quinn et al. (1994).

One hundred forty-four (144) fingerlings were used in the experiment to test the effectiveness of the vaccine. Fish were collected from the Centro Integrado de Recursos Pesqueiros e Aquicultura (CIRPA) of Petrolina , PE, Brazil.

Extraction of S-layer proteins

S-layer proteins were extracted according to the methodology described by Fujimoto et al. (1991) and McCoy et al. (1976), with small modifications. The isolates were seeded in a TSA culture medium (*Tryptic Soy Agar*) and placed in a laboratory incubator at 28ºC for 48 h. After that, the isolates were suspended in 10 mL of 0.2 M glycine (pH 2.2) until obtaining final concentration of 5 x 10^9 CFU mL^{-1}. Soon after, the samples were placed in a vortex (20 min) at ambient temperature, and the cells were collected through centrifugation (5000 x *g* for 30 min). The pH of the supernatant was adjusted to 7.5 with NaOH (4 N), and the proteins were precipitated (overnight at 4°C) through addition of $(NH_4)_2 SO_4$ (5 g $10mL^{-1}$). After centrifugation (5000 x *g* for 1 h), the proteins precipitated were resuspended in 500 µL of 50 mM Tris-HCl pH 7.5 and stored at -20°C. The protein concentration was determined by the Bradford method (Bradford, 1976) using bovine albumin (1mg mL^{-1}) as a standard.

The proteins were visualized in 12% polyacrylamide gel with denaturing conditions (SDS-PAGE) according to the methodology described by Laemmli (1970). After preparation of the gel, the protein extracts (10 µL) were added to a mix containing 480 µL of sample buffer and 20 µL of β-mercaptoethanol heated in a water bath at 100°C for 5 min before being applied in the gels. After electrophoresis, the gels were stained with Coomassie Blue (Blum et al., 1987) and the protein standards were registered in an image-capturing system.

Vaccine production

The bacterins were prepared according to Normative Instruction 31/2003 of the Ministério da Agricultura Pecuária e Abastecimento (MAPA, 2003) [Ministry of Agriculture]. First, the strains of *A. hydrophlia* were cultivated in TSA medium at 28□C for 24 h. After growth, a suspension of the colonies in sterile saline solution was prepared and the quantity of bacteria was estimated in colony forming units per milliliter (CFU mL^{-1}) comparing a dilution of the suspension with scale 4 of McFarland through the turbidimetric method and through spectrophotometry (580 nm), optical density of 1.730; corresponding to 1.2 x 10^9 CFU. Then, 10% of this bacterial suspension was added in saline solution (NaCl 0.85%) in *Tryptic Soy Broth* (TSB) medium for cultivation in a shake flask (180 rpm) at 28ºC for 8 h. Ten mL of the vaccine culture was then removed for bacterial count, preparing decimal dilutions up to 10^6 added to TSA medium at 28ºC for 24 h. At the same time, 0.6% of formol P.A. was added to the vaccine culture for inactivation and placed once more in a shake flask (180 rpm) at 28ºC overnight. An aliquot of the treated culture was seeded in BHI (*Brain Heart Infusion*) medium and thioglycolate to confirm inactivation of the bacterial cells. After confirmation of inactivation, 15% aluminum hydroxide was added to the bacterins as an adjuvant. The bacterins were then seeded in BHI culture medium and incubated at 28ºC for 24 h to check for their safety. The bacterins were subsequently kept at 4ºC, according to the methodology of Grabowski et al. (2004).

Evaluation of the effect of the vaccine on Nile tilapia inoculated with *A. hydrophila*

To test the bacterins effect on fish, an *in vivo* experiment was carried out using 144 Nile tilapia fingerlings, sexually reversed, with a mean weight of 8.925 g and distributed in 24 aquariums of 60 L useful volume in a completely randomized experimental design The treatments consisted of six groups: two of them unvaccinated (one inoculated with saline solution and other inoculated with *A. hydrophila*); two vaccinated groups (one vaccinated with the bacterin 1 and the other with the bacterin 2) and inoculated with saline solution, and other two vaccinated groups (one vaccinated with the bacterin 1 and the other with the bacterin 2) and challenged with *A. hydrophila*, in a total of six treatments and four repetitions. The aquariums had constant aeration through air stones connected to mini air compressors. Experimental management consisted of daily siphoning in the morning (8:00) and afternoon (15:00), with the removal of around 40% of the water, which in addition to exchanging the water, also removed feces and possible leftover feed. The internal tank walls were cleaned weekly to avoid the rise of periphyton. The fish were given *ad libitum* access to commercial feed for omnivorous fingerlings.

Table 1. Parameters of hepatic histology of vaccinated and unvaccinated Nile tilapia after challenge with A. hydrophila.

Group	Parameter
Fish not vaccinated and not inoculated with *A. hydrophila*	0
Fish vaccinated with bacterin 1 and inoculated with *A. hydrophila*	1 and 2
Fish vaccinated with bacterin 2 and inoculated with *A. hydrophila*	1, 2, and 3
Fish not vaccinated and inoculated with *A. hydrophila*	3 and 4

For inoculation of the bacterins, the fish were anesthetized by immersion in benzocaine (100 mg L^{-1}) and then the vaccines were injected in the peritoneal cavity, one centimeter below the pelvic fin. The fish remained without food for 24 h before the injection and they were challenged with *A. hydrophila* 15 days after immunization. The Nile tilapia were inoculated with *A. hydrophila* through a bacterial inoculum preparation with dilution in sterile saline solution at a concentration of 2.8×10^9 CFU mL^{-1}. This solution was applied through intramuscular injection, right laterodorsal, in each experimental fish, just as the pure saline solution (control), at the proportion of 0.2 mL^{-1} for animal. After this challenge, the fish were observed every 12 h over a period of five days in regard to mortality and the occurrence of clinical signs. They were also observed for the appearance of lesions and other pathological changes brought about by *A. hydrophila.*

Statistical analysis

In order to verify the effect of the vaccine on fish infected with *A. hydrophila*, the zero inflated binomial (ZIB) model was used for statistical analysis. The ZIB model is a parametric model which properly accommodates the overdispersion caused by count data with excess zeros, as the case of our experiment. A detailed discussion regarding zero inflated models can be found in Hall (2000) and references therein. Let Y_i represent the number of dead fishes observed in the i-th tank. Then, since there are 6 fishes in each tank at the begining of the experiment, according to the ZIB model, the probability of observing Y_i dead fishes is given by:

$$P(Y_i = y_i) = \begin{cases} w + (1-w)(1-\pi_i)^6, & y_i = 0 \\ (1-w)\binom{6}{y_i}\pi_i^{y_i}(1-\pi_i)^{6-y_i}, & y_i = 1, 2, \cdots, 6 \end{cases},$$

in which w corresponds to the probability of the number of dead fishes being equals to zero, regardless of the conditions of the experiment, and π_i is the probability of those fishes subject to the conditions, that is type of vaccine (placebo, vaccine 1 or vaccine 2) and bacteria inoculation (inoculated or not inoculated) dying, given by:

$$\pi_i = \frac{\exp(\beta_0 + V_{1i}\beta_1 + V_{2i}\beta_2 + I_i\beta_3)}{1 + \exp(\beta_0 + V_{1i}\beta_1 + V_{2i}\beta_2 + I_i\beta_3)},$$

Where,

$$V_{1i} = \begin{cases} 1, & \text{if the fishes in the i-th tank receive vaccine 1} \\ 0, & \text{otherwise} \end{cases},$$

$$V_{2i} = \begin{cases} 1, & \text{if the fishes in the i-th tank receive vaccine 2} \\ 0, & \text{otherwise} \end{cases},$$

and

$$I_i = \begin{cases} 1, & \text{if bacteria were inoculated in the fishes in the i-th tak} \\ 0, & \text{otherwise} \end{cases}.$$

Histological analysis

Livers collected from an animal of each group were fixed in 10% formalin and received routine histological treatment. They were included in paraffin, cut, and stained through a modification of the Masson-Goldner Trichrome method (Pierce et al., 1978). Photomicrographs were taken of the slides obtained with 40x magnification using the IS Capture® software.

A histological classification system was used to objectively estimate the histological changes or lesions identified in liver samples of the fish evaluated (Table 1). Using this classification system, adapted from Pierce et al. (1978), a numerical value was attributed to each liver according to its histological characteristics. This system clearly differentiates between normal hepatic histology and histological changes.

RESULTS

Extraction of S-layer proteins

Quantification of the S-layer proteins of the 20 isolates of *A. hydrophila* allowed observation of concentrations that ranged from 2.954 to 3.560 µg mL^{-1}.

Differences in the protein patterns among the 20 isolates of *A. hydrophila* were visualized in SDS-PAGE. In all, eight distinct protein patterns were observed, with the presence of protein bands with predominant apparent molecular weight of 52 and 70 kDa. Eight isolates representing these profiles were then chosen to make up two vaccine groups. In electrophoresis, it may be observed that profile 1 represented four isolates with proteins bands of 52 and 72 kDa; profile 2, three isolates with 72 kDa; profile 3, two isolates with bands of 48, 52, and 70 kDa; profile 4, one isolate with 48, 52, 72, and 90 kDa; profile 5, three isolates with 48, 52, and 70 kDa; profile 6, two isolates with 52 and 70 kDa; profile 7, four isolates with 52 and 70 kDa; and profile 8, one isolate with 52 and 70 kDa. The profile of SDS-PAGE electrophoresis protein patterns of the eight isolates of *A. hydrophila* is shown in Figure 1.

Evaluation of the effect of vaccine in Nile tilapia inoculated with *A. hydrophila*

In the *in vivo* experiment, the two bacterins produced

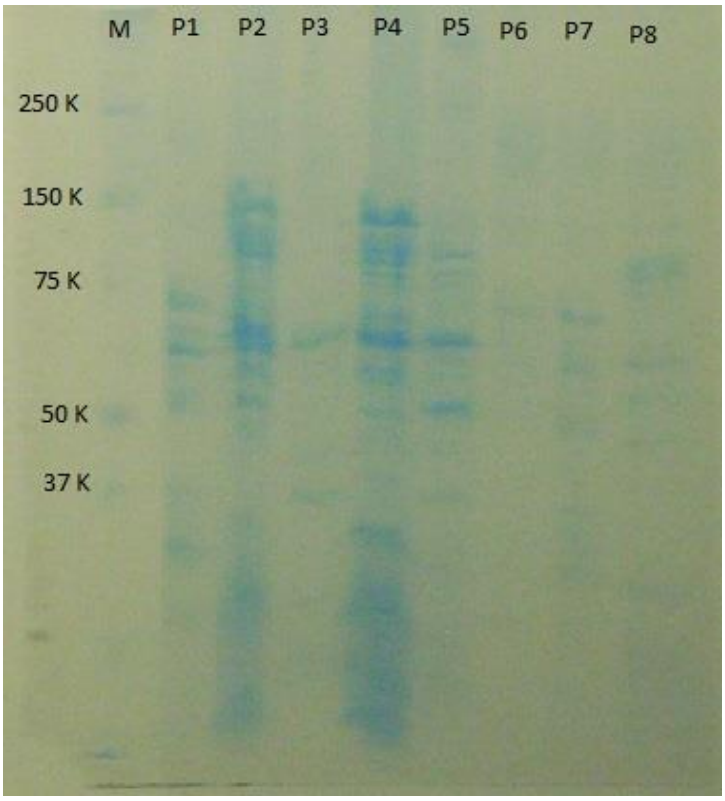

Figure 1. SDS-PAGE of eight distinct electrophoretic profiles of S-layer proteins of *A. hydrophila*: letter M refers to the molecular weight marker proteins Precision Plus Protein Dual Color Standards 161-0374 (Bio Rad) followed by profiles observed.

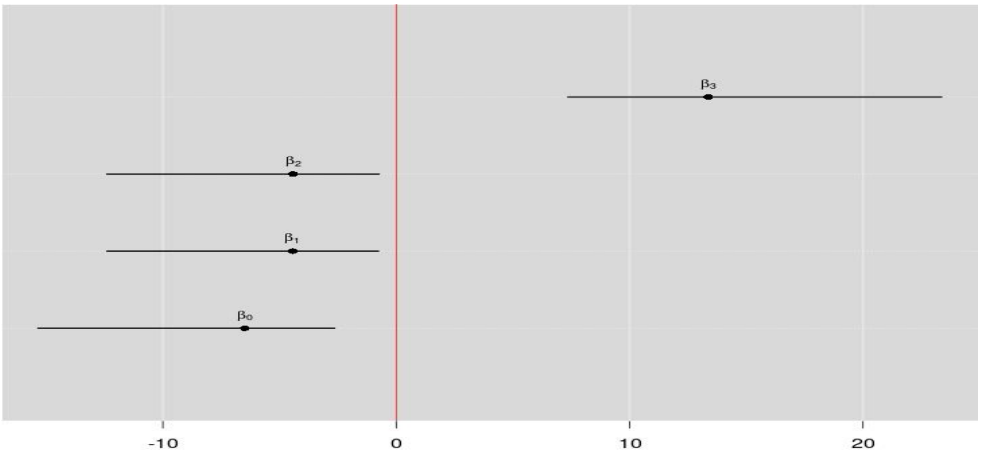

Figure 2. 95% credible interval for the regression coefficients: (β0) control group, fish not vaccinated and not inoculated with A. hydrophila, (β1) effect of vaccine 1, (β2) effect of vaccine 2, and (β3) effect of inoculation of the bacteria.

significantly reduced the probability of death of the vaccinated fish in relation to the unvaccinated ones after the challenge with *A. hydrophila*, as shown in Figure 2.

It is also observed that the probability of death of the vaccinated fish inoculated with *A. hydrophila* reduced, whereas the probability of death increased in the unvaccinated and inoculated fish. All the unvaccinated fish inoculated with *A. hydrophila* died within 24 h after inoculation. There was no statistical difference between the effect of the two vaccines in the vaccinated and

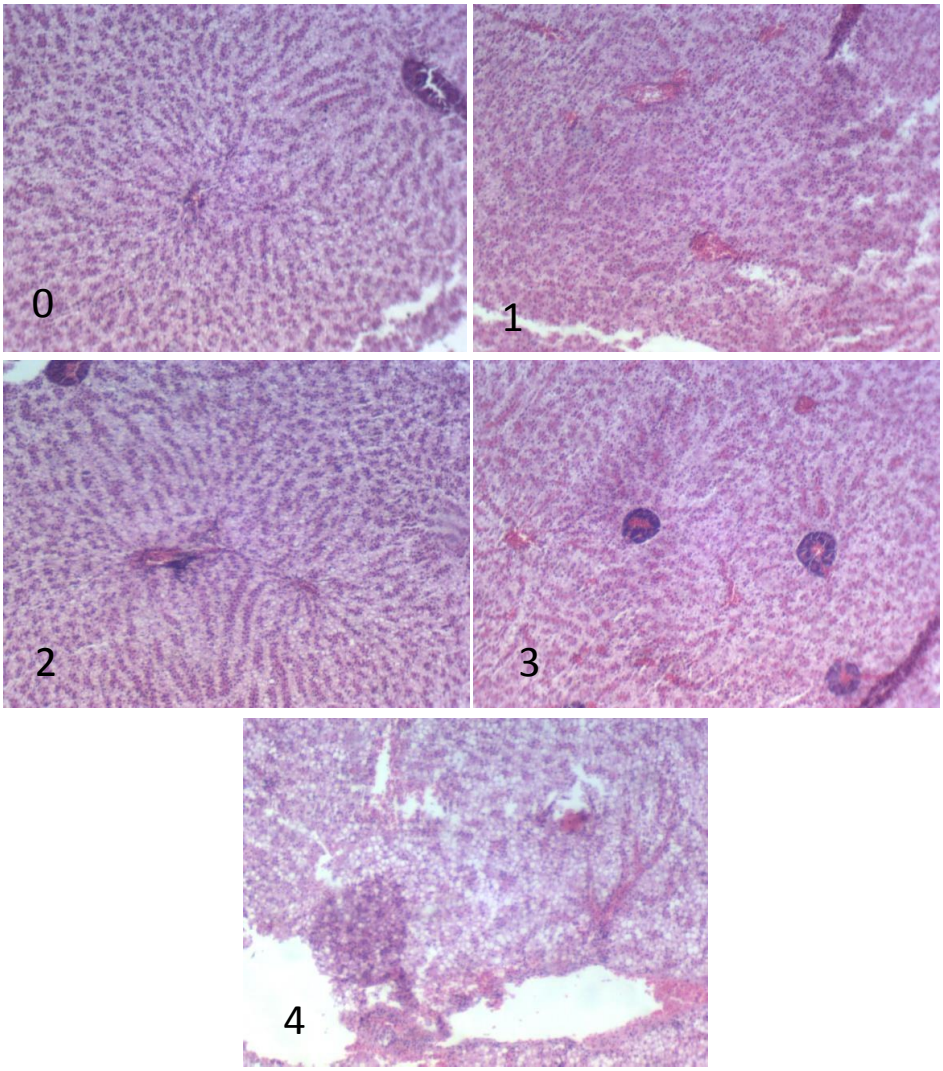

Figure 3. Hystological results of liver. Numbers: parameters described in Table 1. *Liver congestion; ** oss of hepatic cord structure.

inoculated fish. The fish that were vaccinated and that survived the inoculation showed lesions characteristic of the bacteriosis, such as redness in the ventral region, loss of scales, and hemorrhages at the base of the fins and integument, with exposure of muscle tissue in some. All the unvaccinated fish inoculated with *A. hydrophila* that died within 24 h after the challenge showed signs of systematic infection by *A. hydrophila,* such as swelling of the body, ulcerous skin lesions, and hemorrhages.

Histological analysis

Hepatic histology of the animals of the control group showed normal parameters, and these animals were classified in parameter 0, described in Table 1. The livers of the animals that were inoculated and that did not receive the bacterins showed classification 3 (25%) and 4 (75%). The major alteration was the congestion of hepatic blood vessels and loss of hepatic cord structure. It may be observed that the use of the vaccines was able to revert these changes. The inoculated animals vaccinated with bacterin 1 showed classification 1 (50%) and 2 (50%), whereas those vaccinated with bacterin 2 showed classification 1 (50%), 2 (25%), and 3 (25%) (Figure 3). These samples demonstrate a decrease in hepatic vessel congestion and increase in hepatic cord structure.

DISCUSSION

S-layer proteins are common structures in *Aeromonas* species (Esteve et al., 2004) and are correlated with the

pathogenicity of these bacteria. In this study, the concentration of the S-layer proteins extracted from 20 isolates of *A. hydrophila* showed values that ranged from 2.954 to 3.560 µg mL^{-1}, which were considered good results since such proteins represent around 10% of the total of cell proteins in bacteria (Avall-Jaaskelainen and Palva, 2005).

It is known that electrophoresis undertaken in polyacrylamide gel is a refined technique that allows determination of protein profiles of bacterial cells or extracellular products of important pathogens such as *Aeromonas* and *Escherichia coli* (Figueiredo and Leal, 2008). In this study, eight distinct electrophoretic profiles were visualized in the 20 isolates of *A. hydrophila* analyzed with protein bands with apparent molecular weight of 52 and 70 kDa. According to Ewing et al. (1960), through these profiles, the difference of antigenic structures or total proteins of isolated groups or of species may be compared.

Dooley and Trust (1988) reported that highly virulent isolates of the species *A. hydrophila* are capable of producing S-layer proteins with apparent molecular weight of 52 kDa. Other reports in the literature show that S-layer proteins in *A. hydrophila* may show variation in the molecular weights described, which are approximately 51.5 kDa (Yan et al., 1996) and 91 kDa (Rahman, 1998), depending on the isolate. Our findings indicate a variation in the molecular weight of the S-layer proteins of *A. hydrophila*, although they show predominantly a protein with apparent molecular weight of approximately 52 kDa. Data from the literature show that the S-layer proteins responsible for the main virulence factors in *A. hydrophila* are found around this apparent molecular weight (Poobalane et al., 2008; Yeh and Klesius, 2011).

According to Fagan and Fairweather (2014), bacteria that exhibit the S layer are more virulent since the presence of these proteins are implicated in different biological roles, whose functions are to act as a water and nutrient reserve, increase adherence to the surface through formation of biofilms and of force of infection, increase the invasive capacity of the pathogenic bacteria that easily escape the action of the phagocytes, and increase microbial resistance to antimicrobial agents (Kinns and Howorka, 2008; Fagan and Fairweather, 2014). The loss of ability to produce the S layer reduces the virulence of *Aeromonas* spp. (Merino et al., 1995; Noonan and Trust, 1997).

The presence of these proteins with different protein profiles in association with the antigenic variation in *A. hydrophila* isolates is still little reported in the literature. Kostrzynska et al. (1992) found antigenic diversity upon analyzing the S-layer proteins of pathogenic isolates of *A. hydrophila* and of *A. veronii* biotype *sobria,* revealing the antigenic complexity of these proteins present in these isolates. One of the possible explanations for this diversity is the great variation in gene expression among the different isolates, which, for its part, leads to different levels of expression of the virulence factors, such as surface proteins, especially the S-layer proteins (Chu et al., 1993; Boot and Pouwels, 1996; Engelhardt, 2007). As the S-layer proteins in the species of *Aeromonas* are characterized through being a common antigen found in all the isolates (Poobalane et al., 2010), interest grows in utilizing this antigen for vaccine production.

Studies related to fish immunology are in full development; nevertheless, it is known that the use of vaccines against bacterial and viral diseases, though of recent origin, has had good results in relation to scientific and economic aspects upon minimizing the use of antimicrobial drugs in the growth environment, especially due to the projections made in regard to growth of Brazilian and world aquaculture. Immunization programs against diseases and products for preventive use that are ecologically safe for maintaining the health of aquatic animals will be necessary (Aoki et al., 2008; Figueiredo and Leal, 2008; Ismail et al., 2010; Silva et al., 2013). With a view toward their clinical and economic importance, different types of vaccines have been developed against *A. hydrophila* for use in fish (Poobalane et al., 2010; Fernandez et al., 2014). Although these different vaccines show varied degrees of protection in fish, there is still not a commercial vaccine available for *A. hydrophila* (Fang et al., 2004). This is related to the inability of the vaccines in offering cross protection against different isolates of *A. hydrophila* since this bacteria is very heterogeneous in nature, both biochemically and serologically (Poobalane et al., 2010; Fernandez et al., 2014).

The bacterins produced based on the electrophoretic profiles of the *A. hydrophila* isolates in this study reduced the probability of death of the vaccinated fish compared to the unvaccinated fish. It should be noted that the challenge offered through *A. hydrophila* in the Nile tilapia fingerlings that were vaccinated and inoculated was made at a high concentration of the bacterial inoculum, with $2.8x10^9$ CFU/mL, which is considered a lethal dose. The fish that were not vaccinated but only inoculated were dead 24 h after the challenge, which confirms the large potential of pathogenicity of *A. hydrophila* derived from fish and shows its high capacity for causing diseases in these animals, especially because these isolates were obtained from sick fish. In the fish that survived that were vaccinated and inoculated, it was possible to observe lesions 24 hours after the challenge. These lesions showed characteristics common to those caused by *A. hydrophila,* and as of the third day, constancy was observed in the number and in the appearance of the lesions, lasting up to the fifth day. These findings are similar to those described for septicemic infections brought about by *Aeromonas* spp. (Boijink et al., 2001, Boijink and Brandão, 2004).

Although the vaccine was produced from the bacterial culture in broth and inactivated with formalin, it provided

protective capacity to the Nile tilapia fingerlings against the highly virulent isolate of *A. hydrophila*, which corroborates the results of Dehghani et al. (2012), who evaluated the effectiveness of vaccines inactivated by formalin and by heat, and vaccines constituted by lipopolysaccharides against *Aeromonas hydrophila*. Prasad and Areechon (2010), upon analyzing the effectiveness of a vaccine inactivated with formalin against *A. hydrophila* and *Streptococcus* sp. in red tilapia, obtained good results in stimulation of specific humoral immunity.

Although it is not possible to observe a statistical difference between the effects of the two bacterins, they confer protection to the vaccinated fish regardless of the surface protein profile. Studies indicate that the S-layer proteins together with the other surface proteins are important for protection against infection by *A. hydrophila* since these proteins are very important for initial colonization of the bacteria (Noonan and Trust, 1997). In the same way, Poobalane et al. (2010), in Japan, obtained success in effectiveness of the recombinant vaccine with S-layer protein of 45 kDa purified against different virulent isolates of *A. hydrophila* in common carp, emphasizing the importance of the use of this antigen in production of vaccines for protection against this disease. This information is extremely important for future studies with a view toward purifying and using these proteins as a common antigen, seeking satisfactory results in the immunological response of the fish when exposed to diverse species of *Aeromonas* spp., including *A. hydrophila.* The protein characterization of the isolates of *A. hydrophila* of the region will allow the proposal of a possible vaccine against these agents in fish, which may be produced efficiently, and considered alternative measures to be used by fish producers in their farming systems.

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