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Aloe vera enhances the innate immune response of pacu (*Piaractus mesopotamicus*) after transport stress and combined heat killed *Aeromonas hydrophila* infection

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ABSTRACT

In this study, pacu (*Piaractus mesopotamicus*) were fed with diets containing *Aloe vera* for 10 days prior to transport stress and infection with heat killed *Aeromonas hydrophila*. *A. vera* is popular around the world due to its medicinal properties, including immunostimulatory effects which was observed in this study. The results show that transport causes immunosuppression, an effect that was prevented by *A. vera*. Specifically, *A. vera* prevented reductions of both leukocyte respiratory burst and hemolytic activity of complement system caused by transport. Further, fish fed with *A. vera* also showed significantly higher leukocyte respiratory burst, serum lysozyme concentrations and activity of complement system 24 h after bacterial infection. Additionally, we observed that *A. vera* may modulate the innate response through activation of complement system during bacterial immune stimulation. In summary, *A. vera* extract enhanced innate immune parameters and consequently the ability of fish to cope with pathogens following transport stress. These findings show that *A. vera* has promise for use in aquaculture and add further evidence that medicinal herbs added to fish feed assist to prevent disease outbreaks.

1. Introduction

The production of fish by aquaculture has increased significantly over the past few decades, jointly with the incidence of fish diseases. The increasing prevalence of diseases has been associated with intensive rearing procedures that cause stress such as netting, grading, transport, crowding and poor water quality, among others, which can affect the fish immune response and/or increase the virulence of pathogens [1]. Thus, studies focused on strengthening the immune system of fish are of great interest to researchers and producers.

Some methods to prevent disease outbreaks that have been used include chemotherapy, vaccination and immunostimulants products. However, the use of antibiotics and chemotherapy has created several problems, including the development of drug resistance [2,3], toxic effects on fish and negative impacts on the environment and human health [4]. Vaccination has been considered a potential treatment. Nevertheless, a single vaccine, effective against only a single type of pathogen [5], also causes stressful handling [6] and can be expensive for widespread use by fish producers [7,8]. Further, the development of vaccines for heterogeneous species or multiple strains is extremely complex [9,10]. Thus, immunostimulants appear to be a viable alternative to these methods in the control of fish diseases, as recently suggested by Hang et al. [11]. Immunostimulants promote the activation of specific and/or non-specific defense mechanisms [12,13], and have been shown to be suitable for use in aquaculture [14]. Among the possible immunostimulants, medicinal plants arise showing great potential. Recent reviews [7,8,15–20] have emphasized the use of herbs as an emerging approach to enhance fish immunocompetence that may have substantial benefits for the aquaculture industry [21]. Finally, herbal extracts are easily obtained, and usually inexpensive, act against a broad spectrum of pathogens [22], and

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may have limited environmental impact due to their biodegradability [23].

Products derived from the *Aloe vera* are popular around the world due to their cosmetic and medicinal properties [24]. Several benefits to human and other animals health have been reported (e.g. chickens [25], mice [26], felines [27], dogs [28] and human beings [29]) including, wound healing, antibacterial, antiviral, antifungal, antidiabetic, anticancer, gastroprotective and immunemodulatory properties [25,30–33]. However, except for few studies [34–41] *A. vera* has been almost unexplored in aquaculture.

Given the potential benefits of *A. vera* extract in aquaculture, the main purpose of this study was to evaluate whether feeding fish with various levels of *A. vera* would affect their immune response to heat killed *Aeromonas hydrophila* injection after they have been subjects to transport stress. Further, we used the fish pacu (*Piaractus mesopotamicus*) as an experimental model because it is a commercially valuable South American species, especially in Brazil where its use in farming is constantly growing.

2. Material and methods

2.1. Experimental animals

We used a total of 240 (71.3 ± 3.3 g) juvenile pacu, obtained from the Centro de Aquicultura of Univ. Estadual Paulista UNESP. The fish were held in 24 fiberglass tanks (100 l; 10 fish per tank) and initially fed with a commercial diet at a ration of 3% of their body mass twice a day. Tanks were supplied with water at a temperature of ~29.5 °C, with oxygen and ammonia levels monitored daily (~6.5 mg l^{-1} and 0.34–0.37 mg l^{-1}, respectively). The photoperiod was14 h light: 10 h dark.

2.2. Experimental design: the effects of A. vera on stress and innate immune responses

After 2 week of acclimation, the fish were fed one of four experimental diets (6 tanks per treatment) for 10 days: one control diet (no *A. vera*), and diets to which 5 g kg^{-1} (0.5%), 10 g kg^{-1} (1%) and 20 g kg^{-1} (2%) of *A. vera* extract (see diet preparation below) were added. On the 11th day, the fish had their blood drawn by caudal puncture (n = 12 per diet), and the remainders were transported in plastic bags for 4 h at a density of 166 g l^{-1}. Immediately at the return to the lab, 12 fish from each diet were sampled. The leftover fish were divided into 3 groups: uninjected (recovery), intra-peritoneally injected (IP) with PBS buffer (sham-injected) and IP with *A. hydrophila*. Fish were then sampled 24 h after injection (n = 8). We measured plasma cortisol and glucose levels, leukocyte respiratory burst, serum lysozyme levels and hemolytic activity of the alternative complement system.

2.3. Preparation of A. vera powder and experimental diets

A total of 250 *A. vera* leaves (final weight of 75 kg) were harvested, had their base cut off and placed in vertical position for 2 h to remove the aloin component by gravity; this component has emetic properties that could interfere with feed ingestion. Thereafter, the leaves were peeled, the whole parenchyma triturated in a food processor, and the *A. vera* gel centrifuged at 10,000×g for 30 min at 4 °C to remove the fiber [42]. The supernatant was then separated and stored at −20 °C. *A. vera* dehydration was performed using a spray drying process, during which a powder was formed from the liquid by rapidly drying it with hot air (±180 °C). Initially, the dry matter content of *A. vera* gel was determined according to AOAC [43]. However, as the amount of dry matter in the *A. vera* gel was low (0.76 ± 0.005%) an excipient had to be added to improve the spray drying process. Then, the inclusion of different levels of Aerosil (Aerosil 200 Pharma, Evonic; Aerosil® colloidal silicon dioxide) were tested (1, 3, 5, 10 and 30%; data not shown), and based on these trials, 3% (w/v) of Aerosil was added to the *A. vera* gel. During the spray drying process, the following parameters were used: inlet temperature 179.2 ± 1.8 °C; outlet temperature 88.5 °C; atomizer was set to 30,000 rpm with a depression of 15 mm CA; and an air flow rate of 8 l h^{-1}. After the dehydration process, we obtained a crude powder that contained 20.75% of *A. vera*. The powder was stored at −20 °C until use.

To prepare the experimental diets, a commercial diet (Fri-Aqua Onivoros: 4–6 mm; 12% moisture; 32% protein; 4.5% fat) was ground into powder. Thereafter, we weighed 24.09 g, 48.19 g and 96.38 g of the crude powder, corresponding respectively to 5 g, 10 g and 20 g of *A. vera,* using the rate of 20.75% obtained above. Then, the various amount of *A. vera* were suspended in 0.5 l of water and sprayed onto the powdered feed. These diets, corresponding to 0.5, 1% and 2% *A. vera* (w/w), were then dried and pelleted. To avoid interference on the palatability, the control diet contained a similar amount of Aerosil.

2.4. Preparation of A. hydrophila and experimental infection

The *A. hydrophila* (strain A135, LAPOA, Jaboticabal, São Paulo, Brazil) was identified by sequencing of the 16S rDNA (the strain used showed a similarity of 97% with GenBank accession # ATCC 7966). Bacteria were stored in TSB (Tryptic Soy Broth, Hi Media) medium with 30% glycerol (sterile) at −80 °C. For use in trials, an aliquot of 20 μl was added into 5 ml of autoclaved TSB medium and incubated in a bacteriological incubator at 28 °C, for 24 h. Thereafter, 700 ml of autoclaved TSB medium was added and incubated again using the same conditions. This bacterial suspension was centrifuged at 12,000 × g for 20 min and the supernatant was discarded. Subsequently, the remaining pellet was washed twice with PBS buffer (0.01 M) and centrifugation steps of 12,000 × g for 20 min. Finally, the pellet was re-suspended in PBS (0.01 M) and adjusted according to the McFarland turbidity standard to obtain a suspension of 3×10^9 cfu ml^{-1}, that was inactivated[1] in a water bath at 40 °C for 30 min [44], prior.

The fish designated to receive IP injections (PBS or *A. hydrophila*) were netted from the holding tanks and anaesthetized in water containing 0.1 g l^{-1} MS-222 (tricaine methanesulfonate; Sigma–Aldrich, São Paulo, Brazil; #E10521) until ventilatory movements ceased. Their mass was recorded and they were given a 4 μl g^{-1} IP injection of *A. hydrophila* or PBS.

2.5. Fish sampling

Fish were anaesthetized and blood samples were drawn from the caudal vessel using syringes without anticoagulant, and dispensed into heparinized microtubes (plasma), microtubes containing the anticoagulant Glistab® (plasma for glucose) and microtubes without anticoagulant (serum). Whole blood was used immediately to measure leukocyte respiratory burst. To obtain plasma, blood samples were centrifuged for 10 min at 3000×g. Glucose levels were determined immediately, whereas the plasma for the analysis of cortisol levels was stored at − 80 °C. To obtain serum for the analysis of hemolytic activity of complement system and serum lysozyme concentration, the blood was allowed to clot

[1] Inactivation of bacteria was chosen to stimulate the pacu's immune system but avoid any significant mortality, since that transport could increase the susceptibility to the bacterial challenge.

at room temperature for 3 h, and then centrifuged at 3000 × g for 10 min. Serum was stored at − 80 °C prior to the analyses.

2.6. Glucose and cortisol plasma assays

Plasma glucose concentrations were determined by enzymatic method (Labtest kit, REF: 1012, São Paulo, Brazil, http://labtest.com.br/en/reagents/glucose-liquiform-vet) using spectrophotometer (Model Genesys 10S, Thermo Scientific Inc., Madison, WI, USA), and cortisol levels were measured using a commercial ELISA kit (DRG International, Inc., USA; Cortisol Enzyme-Linked ImmunoSorbent Assay, EIA 1887) following the manufacturer's instructions.

2.7. Immunological assays

2.7.1. Leukocyte respiratory burst

The production of reactive oxygen species was measured using NBT (nitrotetrazolium blue chloride, Sigma-Aldrich, São Paulo, Brazil; #N6876), following the protocol of Sahoo et al. [45]. Immediately after bleeding, 100 μl of heparinized blood were incubated with an equal volume of NBT buffer (0.2%) at room temperature for 30 min. Thereafter, 1 ml of dimethylformamide (DMF, Sigma-Aldrich, São Paulo, Brazil; #227056) was added to the samples, that were read in a spectrophotometer (Model Genesys 10S, Thermo Scientific Inc., Madison, WI, USA) at wavelength 540 nm, at room temperature.

2.7.2. Serum lysozyme concentration

Serum lysozyme concentration was determined according to Demers and Bayne [46] with modifications by Zanuzzo et al. [36]. Briefly, standard solutions of hen egg white lysozyme (Sigma-Aldrich, São Paulo, SP, Brazil; #L6876) and serum samples were placed into a 96-well plate in triplicate with a suspension of *Micrococcus lysodeikticus* (Sigma-Aldrich, São Paulo, Brazil; #M3770). After mixing, absorbance was measured at 450 nm over 10 min using a microplate reader (Model Multiskan Ascent, Thermo Fisher Scientific Inc., Madison, WI, USA) at room temperature. The rate of decrease in absorbance for each sample was then compared to that obtained with the standard curve.

2.7.3. Hemolytic activity of alternative complement pathway

Serum hemolytic activity of the complement (alternative pathway) was measured according to Zanuzzo et al. [36] with modifications for use with pacu blood as follows. Initially, a sample of rabbit blood was collected and the rabbit erythrocytes (RaRBCs) were washed and isolated according to Zanuzzo et al. [36]. To optimize the assay for pacu, a series of dilutions (1:24, 1:12, 1:8, 1:6 and 1:4 in a final volume of 200 μl) was made by mixing a pool of aliquots of all serum samples with TEA-EGTA-Mg^{2+} buffer (triethanolamine ethylene glycol tetraacetic acid; 8 mM, with 2 mM of Mg^{2+} and 0.1% gelatin, pH 7.4) and the RaRBC suspension, and measuring absorbance at 700 nm using a spectrophotometer (Model Genesys 10S, Thermo Scientific Inc., Madison, WI, USA) at 15, 20, 25, 30, 35 and 40 °C (data not shown). Based on this preliminary work, the assay was carried out with a 1:8 dilution at 25 °C. Hemolytic complement activity (ACH_{50}) for each sample was measured as the time (seconds) required for the initial optical density to be reduced by one-half (50% of RaRBC hemolysis by the alternative pathway). To obtain the activity of complement system per second (reduction of DO sec^{-1}), we used:

$$ACH = \frac{1}{\text{Time to obtain the rate at which 50\% lysis is reached}}$$

2.8. Statistical analyses

This experiment was analyzed as a 4 (diets) x 5 (sampling point) two-way ANOVA, followed by a Duncan's Multiple Range Test. Data were transformed prior to statistical analysis as they failed normality (Cramer Von Mises) and/or homoscedasticity tests (Brown-Forsythe). All analyses were performed using the Statistical Analysis Software (SAS, version 9.2) package with $P < 0.05$ set as the level of statistical significance. Values in the text and figures are means ± 1 standard error (S.E.) of the mean.

2.9. Animal welfare statement

The experimental procedures described were approved by the Comissão de Ética no Uso de Animais (CEUA - Protocol 002111/12) and performed in accordance with the guidelines for the ethical principles in animal experimentation adopted by the Colégio Brasileiro de Experimentação (COBEA, Brasília, Brazil).

3. Results

No mortality was observed during the experimental period. Plasma glucose and cortisol concentrations were approx. 2.5–3-fold higher in all treatments immediately after transport (arrival) compared to the initial sampling ($P < 0.05$), without differences among treatments (Fig. 1). Glucose levels were significantly lower (i.e. by 40–60%) in all fish groups 24 h after transport (recovery) compared to arrival values (Fig. 1A). There were slight differences in plasma glucose levels among groups 24 h after transport, which were probably not biologically significant. Neither *A. hydroplila* nor PBS injection had any effect in fish fed with the control diet ($P < 0.05$, Fig. 1A).

Plasma cortisol levels differed among diet treatments 24 h after transport (recovery), when fish fed with 1 and 2% *A. vera* diets had levels higher than those of control fish ($P < 0.05$, Fig. 1B). Cortisol levels in control fish returned close to the initial values 24 h after transport (recovery; Fig. 1B) in contrast with fish fed with *A. vera*, which remained close to arrival values ($P < 0.05$, Fig. 1B). There was no effect of *A. hydroplila* vs. PBS injection on cortisol levels in fish fed with the control diet. In contrast, *A. vera* treatment generally decreased the levels of cortisol in *A. hydrophila* injected fish but the combination of 1% *A. vera* and IP with *A. hydrophila* resulted in statistically significant lower cortisol values compared to control fish ($P < 0.05$, Fig. 1B).

Leukocyte respiratory burst (LRB) was significantly lower in control fish immediately after transport (arrival) compared to initial control values and those observed 24 h after transport (recovery). In addition, fish fed with the *A. vera* diets had significantly higher (approx. 15%) LRB compared to fish fed with control diet immediately after transport (arrival; Fig. 2A). No significant difference was observed among diet treatments 24 h after transport (recovery) or when fish were injected with PBS (Fig. 2A). Further, the LRB was lower in control fish when injected with *A. hydrophila* compared to PBS injection (~20%). Finally, all fish fed with *A. vera* diets had higher LRB values than control fish when injected with *A. hydrophila* (Fig. 2A).

Serum lysozyme concentrations (SLC) decreased immediately after transport (arrival) in fish fed with 0.5 and 2% of *A. vera* diets. Those fed with 0.5 and 2% *A. vera* diets had higher SLC 24 h after transport (recovery) compared to control fish and those fed with 1% *A. vera* ($P < 0.05$, Fig. 2B). IP injection with *A. hydrophila* led to significant increases in SLC compared to PBS values in all treatments. This response in the *A. hydrophia* group was also affected by

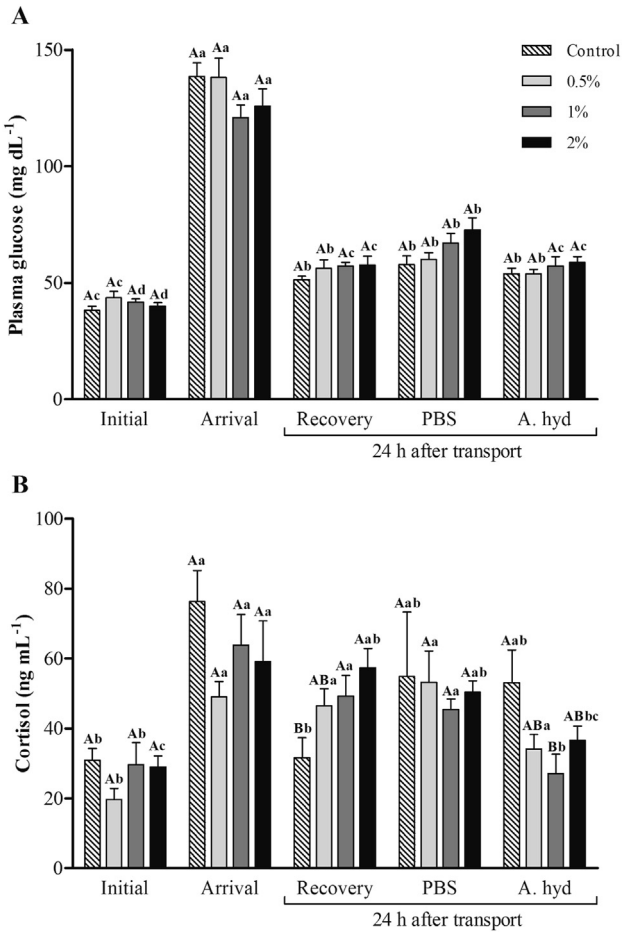

Fig. 1. Plasma glucose (A) and cortisol (B) concentrations in pacu fed with *A. vera* diets (0%; 0.5%; 1%; and 2%) before transport (Initial, n = 12), immediately after transport (Arrival, n = 12), 24 h after transport (Recovery, n = 8) and after they were given an IP injection with PBS or *A. hydrophila* (n = 8). Different capital letters indicate differences among diets at a given time point. Different lower case letters indicate a difference between sampling points within a group ($P < 0.05$). Values are means ± 1 standard error (S.E.).

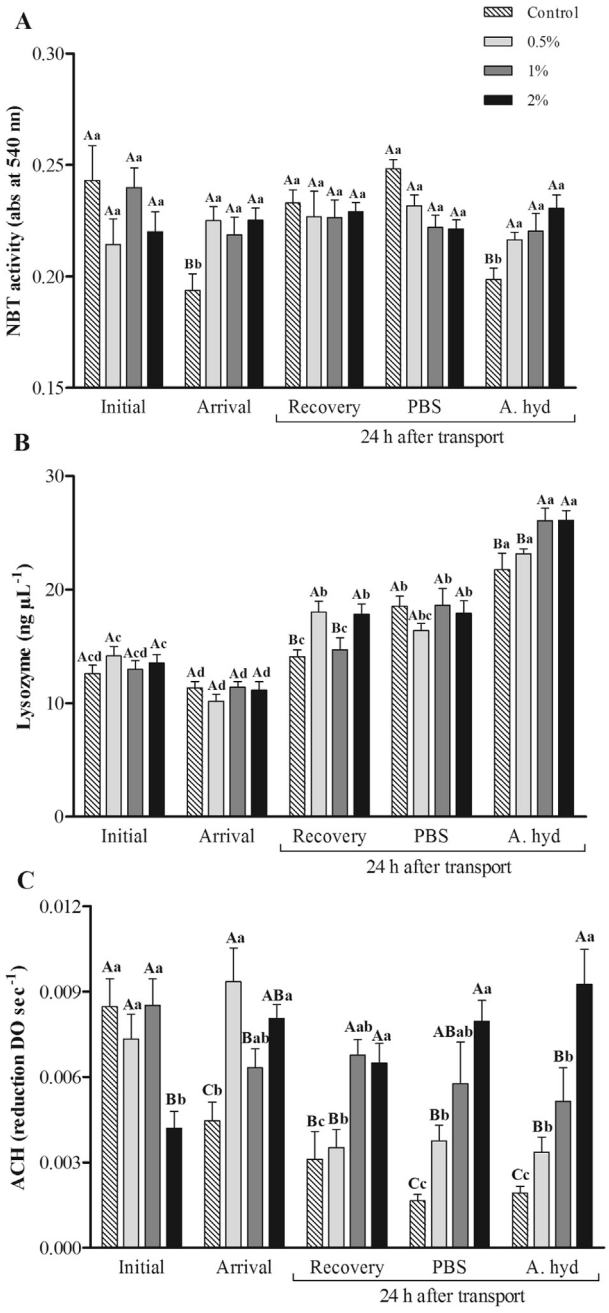

Fig. 2. Leukocyte respiratory burst (NBT activity - A), serum lysozyme concentrations (B) and serum complement activity (ACH - C) in pacu fed with *A. vera* diets (0%; 0.5%; 1%; and 2%) before transport (Initial, n = 12), immediately after transport (Arrival, n = 12), 24 h after transport (Recovery, n = 8) and after they were given an IP injection with PBS or *A. hydrophila* (n = 8). Different capital letters indicate differences among diets at a given time point. Different lower case letters indicate a difference between sampling points within a group ($P < 0.05$). Values are means ± 1 standard error (S.E.).

diet. The SLC was higher (by ~ 4.5 ng μl^{-1}) in fish fed with 1% and 2% *A. vera* diets compared to control fish or those fed with 0.5% *A. vera* ($P < 0.05$, Fig. 2B).

Hemolytic activity of complement system (ACH) was lower in pacu fed with 2% *A. vera* diet compared to all other groups at the initial sampling time ($P < 0.05$, Fig. 2C). Immediately after transport (arrival), control fish showed a significantly reduction in ACH compared to initial values (2-fold). However this reduction was prevented in fish fed with *A. vera* diets as the ACH in these groups was approx. 2-fold higher than control ($P < 0.05$, Fig. 2 – C). The ACH 24 h after transport (recovery) was approx. 2.5-fold higher in fish fed with 1 and 2% *A. vera* diets than in control (Fig. 2 – C). The dietary *A. vera* provoked significant improvement in the concentration-dependent effects on the ACH in PBS and *A. hydrophila* injected fish compared to control values ($P < 0.05$, Fig. 2 – C).

4. Discussion

Recently increasing attention has been given to the use of plants products for disease control in aquaculture, in view of the number of reviews published [7–9,15–22,47–49]. However, *A. vera* has received only a rare mention (e.g. Hai [16] and Vallejos-Vidal et al. [19]), despite the fact that it contains several compounds that may provide this herb with a great potential to improve fish production. Here, we found that dietary *A. vera* supplementation prevented the immunosuppression provoked by the stress of transport, injection and bacterial infection; treatment also enhanced SLC tested after heat killed *A. hydrophila* infection. Furthermore, there was a dose-dependent prevention of the loss of ACH after treatment with *A. vera* diets, which also appears to have modulated the other innate immune parameters when fish were exposed a bacterial

immune stimulation. Finally, our findings confirm that *A. vera* increased the resistance against a pathogen infection and has promise for use in aquaculture, especially for being applied before stressful handling to increase fish protection and prevent disease outbreaks.

Blood parameters such as plasma glucose and cortisol levels are often used as indicators of stress [50]. In our study, plasma glucose and cortisol levels increased immediately after transport confirming the stress caused by the procedure. The results also show that transport impaired LRB and ACH. These deleterious effects on immune responses associated to stressful aquaculture operations are widely known [1,51,52]. Moreover, we found that *A. vera* slowed the recovery of cortisol level from high relative to control fish 24 h after transport (recovery) but reduced cortisol levels relative to control fish 24 h after injection with *A. hydrophila*. At present, we do not have an explanation for this result. Zanuzzo et al. [53] showed that steelhead trout (*Oncorhynchus mykiss*) fed with 0.5% *A. vera* during 8 weeks had resting cortisol levels one-half of those of the control fish. Tilapia (*Oreochromis niloticus)* fed with *A. vera* diets during 60 days also had significantly difference in cortisol level compare to control fish [39]. These data add evidence that this compound may modulate cortisol levels in fish. Nonetheless, additional experiments may confirm/determine the extent of the effects of *A. vera* on the cortisol levels in fish.

Leukocyte respiratory burst plays an essential role in the control of host immune response and resistance to pathogens [54]. Selvaraj et al. [55] observed a dramatically reduced survival *in vitro* of *A. hydrophila* exposed to macrophages obtained from fish with enhanced oxygen burst activity. In this study, LRB decreased immediately after transport in control fish, which would likely result in enhanced susceptibility to disease. However, a reduction of LRB in fish fed with *A. vera* was not observe, suggesting a protective effect triggered by the herb. We also notice that *A. vera* prevented LRB reduction after bacterial immune stimulation. Thus, these results suggest that *A. vera* improved the resistance of pacu against a bacterial infection. In previous study, Zanuzzo, et al. [35] and Zanuzzo et al. [38] also showed that adding *A. vera* to the water prevented the reduction of LRB promoted by the stress (transport and induced spawning, respectively) in matrinxã (*Brycon amazonicus*). Additionally, two dihydrocoumarins isolated from *A. vera* stimulated the *in vitro* phagocytic capacity and the oxygen respiratory burst of macrophages in rat [56]. Some reports also have shown that oral administration of other herbal supplements enhanced the LRB in different fish species, such as tilapia (*O. mossambicus*) fed with diets containing *Eclipta alba* [57], *Astragalus membranaceus* and *Lonicera japonica* [58], and rainbow trout (*O. mykiss*) fed with diets containing *Zingiber officinale* [47].

Lysozyme is an important bactericidal molecule of the innate immune system [59]. In this study, SLC increased in all fish groups following bacterial immune stimulation. Enhanced serum lysozyme activity was also observed in carp (*Cyprinus carp*) infected with *A. punctate* [60] and in Atlantic salmon (*Salmo salar*) experimentally challenged with *A. salmonicida* [61]. In addition, our results show that fish fed with 1 and 2% *A. vera* diets significantly enhanced SLC after a bacterial immune stimulation. Even with different active compounds, other medicinal herbs have also shown positive effects on SLC in fish. Ardo et al. [58] showed that *A. membranaceus* and *L. japonica* significantly enhanced plasma lysozyme activity in tilapia (*Oreochromis niloticus*). Verma et al. [62], using the indigenous plant *Leucaena leucocephala* and *Ficus benghalensis*, also showed increased lysozyme activity in *Clarias gariepinus* after infection with *A. hydrophila.* Tilapia (*O. mossambicus*) fed with diets containing *E. alba* extract had significant increase in serum lysozyme activity [57].

The complement system is an important component of the innate immunity that plays an essential role in detecting the presence of pathogens (for a review see Boshra et al. [63] and Zhang and Cui [64]). The complement system has been also designated as an indicator of fish immune competence because it is down-regulated in many situations of stress [65] and can be activated by immunostimulants [57,66–68]. Several orally administrated herbs have been shown enhance ACH in fish, such as mistletoe extract, *Viscum album coloratum* [69], *Rauvolfia tetraphylla* [70], *E. alba* [57], *Tinospora cordifolia* [71], *Nyctanthes arbortristis* [72], triherbal leaf extract from *Azadirachta indica*, *Ocimum sanctum* and *Curcuma longa* [73] and *Punica granatum* [74]. In this study, the dietary *A. vera* stimulated the ACH that was depressed after transport. Moreover, 24 h after transport *A. vera* also prevented a reduction caused by transport, in a dose-dependent pattern, in fish injected with PBS and heat killed *A. hydrophila*. These results confirm the immunostimulatory property of *A. vera,* in response to injury and its enhancement of resistance against bacterial infection in pacu.

The most interesting findings of this study involved the complement system. Herein, 24 h after transport fish fed with *A. vera* and injected with PBS had higher complement system activity compared to control, in a dose-dependent pattern; however no differences were observed in LRB and SLC. Although, when fish were fed with *A. vera* and exposed to a bacterial immune stimulation, an increase in a dose-dependent pattern were also observed in LRB and SLC. Complement mediates opsonization of the pathogen, allowing the recognition of these microbes by phagocytes and consequently phagocytosis [63]. The activation of phagocytosis induces other antimicrobial mechanisms, such as release of lysosomal enzymes and production of reactive oxygen species [75]. When the outer cell wall of bacteria is disrupted by the complement, exposing the inner peptidoglycan layer of bacteria, then lysozyme becomes effective [59]. Fragments of C3 also have been shown to induce inflammation and stimulating the respiratory burst in leukocytes isolated from rainbow trout [76]. Based on the above statements, these data support an important finding. *A. vera* prevented a reduction of alternative complement system activity, which in combination with bacterial immune stimulation could have modulated LRB and SLC in a dose-dependent pattern. This finding indicates evidence of the mechanism of action of *A. vera* as discussed below. However, even of it is well-known that complement system could mediate innate immunity in fish, due to *in vitro* functional experiments or by homology with the complement system of mammals [77], this is the first evidence *in vivo* of modulation of innate immunity by complement system from a functional point of view in fish. There is limited amount of functional data on the physiological role of the fish complement system [63,64], even the identification of complement system components has been a relatively recent discovery in fish [77].

The responses elicited by *A. vera* are complex, in part due to the composition of the extract, which has more than 75 biologically active compounds with multiple biological activities [31]. However, the immune-modulatory property of *A. vera* has been mainly attributed to their polysaccharide (e.g. aloeride [78]) and glycoprotein fractions, such as mannose (e.g. acemannan [79]). Although, the exact immune-modulatory mechanisms of herbal extracts in fish are still unknown [8,9], it could be speculated that *A. vera* could act through toll-like receptors (TLR) [19,80], a group of structurally related molecules that are expressed on the cell surface and serve as pattern recognition receptors (PRR) to detect the presence of microbial infection [81]. These TLR have been found to recognize immunomodulating substances such as zymosan, BCG cell walls, heat shock protein 60, polymannuronic acid polymers and LPS [82]. Some reports have shown in mammals that polysaccharides isolated from medicinal herbs, such as *Acanthopanax*

senticosus, roots of *A. embranaceus* and *Platycodon grandiflorum,* activated macrophages and B cells proliferation, cytokine release and production of nitric oxide via the activation of TLR-4 [83–85]. The *A. vera* could act via TLR-4 since these receptors bind to mannose molecules, one compound of the *A. vera* extract. Nevertheless, there is a body of evidence indicating extensive cross-talk between TLR and complement signaling pathway [86,87]. These two components are critical for first-line host defense and revealed a marked synergistic interaction between TLR and complement [88]. Kaczorowski et al. [89] demonstrated in a murine macrophage cell line that TLR stimulation leads to synthesis and release of complement components by macrophages, and this effect was TLR4-dependent. However, as mentioned above, *A. vera* modulated the alternative complement system in a dose-dependent pattern, which reinforces the hypothesis that *A. vera* could act through cross-talk between TLR-4 pathway and complement. Thus, when fish fed with *A. vera* were exposed to a bacterial immune stimulation, the higher activity of complement system may cause a higher release of anaphylatoxin molecules, increasing the inflammatory process and up-regulating LRB and SLC. Moreover, the presence of a pathogen could activate other pathways and the recognition of *A. vera* could be also performed by multiple pattern recognition receptors, which could include TLR but also non-TLRs. These results suggest a plausible mechanism of how *A. vera* may promote immune-modulation and assist in understanding the mechanism of action of immunostimulants in fish. Recent studies also reinforce the hypothesis that this mechanism of the immunostimulants is mediated by TLR signaling pathways in fish [90–92].

In conclusion, dietary *A. vera* either improved or prevented loss of innate immune activity in pacu after a stressful handling and a bacterial infection. Recently, increasing efforts have been made to improve fish immunocompetence, and these results are promising, since 1) a possible commercial large-scale process to prepare the herb extract was demonstrated as viable 2) a relevant enhancement or prevention of loss of the innate immune response induced by *A. vera* was observed, 3) oral administration of the extract was employed, which has been shown to be most suitable for aquaculture [8,9] and 4) the medicinal herb used here is easily obtained and inexpensive, can act against a wide range of pathogens and have limited environmental impact. Furthermore, studies on the isolation/identification of *A. vera* bioactive compounds that are responsible for such activities and the exact mechanism by which *A. vera* affects fish immunity are necessary. Finally, the extent of the effects of *A. vera* taking into account dose and duration of exposure should be investigated, since both are potential factors for overdosing [9] which was recent observed by Gabriel et al. [39].

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