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Phagolysosomal activity of macrophages in Nile tilapia (*Oreochromis niloticus*) infected *in vitro* by *Aeromonas hydrophila*: Infection and immunotherapy

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ABSTRACT

The biochemical mechanisms involved in phagocytosis and the intracellular survival of *Aeromonas hydrophila* (*Ah*) in host macrophages (MΦs) are complex processes that affect infection success or failure. Thus, in the present study, we described the *in vitro* infection of Nile tilapia MΦs by a homologous bacterium and tested the effects of anti-*A. hydrophila* immunoglobulin Y (IgY) on the phagolysosomal activity and intracellular survival of the pathogen. The anti-*Ah* IgY modulated lysosomal acid phosphatase (LAP) activity as well as the production of reactive oxygen intermediates (ROIs) and nitric oxide (NO), thereby potentiating phagocytosis and the elimination of *Ah*. Thus, we assume that the specific IgY had a beneficial effect on infection control and postulated the use of the Nile tilapia MΦs as an important *in vitro* experimental model for the functional and therapeutic study of *Ah* infection.

1. Introduction

The main cellular defense mechanism that is effective against pathogenic microorganisms such as bacteria is regulated by phagocytic cells involved in the host innate immune response [1,2]. In teleost fish, due to the non-evolution of the adaptive immune response in this vertebrates, phagocytosis is the main mechanism responsible for the elimination of pathogens and is mediated by neutrophils, thrombocytes and macrophages (MΦs) [3,4]. The phagocytic activity of MΦs is initially related to the production of lysosomal acid phosphatase (LAP), which contributes to acidification of the phagolysosome and the subsequent activation of enzymes responsible for the production of reactive oxygen intermediates (ROIs) and nitric oxide (NO) [5].

Aeromonas hydrophila (*Ah*) is a commensal and cosmopolitan bacterium with the potential to infect a wide variety of species including fish, mammals and humans [6,7]. The bacterium is aquatic and is associated with diseases in fish [8,9] and humans as a food or waterborne pathogen [10]. It causes gastroenteritis, cellulitis, peritonitis, meningitis and pneumonia in animals [11] and immunocompromised humans [12,13]. In underdeveloped countries, *Ah* is one of the main causative agents of diarrhea in children and newborns [14]. In Nile tilapia, *Ah* infection leads to skin ulceration [7], gastroenteritis [15], spreading to vital organs and systemic inflammatory response syndrome, resulting in death of the host [16,17]. Thus, the importance of this pathogen to public and animal health is evident [18,19].

The use of antibiotics for the treatment of aquatic organisms, although effective, generates antibiotic residues in the water [20,21], representing a risk for the development of microbial resistance and affecting aquatic organisms, mammals, birds and humans [22]. This fact explains the resistance to several antibiotics by different strains of *Aeromonas* spp. isolated from fish farms, despite no history of the use of such substances for the prevention or treatment of the disease in that specific location [23]. Consequently, the microbial resistance generated by antibiotic residues in water has a negative effect on public health [24]. In this context, the use of IgY antibodies extracted from the egg yolk of preimmunized laying hens represents a promising alternative in the treatment of bacterial diseases in aquatic organisms [25].

The production and extraction of IgY from the egg yolks of laying hens is economically feasible and meets animal welfare standards, and in addition to the fact that it has a low production cost, IgY can be

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produced in large quantities and can be obtained without euthanizing hens or subjecting them to cruel methods [26,27]. An egg generates on average 100 mg of IgY in the yolk; as laying hens produce more than 20 eggs per month, the IgY production in 30 days can reach values greater than 2 g per animal [28]. The use of IgY neutralizes bacterial adhesion to target cells and tissues as shown in the prophylaxis of infectious diseases such as diarrhea of piglets caused by *Escherichia coli* [29], gastric ulcer caused by *Helicobacter pylori* [30], mycoses caused by *Candida albicans* [31], canine parvovirus infection [32] and aflatoxin toxicity [33]. Oral treatment with IgY antibodies specific for *Vibrio anguillarum* antigens conferred protection in experimentally infected tongue sole [34]. In shrimp, passive immunization with IgY antibodies against the white spot syndrome virus resulted in protection against infection [35].

Therefore, the use of IgY in infectious disease immunotherapy is a promising alternative in the treatment and prophylaxis of bacterial diseases in aquatic organisms due to its specificity and lack of residual effect in water, predator or human consumer. Therefore, the present work aims to evaluate the *in vitro* effect of anti-*Ah* IgY on the phagolysosomal activity and intracellular survival of *Ah* in Nile tilapia MΦs as well as the potential use of these cells as a model for bacterial infections.

2. Materials and methods

2.1. Anti-Ah IgY production and characterization

IgY specific against the *Aeromonas hydrophila* strain (GenBank accession number: MH305534.1) and nonspecific IgY were produced, purified and previously validated by Fernandes et al. [36].

2.2. Ah neutralization by ELISA assay

An indirect enzyme-linked immunosorbent assay (ELISA) was used for this purpose according to Shi et al. [37]. A 96-well flat bottom microplate was used (Corning Costar Corporation, Cambridge, USA) and was presensitized with live bacterium at a concentration of 1.5×10^7 CFU/mL in 0.05 M carbonate-bicarbonate buffer (CBB) pH 9.6 adsorbed in a volume of 200 µl/well and incubated at 4 °C for 18 h. IgY was tested in a volume of 100 µL and at the concentrations of 50, 100, 150, 200, 250 and 300 µg/mL per well. The plate incubated for one hour at room temperature. The plate was then washed with 0.05% PBS-Tween 20, and 100 µl/well of peroxidase-conjugated rabbit anti-IgY IgG (1:500 v/v dilution) was added, followed by incubation for one hour at room temperature. The reaction was developed with substrate/chromogen (H_2O_2/ABST). The reaction was read at 640 nm on a microplate reader (Molecular Devices SpectraMax, Molecular Devices, Sunnyvale, CA, USA).

2.3. In vitro neutralizing activity of anti-Ah IgY

The *Ah* was grown in tryptone soy broth (TSB) (Difco, Detroit, MI, USA) and incubated for 24 h at 28 °C, after which the culture was centrifuged for 10 min at 10,000 × *g* and the pellet was washed and suspended in 0.15 M phosphate buffer saline (PBS) pH 7.4 and adjusted to a concentration of 1.5×10^7 CFU/mL. The bactericidal activity of IgY was determined by incubating 100 µL of this diluted *Ah* suspension with 100 µL of anti-*Ah* IgY at the concentrations of 50, 100, 200 and 300 µg/mL and nonspecific IgY at 300 µg/mL in a microtube for one hour at 28 °C. In the bacterial control group, PBS was used instead of IgY. After incubation, the number of viable bacteria was determined by counting the number of colonies grown in Petri dishes containing tryptone soy agar (TSA) (Difco, Detroit, MI, USA) for 24 h at 28 °C.

2.4. Cell isolation and culture

Nile tilapia (304.5 ± 7 g) (n = 12) were anesthetized by immersion in benzocaine solution (Sigma-Aldrich Laboratory, Steinheim, Germany) (1: 20,000 v/v) diluted in 98% ethanol (0.1 g/mL) and injected with 50 µg/mL of purified *Ah* membrane proteins (MPs) in their swim bladders, according to Fernandes et al. [36]. After 72 h, the fish were euthanized by immersion in benzocaine (1: 500 v/v), and the leukocyte cells were collected. For this purpose, the fish were injected with 0.5 mL of RPMI-1640 medium (Sigma-Aldrich, St. Louis, USA) with 1% heparin in their swim bladders and its content was aseptically collected and centrifuged at 400 × *g* for 10 min; the resulting pellet was washed twice. The cells were resuspended in RPMI-1640 medium containing 100 IU penicillin-streptomycin (Sigma-Aldrich, St. Louis, USA) and 5% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, USA) in a tissue culture plate under 5% CO_2 at 28 °C. After 24 h, the supernatant was removed, and the non-adherent cells were discarded, with the MΦs remaining in the plate. Antibiotic-free RPMI-1640 medium and FBS were added for the following tests.

2.5. Cell phenotyping

The MΦs were phenotyped by immunocytochemistry (IC). The cell culture was readjusted to 3.0×10^7 cells/well and distributed in triplicate into a 12-well plate containing round coverslips (13 mm) for cell adhesion and incubated at 28 °C in a controlled atmosphere at 5% CO_2. Afterwards, the coverslips were collected and fixed for 15 min in 4% formaldehyde, washed three times with 0.05% PBS-Tween, and permeabilized with 0.25% Triton X-100. The nonspecific sites were then blocked. The primary antibodies produced in rabbit anti-MHC class II (1: 1000 dilution, Abmart/X1-H9B8H2), anti-CD68 (1: 1000 dilution, Abmart/X-I3KF76-N), anti-iNOS (1: 1000 dilution, Neomarkers/RB-1605-P) and anti-TNF-α (1: 1000 dilution, Anaspec/AS-55383) containing 1% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, USA) were incubated for 18 h at 4 °C, and the sample washed twice in 0.05% PBS-Tween. The anti-rabbit IgG secondary antibody (Dako/K4061) diluted according to the manufacturer's instructions was incubated for one hour at 4 °C and the reaction was developed with diaminobenzidine (DAB, Sigma-Aldrich, St. Louis, USA) and Harris hematoxylin counterstaining. The slides were mounted with Erv-mount (Erviegas EasyPath, DuraEdge, USA) and analyzed under light microscopy, and the images were captured using the Olympus Stream Software (Olympus Life Science, Center Valley, PA, USA).

2.6. Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) was performed as described by Ibrahim-Granet et al. [38]. Briefly, the MΦs were readjusted to a concentration of 3.0×10^7 cells/well and distributed in triplicate in a 12-well plate and infected with live *Ah* at a concentration of 1.5×10^9 on round coverslips (13 mm). The coverslips were fixed in 2.5% glutaraldehyde and washed with 0.2 M phosphate buffer. Next, the coverslips were dehydrated with acetone and desiccated in a Critical Point Dryer (Bal-Zers CPD030). The coverslip were mounted in a support, and the material was sputtered with gold in a Sputter Coater (Bal-Zers SCD050). The images were captured by SEM (JSM 6060, Jeol).

2.7. Transmission electron microscopy (TEM)

Ultrastructural processing and analysis were performed according to Esteban et al. [39]. The distribution and concentration of MΦs and *Ah* were the same as those of SEM. The cells were detached and centrifuged at 400 × *g* to form a pellet, which was resuspended and fixed in Karnovsky's solution (2.5% glutaraldehyde; 2% formaldehyde in 0.1 M phosphate buffer pH 7.4) for 2 h at 4 °C. The pellet was then washed three times in 0.1 M phosphate buffer and fixed with 1% osmium

tetroxide in cacodylate buffer for one hour, and the images were captured by transmission electron microscopy (Jeol-100CXII).

2.8. Electrophoresis and western blot

For the western blot assay, the proteins in the supernatant of the swim bladder inflammatory exudate were quantified using the method described by Bradford [40], separated on 12% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) according to Laemmli [41] and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membrane was then blocked with PBS-milk (Molico, Nestlé Brazil, São Paulo, Brazil) at 10% 0.25 M pH 7.2 at room temperature for two hours, washed with 0.1% PBS-Tween 20 and incubated with anti-TNF-α (Anaspec/AS-55383) in a 1:1000 dilution under agitation for two hours and washed with 0.1% PBS-Tween 20. The conjugate (Dako/K4061) was diluted according to the manufacturer's instructions, added to the membrane and incubated for one hour at room temperature. The reaction was developed with diaminobenzidine substrate (DAB, Sigma-Aldrich, St. Louis, USA) and blocked by adding deionized distilled water, and the membrane was scanned and analyzed with the Image Lab software (Bio-Rad, Hercules, CA, USA).

2.9. Experimental design

For evaluation of *in vitro* cell infection and IgY immunotherapy, the MΦs were adjusted to a concentration of 3.0×10^5 cells/well distributed in triplicate and the culture was incubated at 28 °C in a 5% CO_2 atmosphere. The negative control (cells + PBS), positive control (cells + bacteria), specific IgY (cells + bacteria + specific IgY) and nonspecific IgY (cells + bacteria + nonspecific IgY) at the concentration of 300 μg/mL were distributed in a 96-well tissue culture plate. The cellular activity parameters were measured at preset times 6, 24 and 48 h after infection with 1.5×10^7 live *Ah*/well. The experimental design was maintained for the assays below.

2.10. Acid phosphatase (AP) activity

The AP activity was measured according to methods described by Yang et al. [42]. For this purpose, the MΦs were readjusted, and the treatments were distributed. Next, the wells were washed and 100 μl/well of a solution containing 0.1 M sodium acetate pH 7.2, 0.1% Triton X-100 and 5 mM *p*-nitrophenyl phosphate (Sigma-Aldrich, St. Louis, USA). The reaction was incubated for two hours and blocked with 10 μl/well of 1 M NaOH solution, and the absorbance was measured at 405 nm in a microplate reader.

2.11. Respiratory burst activity

The nitro blue tetrazolium (NBT) assay (Sigma-Aldrich, St. Louis, USA) was performed according to methods described by Grayfer et al. [43]. The predetermined treatments were infected with live *Ah,* and PMA (phorbol-12-myristate-13-acetate) at 100 ng/mL (Sigma-Aldrich, St. Louis, USA) was added. After the incubation period, 100 μl/well of 2 mg/mL NBT (Sigma-Aldrich, St. Louis, USA) was added, and the mixture was incubated for two hours. Finally, the plate was centrifuged at 400 × *g* for 10 min, the supernatant aspirated and 140 μl/well of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, USA) and 120 μl of 2 M KOH were added, and the reaction was scanned at 630 nm in a microplate reader.

2.12. Production of nitric oxide (NO)

NO production was determined using the Griess reaction described by Stafford et al. [44]. To that end, 75 μl/well of supernatant from the MΦs culture was transferred to a new 96-well tissue culture plate, the volume of the supernatant was conjugated in an equal volume of the Griess reagent (1% sulfanilamide, 0.1% of N-naphthyl ethylenediamine and 2% phosphoric acid (Sigma-Aldrich, St. Louis, USA)), and the reaction was incubated for 10 min and its absorbance read at 540 nm in a microplate reader.

2.13. Direct immunofluorescence (IMF)

For the IMF analysis, the MΦs were cultured at a concentration of 3.0×10^7 cells/well at a final volume of 1 mL/well, distributed in triplicate in a 12-well plate containing a round coverslip (13 mm) per well for cell adhesion and incubated at 28 °C under a controlled atmosphere at 5% CO_2. For each experimental treatment and period after infection with 1.5×10^9 live *Ah,* a sample was collected on a coverslip and fixed, the plasma membrane was permeabilized, and nonspecific sites were blocked as described for the immunocytochemistry assay in item 2.5. Then, the MΦs were incubated for 30 min at room temperature with fluorescein isothiocyanate-conjugated anti-*Ah* IgY (Sigma-Aldrich, St. Louis, USA) (FITC anti-*Ah* IgY) according to Fernandes et al. [36]. For nuclei detection, 10 g/mL of 4,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, USA) was added for 10 min at room temperature. The coverslips were mounted with Fluoromount (Sigma-Aldrich, St. Louis, USA) and analyzed under a fluorescence microscope (Olympus BX56 series; Olympus Life Science, Center Valley, PA, USA).

2.14. Flow cytometry

The MΦs and treatments were adjusted, distributed and incubated according to item 2.9. The supernatant from the MΦs culture was removed, and the wells washed once with PBS pH 7.4 and the plate centrifuged at 400 × *g* for 4 min at 4 °C. The supernatant was aspirated and 0.2% lysozyme was added, which was incubated at 28 °C for 20 min. Then, the wells were washed and the plate centrifuged, followed by the addition of 0.1% Triton X-100 and incubation for one hour. The plate was then centrifuged, the supernatant discarded and the pellet resuspended and transferred to a new 96-well plate. Then, 5 μg/mL propidium iodide (PI) (Sigma-Aldrich, St. Louis, USA) was added and the samples injected into a flow cytometer (BD FACSCanto I). Data were processed using the FCS Express 6 Plus software.

2.15. Intracellular bacterial viability in macrophages

In parallel with item 2.14, pellet aliquots from each treatment were transferred to a 96-well plate and seeded by the *pour plate* technique in Petri dishes containing TSA; the cells were incubated at 28 °C for 24 h, and the number of colonies were counted using colony counters (Phoenix CP608, BR).

2.16. Mitochondrial activity

The mitochondrial activity of the MΦs was determined using the method described by Mosmann [45]. After the incubation period of each experimental time and treatment, 20 μL/well of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (Sigma-Aldrich, St. Louis, USA) was added, and the reaction incubated for 4 h. Next, the supernatant was removed, 200 μl/well DMSO was added for solubilization of the formazan crystals, and the absorbance was measured at 595 nm.

2.17. Statistical analysis

Data were analyzed by ANOVA and Tukey's test at a significance level of 5% [46].

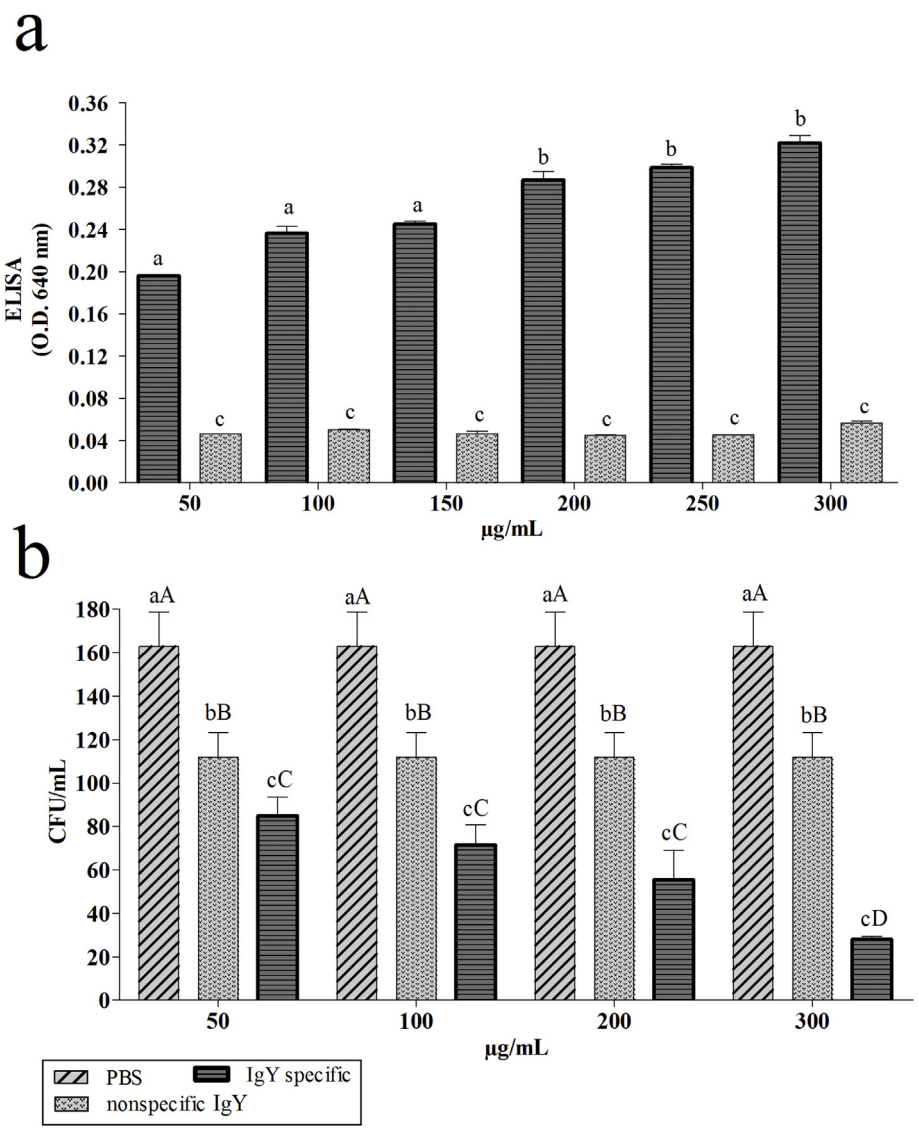

Fig. 1. Functional characterization of anti-*Aeromonas hydrophila* IgY *in vitro*. (a) *Ah* neutralization by ELISA and (b) bacterial growth neutralizing activity. *A. hydrophila* at a concentration of 1.5×10^7 CFU/ml, phosphate buffered saline (PBS) control group, and placebo (nonspecific IgY) tested at constant concentration of 300 µg/ml. Means in triplicate and respective standard deviation, lowercase letters compare the different treatments at the same concentration, and uppercase letters compare the different concentrations, with different letters representing significant difference by Tukey's test ($p < 0.05$).

3. Results

3.1. Functional evaluation of IgY in vitro

The concentration of specific IgY necessary to neutralize the pathogen was between 200 and 300 g/mL relative to the infective dose of *Ah* at a concentration of 1.5×10^7 CFU/mL. Nonspecific IgY had no significant reactivity compared with the specific IgY (Fig. 1a). At the time at which the bacterial growth inhibition potential of IgY was evaluated, all concentrations had an inhibitory effect *in vitro* when compared with the control group (PBS) and nonspecific IgY (placebo effect) at the constant concentration of 300 µg/mL (Fig. 1b). However, the specific IgY concentration of 300 µg/mL was more effective at neutralizing bacterial growth, reducing it to approximately 28 CFU/mL compared with the nonspecific IgY (112 CFU/mL) and PBS (163 CFU/mL) controls.

3.2. Macrophage culture

After 72 h of *Ah* membrane protein (MP)-induced aerocystitis, the adherent cells exhibited cytomorphological features of MΦs, with large and irregular nuclei with abundant cytoplasm and cytoplasmic vacuoles. In the same time period, the proteinogram of the exudate supernatant indicated a pattern of inflammatory proteins and the immunoblot confirmed the production of TNF-α (molecular mass of 18.6 kDa) (Fig. 2a). Immunocytochemistry showed cells positive for MHC class II and CD68, characterizing the phenotype and culture of MΦs as well as the production of TNF-α proving the involvement of these cells in the inflammatory process and of the iNOS in the phagolysosomal activity (Fig. 2b). After isolation and culture of *Ah* and MΦs, the ultrastructural features evaluated by SEM of *Ah* culture cells revealed a rod shape with presence of pili (Fig. 2c), whereas the *Ah*-infected MΦs culture showed ameboid-shaped cells and engulfed rod-shaped structures (Fig. 2d). The TEM results confirmed the phagocytosis by the MΦs, with the anchoring of the pseudopodia, engulfment of the bacterial cell and fusion of the lysosome to the phagosome, forming the phagolysosome (Fig. 2d).

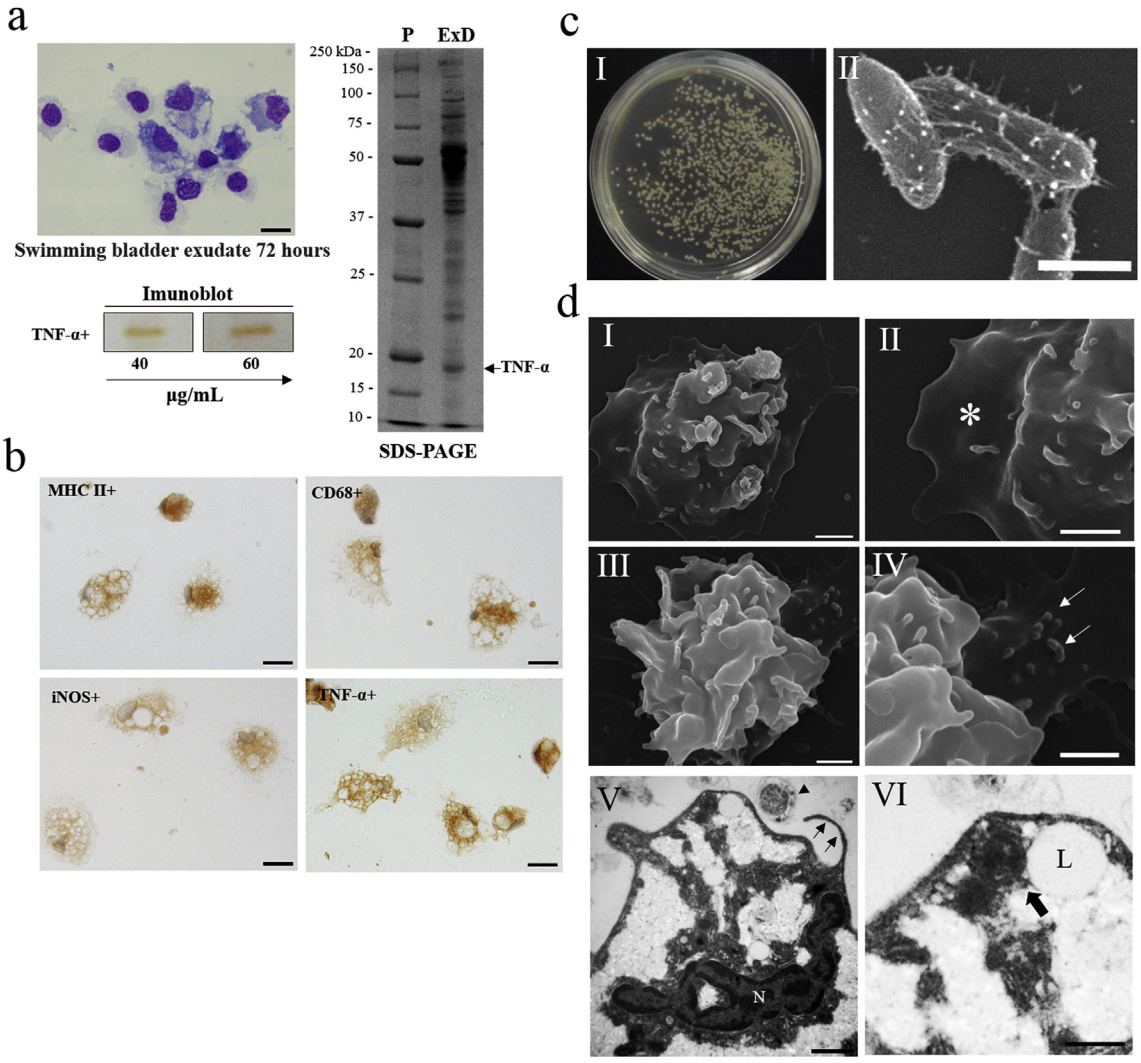

Fig. 2. Cellular and inflammatory characterization of MΦs isolated from the swim bladder of Nile tilapia. (a) Cytology and protein profile of inflammatory exudate (ExD); (b) immunocytochemistry (IC) of the MΦs culture; (c) (I) bacterial culture and (II) scanning electron microscopy (SEM) of *Aeromonas hydrophila (Ah)* and (d) (I-II) negative control and (*) no phagocytic activity, (III-IV) MΦ in phagocytic activity, with arrows indicating engulfed rods, and (V) transmission electron microscopy (TEM), with arrowhead showing the *Ah* and arrows showing the formation of the pseudopod of the MΦ and (N) nucleus; and (VI) arrow indicates the phagosome and (L) lysosome. Wright and Giemsa bars (cytology): 10 µm; IC: 10 µm, Harris hematoxylin and 3′-diaminobenzidine; SEM and TEM: 0.5–1 µm.

3.3. Effect of IgY on the phagolysosomal activity of Ah-infected macrophages

FITC anti-*Ah* IgY was able to identify *Ah* bacterial cells by direct immunofluorescence and confirmed the neutralization of the bacterial cells by the antibody (Fig. 3a). In the images representative of the 48-h time-point, the ability of MΦs to engulf the *Ah* was evidenced in all the treatments; however, the treatment with specific IgY seems to have facilitated the phagocytic process of the bacterial cells when compared with the other treatments (Fig. 3b). In addition, when the phagolysosomal process was evaluated, the specific IgY had a modulatory effect on LAP activity, reducing the activity of this enzyme at 24 and 48 h after infection compared with the other treatments (Fig. 3c). Moreover, the negative control (cells + PBS) had baseline activity levels as expected since the phagocytes were previously activated in the swim bladder with *Ah* MPs; the positive control (cell + bacteria) showed an increase in the period between 6 and 48 h after infection compared with the other treatments; and the nonspecific IgY increased during the period between 6 and 48 h. The production of ROIs started 6 h after infection, and the highest production level was observed at 24 and 48 h for the specific IgY treatment and positive control (cells + bacteria) (Fig. 3d). The production of NO increased at all experimental time points the for treatments infected with *Ah*; however, at 48 h, although the positive control (cells + bacteria), specific IgY and nonspecific IgY increased, only the specific IgY presented a significant difference compared with the other times and treatments (Fig. 3e).

3.4. IgY in Ah neutralization and survival in macrophages

The specific IgY reduced the intracellular survival of *Ah* in MΦs, with the flow cytometry histogram showing increased labeling of the bacterial DNA by PI at 6, 24 and 48 h after infection compared with the other treatments (Fig. 4a), and the statistical analysis confirmed these findings (Fig. 4b). When the intracellular content of MΦs was seeded in a tissue culture plate, the number of viable bacterial colonies for specific IgY treatment was zero between 6 and 48 h after infection, and minimal growth was observed during the 24-h period (Fig. 4c). Fig. 4d shows representative images of the plating of the cytoplasmic content.

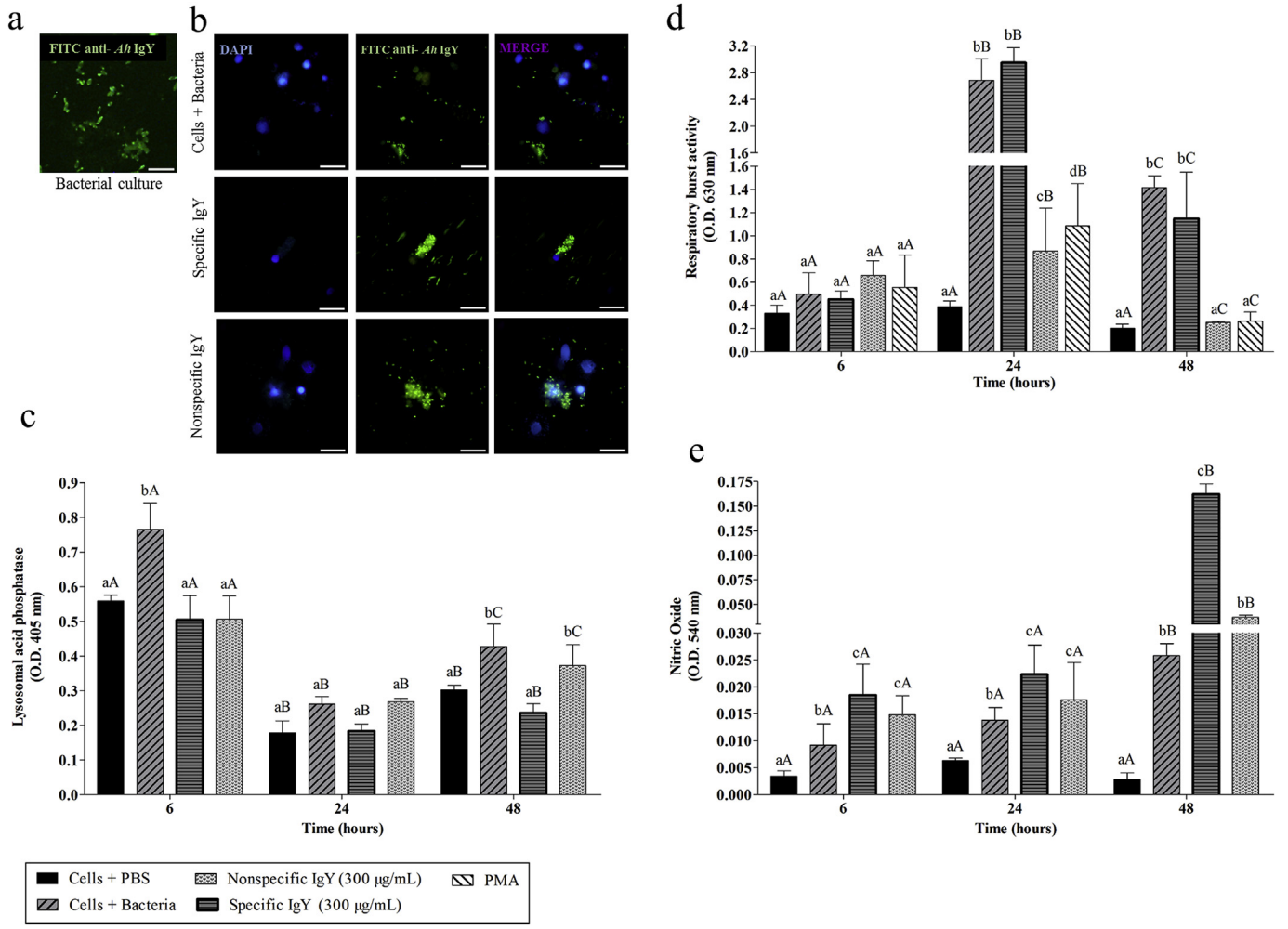

Fig. 3. Effect of immunoglobulin Y on the phagolysosomal activity of MΦs of Nile tilapia. (a) Detection of *Aeromonas hydrophila* (*Ah*) with fluorescein isothiocyanate-conjugated specific IgY (FITC anti-*Ah* IgY); (b) direct immunofluorescence showing *Ah* phagocytosis by MΦs at 48 h with respective IgY treatments and labeled with FITC anti-*Ah* IgY; (c) lysosomal acid phosphatase; (d) respiratory burst activity and (e) nitric oxide production. Bars: 10 µm. Means in triplicate and respective standard deviation, lowercase letters compare the different treatments at the same time-point, and uppercase letters compare the different time-points, with different letters representing significant difference by Tukey's test ($p < 0.05$).

3.5. Effect of IgY on the survival and proliferation of Ah-infected macrophages

The infectious process of *Ah* in the MΦs culture is illustrated in the complementary video, where it is possible to observe the bacterial and extra/intracellular movement in the phagocyte phagosome. The activation of MΦs is shown in Fig. 5a and b, and the adhesion of *Ah* to the cell, as well as its cytolytic effect, is shown in Fig. 5c and d. When the mitochondrial activity was evaluated, the specific and unspecific IgY had a similar effect with increased mitochondrial activity by MΦs during the period between 6 and 24 h, with a reduction in these levels at 48 h compared with the positive control (cells + bacteria) (Fig. 5e).

4. Discussion

The anti-*Ah* IgY was previously characterized and assessed for its specificity against *Ah* cytoplasmic and membrane proteins and the ability to detect and study the kinetics of infection by the homologous bacterium in infected Nile tilapia tissues [36]. In the present study, the potential of IgY to neutralize *Ah in vitro* was predetermined, and the ratio between the ideal amount of specific IgY required for the saturation of the binding sites of the bacterial wall and cell membrane proteins was 300 µg/mL relative to the infective dose of 1.5×10^7 CFU/mL *Ah*. In addition, the same IgY concentration was effective for neutralizing and inhibiting bacterial growth *in vitro*, confirming previous findings. This *in vitro* neutralizing effect of IgY is postulated in the scientific literature, with similar results being observed for *Vibrio anguillarum* [25], *Staphylococcus aureus* [47,48] and *Clostridium difficile* [49]. The nonspecific IgY tested had a neutralizing effect at baseline levels possibly arising from nonspecific binding and cross reactions.

The collection of peritoneal MΦs by prestimulation of the peritoneal cavity and subsequent aspiration of these cells is a method that has been postulated in mammals [50–52] and fish [53–55]. The collection of leukocytes from the swim bladder in fish, because it is a hollow organ, provides a sterile microenvironment [56,57] and the absence of other contaminating tissues such as the intestines, in the case of the peritoneal route, thus avoiding failures in the cell culture of MΦs, enabling through the chronology and cellular kinetics of the inflammatory process different cell types, such as neutrophils at 24 h [58,59] and MΦs after 72 h, to be obtained, as observed in this study. In addition, the aerocystitis induced with *Ah* PMs allowed the presensitization and migration of the leukocytes and subsequent collection of the MΦs, mimicking a natural infection. Some of these proteins have chemotactic potential such as their use as microbial ligands for phagocytic receptors including proteins, glycoconjugates and complex lipids, e.g., mycobacterial lipopolysaccharides and lipoteichoic acids [60]. The

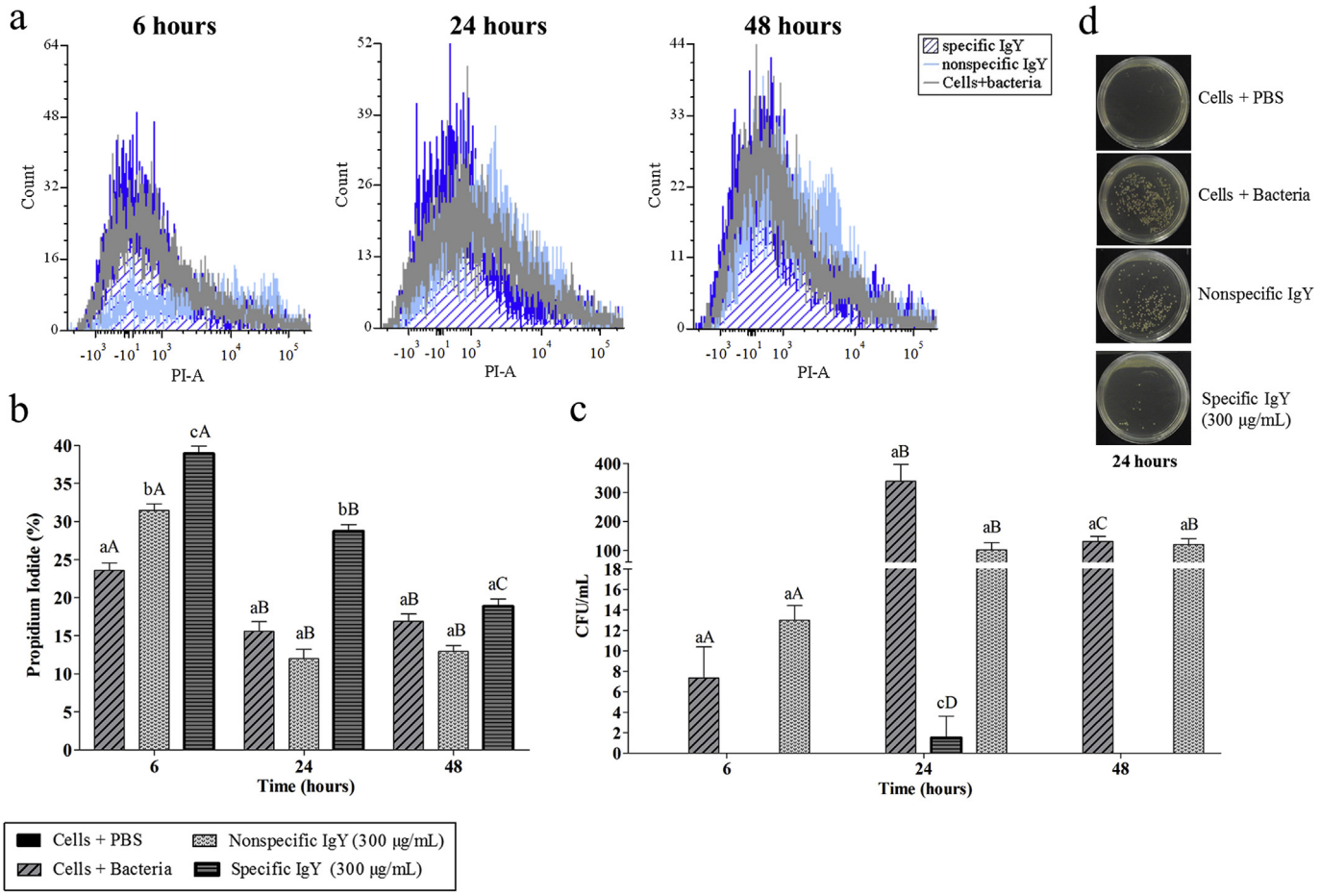

Fig. 4. Effect of immunoglobulin Y on the intracellular viability of *Aeromonas hydrophila* (*Ah*) in MΦs of Nile tilapia. (a) Analysis of *Ah* DNA fragmentations in MΦs culture and labeled with propidium iodide (PI); (b) percentage of nonviable bacterial cells; (c) plating of the cytoplasmic contents of MΦs infected with *Ah*; and (d) representative image of respective plating 24 h after infection. Means in triplicate and respective standard deviation, lowercase letters compare the different treatments at the same time-point, and uppercase letters compare the different time-points, with different letters representing significant difference by Tukey's test ($p < 0.05$).

production of preactivated MΦs in the swim bladder and the use of cellular bioenergetics for migrating to the inflammatory focus establishes an important parameter and allows for evaluation of this cell in its pathophysiological state, together with all the inflammatory mechanisms prior to the phagocytic process of the microorganism. Although the use of MΦs obtained from the head kidney [61,62] and blood monocytes [63,64] is very widespread and the *in vitro* activation of the monocytes with PMA [65] and LPS [66] is possible, the energy cost in these cells used for migration cannot be measured, and these activators do not have the same properties as do whole bacterial proteins. In our study, the production of TNF-α in the supernatant of the swim bladder exudate and its expression by the MΦs in the culture

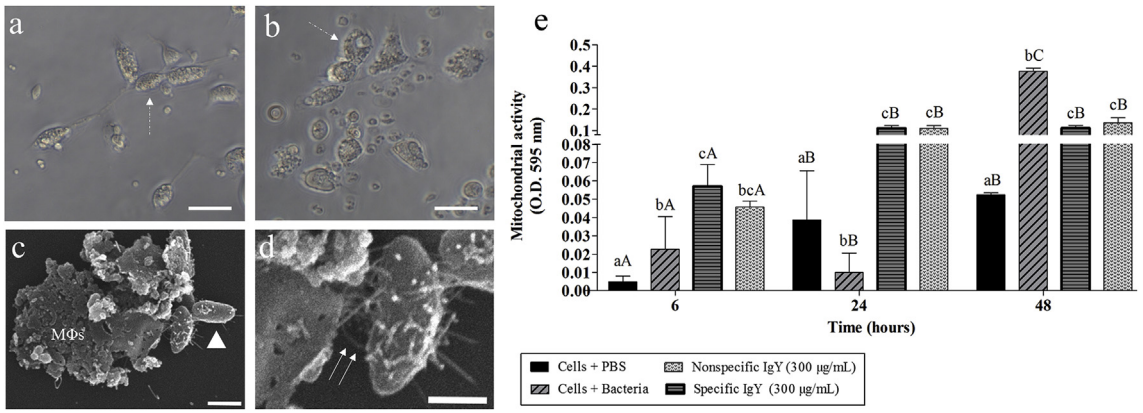

Fig. 5. Mitochondrial activity of MΦs infected with *Aeromonas hydrophila* (*Ah*). (a–b) MΦs culture, dotted arrows indicate active cells; (c) adhesion of *Ah* to the necrotic body of a MΦ (arrowhead); (d) adhesion of the pili to the cell (arrows) and (e) measurement of the mitochondrial activity of the MΦs infected with *Ah*. Inverted microscopy bars: 10 µm; SEM: 0.5–1 µm. Means in triplicate and respective standard deviation, lowercase letters compare the different treatments at the same time-point, and uppercase letters compare the different time-points, with different letters representing significant difference by Tukey's test ($p < 0.05$).

confirmed the onset of the inflammatory process [67], migration and the participation of these cells in inflammation. In addition, the cytomorphology and expression of MHC class II and CD68 characterized a cellular phenotype compatible with MΦs, whereas the iNOS production was involved in the phagolysosomal activity of these cells.

The main virulence factors associated with *Aeromonas* spp. are capsule, lipopolysaccharide, glucan, S-layer, exotoxins and extracellular enzymes, secretion systems, fimbriae, motility and flagella [68]. The presence of pili and the integrity of the other bacterial structures observed in the ultrastructural analysis of *Ah* as well as the ability to infect the host observed in the assay with the pre-established lethal dose 50 (LD50) (data not shown) confirm the virulence of this strain. The ability of *Ah* to infect MΦs and the phagolysosomal process were evidenced by SEM and TEM. The IgY, because it has multiple specificity for distinct antigenic epitopes present in *Ah* membrane and cytoplasmic proteins [36], seems to have neutralized the bacterial cell via surface proteins and virulence factors related to the blockage of the phagocytic process, such as the superoxide dismutase enzyme (SOD), which dismutes superoxide to oxygen and hydrogen peroxide, neutralizing the ROIs production pathway [69]. Moreover, the neutralization/opsonization of anti-*Ah* IgY was proven in the direct immunofluorescence assay of the *Ah* culture. This finding indicates the possible effect of IgY on the physical blockage of bacterial motility, facilitating adhesion and envelopment by MΦs, as indicated in the same assay by the increase in these parameters for the group treated with specific IgY after 48 h. In addition, the importance of bacterial motility is related to the evasion of the phagosome [70]. Immunoelectron microscopy revealed that the neutralization of IgY specific to the bacterial surface results in structural alterations of the cell wall, facilitating the phagocytic process [71] and possibly conferring higher surface hydrophobicity [72,73].

Given its neutralizing effects, IgY had an intracellular action on the phagolysosome, modulating the AP activity at all experimental times by reducing the activity of this enzyme. The baseline levels of AP in the negative control (cells + PBS) group were expected, since the cells were preactivated with *Ah* MPs. Chung and Secombes [74] observed an increase in AP in MΦs preactivated with inactivated *Aeromonas salmonicida* emulsified with incomplete Freund's adjuvant. This finding explains the increase in the positive control (cells + bacteria) at 6 h after infection. Dai et al. [75] showed that *Francisella* spp. has at least four APs Acp (-A, -B, -C, and Hap) and suppresses the oxidative burst after the bacterium is phagocytosed by MΦs of the host [76]. The effects of APs allowed the survival of *Francisella* spp., avoiding intracellular death in the MΦ phagosome [77]. Similar effects were observed at the beginning of the infection (6 h) with increased AP activity in the positive control treatment (cells + bacteria), which can be related to the bacterial APs, given the hypothesis that *Ah* uses mechanisms similar to those of *Francisella* spp., and the fact specific IgY seems to have blocked the activity of this bacterial enzyme selectively, as it has reactivity for *Ah* cytoplasmic proteins [36]. However, this increase may be associated with the production and activity of LAP in response to the pathogen [78]. Therefore, the baseline levels of nonspecific IgY treatment were partially similar to those of the negative control (cell + PBS) proving the inactivity of the IgY for the AP of tilapia MΦs. Thus, specific IgY had an indirect and modulatory effect on LAP activity of the host phagocyte, given the lack of information on the phagolysomal AP activity in the phagocytic process of fish MΦs, as well as the action of IgY on this enzyme. Our findings open new opportunities for understating this mechanism in its natural infectious course, as observed in the positive control (cells + bacteria) and in the intervention of IgY applied in immunotherapy.

The fact that AP increased at the beginning of the 6 h time-point for all treatments was expected, since AP is involved in altering the phagolysosomal pH for subsequent activation of the other enzymes associated with the production of the superoxide anion [79] and nitric oxide synthase [80]. This is shown by the production of the superoxide anion at 24 and 48 h, proving the role of the AP on the production of these reactive species. However, NO production was later increased at 48 h after infection for the treatment with specific IgY, leading to intracellular clearance of *Ah,* as will be discussed later. Qin et al. [73] produced anti-*A. hydrophila* IgY in chickens immunized with inactivated *A. hydrophila* and reported the efficacy of the antibodies in the prophylaxis and treatment of *A. hydrophila* infection in *Megalobrama amblycephala*. The results showed that anti-*A. hydrophila* IgY in head kidney MΦs challenged with *A. hydrophila* had increased production and release of NO and superoxide anion 60 min after *in vitro* infection. Our results, although different, are complementary to the conclusions of those authors, who used a different method for the sensitization and isolation of the MΦs. In addition, it has been shown that these cells used metabolic energy in chemotaxis, diapedesis and migration to the inflammatory focus [81] and are involved in the chronological course of infection, evidencing a chronic stage of the inflammatory process and critical stage of the phagocytic response to *Ah*.

When the intracellular viability of *Ah* was evaluated, the effects of the phagolysosomal activity discussed above appeared to converge and the specific IgY reduced the intracellular survival of the pathogen at 6 h, but growth of the *Ah* was observed at 24 h, suggesting a possible attempt at adaptation through the blocking of the phagocytic mechanisms by *Ah*. To some extent, this result supports the hypothesis that intracellular survival is a strategy to subvert immunological defense mechanisms and to avoid extracellular bactericidal agents such as antibiotics that can lead to chronic infections or relapses [82]. At 48 h, the specific IgY neutralized the infection, as shown in flow cytometry by bacterial DNA labeling and confirmed by plating and negative growth of the cytoplasmic content of MΦs. IgY can be a powerful tool against aeromonas infections, thus replacing the use of antibiotics for treatment, since it does not cause side effects, disease, microbial resistance, or toxic residues [37,83,84].

The mitochondrial activity was measured to evaluate the cellular activity against *Ah* infection; the results showed an MΦs activity at 6 and 24 h, and this activity was reduced at 48 h in specific and nonspecific IgY treatments compared with the increase of this activity at 48 h in the positive control (cells + bacteria), which despite being a response to infection, leads to excessive free radical production causing tissue damage and cellular energy expenditure [85]. In contrast, specific and nonspecific IgY treatments used less of this cellular energy during this time period, and in the case of specific IgY, infection was controlled by the ability to neutralize and block *Ah* virulence factors. Although these treatments showed similar activity levels, in the case of nonspecific IgY, we suggest the use of a cross-reaction and presensitization given that it is an endogenous protein that is promoting this effect. Using higher levels of mitochondrial energy suggests that *Ah* was partially cleared and survival factors were active, preventing MΦs from fighting infection and forcing the cell to adapt through increased mitochondrial activity. Qin et al. [86] described the importance of the *Ah* flagella in infection, showing that mutant strains lacking a flagellum known as B11 were able to invade the MΦs of *Anguilla japonica in vitro* but survived in the MΦs for only 24 h, suggesting that flagellar motility contributes to the invasion and survival of *A. hydrophila* in host MΦs. The S-layer performs several biological functions such as adhesion and protection against attack by the complement system and phagocytes [87,88]. The *Ah* active cytotoxic enterotoxin binds to a target cell surface glycoprotein, forming pores in the host cell membrane and triggering early cell signaling in eukaryotic cells with important action on infection and production of inflammatory mediators in MΦ and epithelial cells [68,89,90]. In addition, it is an important contributor to apoptosis [91]. In agreement with other authors, the presence of pili and the integrity of the capsule reported as well as the extra/intracellular motility in the MΦs of tilapia (complementary video), in addition to the adhesion of *Ah* to a necrotic cell body, prove the infective ability and cytolytic action of the bacteria. In view of this, we suggest that the specific IgY had an indirect modulatory effect on the mitochondrial activity of the MΦs, reducing the energetic expenditure

of the cellular response during the course of inflammation, and the *Ah* MPs had a basal effect on the mitochondrial activation observed in the negative control (cells + PBS).

The recognition and adhesion of MΦs in the opsonized and non-opsonized bacteria are mediated by receptors for the Fc fraction and for standard molecules of microorganisms, respectively, and the changes in the physicochemical conditions of the bacterium potentiate the phagocytic activity [92]. Due to the phylogenetic distance between birds and fish [93] and the structural difference between the IgY and IgM molecules in fish, which is the isotype predominant in the serum of this species due to the involution of the humoral immune response [94], important questions are raised about which receptor the Fc fraction of IgY in birds interacts with in the MΦs of fish or other phylogenetically distant hosts, and this topic requires further studies. Gray et al. [95] suggest that although the activation of the Fc receptors is important for the increase in lysosomal capacity, through the degradation of the transcription factor EB (TFEB)-dependent form, a gene responsible for the induction and activation of the lysosome, non-opsonized *E. coli* bacterial cells also have this ability, which shows the independence of this receptor for activation of this pathway, further proving that MΦs preinfected with *E. coli* have increased phagocytic capacity. This proves that marked activity of specific and moderate activity of nonspecific IgY may be associated with the activation of receptors other than the Fc ligands of fish MΦs, resulting in the neutralization of *Ah* in the case of the specific IgY and in the sensitization of other receptors for non-specific IgY.

Promising studies have shown the bactericidal potential of IgY against gram-negative bacteria in immunotherapy and prevention against infectious diseases, including *Pseudomonas aeruginosa* infection in cystic fibrosis [92], *Acinetobacter baumannii* infection in hospital-acquired sepsis and pneumonia [37], *Porphyromonas gingivalis* infection in periodontitis [96], *Vibrio parahaemolyticus* and *Vibrio vulnificus* infection associated with gastroenteritis in humans [97] and *Shewanella marisflavi* infection in skin ulceration syndrome in sea cucumbers (*Apostichopus japonicus*) [98]. According to Li et al. [25], MΦs were isolated from ayu fish head kidney were treated with specific IgY, infected with FITC-labeled *V. anguillarum* and analyzed 30 min after infection. The results showed that the phagocytic percentage of MΦs from the group treated with specific IgY was higher than that from the group treated with nonspecific IgY. Previous studies have reported that specific IgY exhibits bactericidal and bacteriostatic activities through opsonization of the bacteria for phagocytosis of MΦs or for inhibition of bacterial growth [99–101].

Finally, it was possible to conclude that the method of sensitization and isolation of the MΦs via the swim bladder was effective, enabled obtaining selective cultures and provided a functional model for studying inflammatory and infectious processes. In addition, the IgY produced had a neutralizing action on bacterial surface proteins and proteins preformed in the cytoplasm by blocking adhesion, facilitating opsonization and phagocytosis, and favoring intracellular *Ah* survival mechanisms. Modulatory action was observed in the phagocytic process through the production of AP and consequently of ROIs, controlling the infection without excessive production of these free radicals, thus avoiding tissue damage to the host. In addition, the importance of NO in the bactericidal action of *Ah* was shown, as well as the natural route of infection in a chronic stage. The combination of these effects in the neutralization of *Ah* provided a reduction in the energy expenditure of the MΦs and confirmed the therapeutic action of IgY in *Ah* infection.

Ethics committee

The experiment was carried out in accordance with the regulations of the National Council for the Control of Animal Experimentation (CONCEA) and approved by the ethics committee on the use of animals under protocol number 017204/15.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.12.074.

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