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Infecção experimental de tambaqui (*Colossoma macropomum*) por *Aeromonas hydrophila*: avaliação de antimicrobianos e da resposta imune do hospedeiro

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Médica Veterinária

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SUMÁRIO

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RESUMO

As enfermidades têm se destacado como um dos principais entraves para o desenvolvimento da aquicultura, e apesar do tambaqui *Colossoma macropomum* ser a espécie nativa mais produzida na América do Sul, pouco conhecemos sobre as enfermidades que o afetam e como o seu mecanismo imune reage frente às infecções. A septicemia hemorrágica é causada pela bactéria *Aeromonas hydrophila*, e se destaca como uma das principais enfermidades na aquicultura, principalmente em espécies tropicais. Por isso, confirmamos a patogenicidade de *A. hydrophila* pelo Postulado de Koch e estabelecemos as doses letais até 80%, estimando a DL_{50} em 5, 57 x 10^7 a 1, 41 x 10^8 UFC/ml. Não recomendamos o verde malaquita como recurso profilático e indicamos ceftriaxona, florfenicol, oxitetracyclina, sulfazotrim e tianfenicol como potenciais antimicrobianos para o controle desta bactéria, mas consideramos os óleos essenciais de cravo *Eugenia caryophyllata* e canela *Cinnamomum zeylanicum* como melhores opções para potencial tratamento da bacteriose, com forte atividade inibitória. Descrevemos um perfil leucocitário incomum de severa leucopenia em período agudo de infecção, devido à liberação de armadilhas extracelulares pelos leucócitos (ETosis). ETosis é um mecanismo de suicídio leucocitário, ainda não descrito para a maioria dos peixes, e que pode ser considerado como um dos últimos recursos imunes adotado pelo hospedeiro para tentar conter a infecção. Este mecanismo pode ser visualizado através de uma metodologia de baixo custo e de fácil execução em microscopia eletrônica de varredura, desenvolvida nesta tese. Como os estudos genéticos beneficiam substancialmente o desenvolvimento da criação de uma espécie, a sequência parcial genética de peptídios antimicrobianos (HSP70, lisozima e proteínas do sistema complemento), receptor de citocina e citocinas (IRAK 1, IL-1β e IL-10) foram descritas. Padrões de expressões

genéticas foram caracterizadas quanto a modulação de cada gene, em resposta às diferentes fases de infecção por *A. hydrophila.* A modulação das expressões dos genes imunes mostrou-se aumentada em peixes infectados, tanto na fase inicial da infecção (principalmente em torno de 6h e 24h) quanto na fase crônica (7d e 14d). Apenas o gene associado à regulação térmica, HSP70, mostrou-se negativamente modulado em peixes infectados. Destacamos o aumento na expressão dos genes de citocinas e lisozima, evidenciando atividade pró e antiinflamatórias. Porém, o maior destaque é dado para os genes HSP70 e proteínas do sistema complemento: C3 e C4. A modulação dos genes de HSP70 foi afetada negativamente em peixes infectados, sugerindo que peixes afetados por *A. hydrophila* ficam mais propícios à sensibilidade térmica e às infecções secundárias. Com relação às proteínas do sistema complemento, apesar da regulação aumentada na expressão do gene C4, seguindo a tendência pró-inflamatória, o gene C3 surpreendentemente não foi expresso na maioria dos peixes saudáveis e infectados. Adicionalmente, na análise de do sistema complemento no soro, a ausência de atividade hemolítica corrobora com os resultados de expressão gênica, sugerindo provável deficiência no sistema complemento desta espécie, nas condições testadas. A ausência de dados na literatura e as possíveis razões para a regulação da expressão gênica e associação com doenças de peixes são abordadas nesta tese.

Palavras-chaves: aquicultura, bacteriose, imunidade inata, peixe, peixe nativo, septicemia hemorrágica

ABSTRACT

Diseases have emerged as one of the main obstacles to aquaculture development, and although tambaqui *Colossoma macropomum* is the most produced native species in South America, little is known about the diseases that affect this fish and how its immune mechanism reacts to infection. Hemorrhagic septicemia is caused by the bacterium *Aeromonas hydrophila*, and stands out as one of the major diseases in aquaculture, especially in tropical species. Therefore, we confirmed the pathogenicity of *A. hydrophila* by the Koch's Postulate and established the lethal doses up to 80%, estimating the LD_{50} at 5.57 x 10^7 to 1.41 x 10^8 CFU/ml. We do not recommend malachite green as a prophylactic resource and we recommend ceftriaxone, florfenicol, oxytetracycline, sulphazotrin and thiamphenicol as potential antimicrobials for this bacterium control, but we consider the essential oils of clove *Eugenia caryophyllata* and cinnamon *Cinnamomum zeylanicum* as the best options for potential treatment of bacteriosis, with strong inhibitory activity. We describe an unusual leukocyte profile with severe leukopenia during acute infection due to the release of leukocyte extracellular traps (ETosis). ETosis is a mechanism of leukocyte suicide, not described yet for most of fish species, and it can be considered as one of the last immune mechanisms adopted by the host to try to contain the infection. This mechanism can be visualized through a low-cost and of easy-execution methodology, developed in this thesis. As the genetic studies substantially benefit the development of a species farming, the genetic partial sequence of antimicrobial peptides (HSP70, lysozyme and complement system proteins), cytokine receptor and cytokines (IRAK 1, IL-1β and IL-10) were described. The patterns of gene expression were characterized for of each gene in response to the different stages of *A. hydrophila* infection. The immune gene expression was shown to be increased

in infected fish, both in the initial phase of infection (mainly around 6h and 24h) and in the chronic phase (7d and 14d). Only the gene associated with thermal regulation, HSP70, was shown to be down-regulated in infected fish. We highlight the modulation of cytokine and lysozyme genes, evidencing pro and antiinflammatory activities. However, a great prominence is given to the genes related to HSP70 and complement system proteins: C3 and C4. The HSP70 gene was down-regulated in infected fish, suggesting that fish affected by *A. hydrophila* are more susceptible to thermal sensitivity and secondary infections. Regarding complement system proteins, although C4 gene regulation follows a pro-inflammatory trend, the C3 gene was surprisingly not expressed in most healthy and infected fish. Additionally, in the analysis of the complement system in the serum, the absence of hemolytic activity corroborates with the results of gene expression, suggesting a probable deficiency in the complement system of this species, under the conditions tested. The lack of data in the literature and the possible reasons for the regulation of gene expression and association with fish diseases are addressed in this thesis.

Key-words: aquaculture, bacteriosis, fish, hemorrhagic septicemia, innate immunity, native fish.

Capítulo 1

1. Revisão bibliográfica

1.1. Aquicultura: cenário atual e tendências para a produção de tambaqui

Os últimos dados divulgados pela FAO (Food and Agriculture Organization of the United Nations) em Sofia (2018) demonstram que o continente Americano é o segundo maior produtor mundial de peixes, atrás apenas do continente Asiático (composto por países como a China, que é o maior produtor mundial) (Sofia, 2018). Por isso, se excluirmos as regiões geográficas abrangidas pelo continente Asiático, a América do Sul passa a ser a maior produtora mundial de peixes (Sofia, 2018). E, dentre os países Americanos, o Brasil se destaca como o 13° maior produtor mundial. Ainda, se considerarmos apenas as espécies produzidas em águas continentais, o Brasil passa a ser o 8° maior produtor mundial (Sofia, 2018) e principal produtor do continente Americano (Valladão *et al.*, 2018).

A piscicultura no Brasil é favorecida pela diversidade de espécies de peixes, disponibilidade de recursos naturais, condições climáticas propícias para a produção das espécies continentais, além da disponibilidade de insumos em quase todo o país. Nos últimos dados divulgados pelo IBGE (referentes à 2017), o Brasil produziu mais de 485 mil toneladas de peixes no ano, correspondendo quase 70% da produção aquícola. Dentre uma enorme variedade de peixes exóticos e nativos com potencial para a aquicultura, o tambaqui, *Colossoma macropomum* se destaca por ser o peixe nativo mais produzido no Brasil (Valladão *et al.*, 2018), totalizando 18,2% da piscicultura nacional (IBGE, 2017). No Brasil, espera-se que nos próximos anos, a tendência de crescimento na produção de espécies nativas ultrapasse a de espécies exóticas, assim como ocorreu historicamente em outros países representativos em produção de peixes (Valladão *et al*., 2018).

O tambaqui *C. macropomum*, descrito como black pacu nas estatísticas da FAO, ou conhecido por cachama em diversos países da América Latina, é um peixe caraciforme,

nativo da Bacia Amazônica. Adaptado às águas quentes, é um animal sensível às variações térmicas (Hashimoto *et al.*, 2012; Fernandes *et al.*, 2018; Valladão *et al.*, 2018), mas se destaca quanto ao potencial produtivo devido seu hábito zooplanctófago, fácil reprodução (Moro *et al.* 2013) e por ser resistente às situações de hipóxia extrema e baixo pH (Wood *et al.*, 2017). Por ter a carne muito apreciada pelos consumidores, este peixe apresenta um alto valor cultural e comercial para diversos países da América do Sul, com destaque para o Brasil, Colômbia, Peru, Venezuela, Bolívia e Equador (Valladão *et al.*, 2018). A produção do tambaqui cresce anualmente no país, com destaque para o estado de Rondônia, que é o maior produtor, seguido do Amazonas, Maranhão, Roraima, Pará e Tocantins, e atualmente, sua criação tem se expandido para outros estados, principalmente aqueles de clima quente, como Norte, Nordeste e Centro-Oeste (IBGE, 2017).

Como um fato comum e histórico em diversos países, a tendência futura é de que a produção de peixes nativos ultrapasse a dos peixes exóticos na América do Sul (Valladão *et al.*, 2018), assim como a produção de tambaqui que já ultrapassou a dos ciprinídeos no Brasil e em breve poderá alcançar a produção da tilápia-do-Nilo *Oreochromis niloticus*. No entanto, com a intensificação da produção do tambaqui, assim como de qualquer outra espécie, ocorre o surgimento de problemas sanitários que podem afetar economicamente a produção, inviabilizando sua comercialização dentro e fora do país.

Pesquisadores concordam que o aparecimento de enfermidades infecciosas na aquicultura está frequentemente associado a elevada quantidade de matéria orgânica nos viveiros de produção, decorrente da alta densidade de estocagem e elevado arraçoamento (uso massivo de ração comercial), além de ser facilitado pelo manejo inadequado e estressante. Assim, baseado nos aspectos atuais da produção do tambaqui e as perdas ocasionadas pelas doenças, o conhecimento sobre agentes etiológicos e a relação destes com o hospedeiro se faz necessário.

1.2. Principais enfermidades de peixes de produção no Brasil: destaque para o tambaqui

As doenças desempenham um papel importante nas perdas em aquicultura. No Brasil, temos relatos de dois principais grupos de patógenos afetando peixes de criação: parasitos (Valladão *et al.*, 2018; Tavares-Dias e Martins, 2017) e bactérias (Sebastião *et al.*, 2015; Valladão *et al.*, 2018; Tavares-Dias e Martins, 2017). Os patógenos que acometem os peixes são considerados primários ou oportunistas. O agente patogênico é considerado primário quando é capaz de causar a doença no peixe, independentemente de sua microbiota ou do estado de seu sistema imunológico, já o patógeno oportunista, causa a doença em situações favoráveis para seu desenvolvimento, como, por exemplo, quando há comprometimento da microbiota normal, das barreiras protetoras do corpo ou do sistema imunológico.

Entre os patógenos já diagnosticados, uma quantidade significativa de parasitos são reportados para diferentes espécies de peixes no Brasil. Alguns parasitos têm se destacado por serem capaz de causar doença com elevadas perdas econômicas no tambaqui, como os protozoários *Ichthyophthirius multifiliis* (Eiras *et al.*, 2012; Santos *et al.*, 2013; Valladão *et al.*, 2018) e *Piscinoodinium pillulare* (Eiras *et al.*, 2012; Santos *et al.*, 2013), além de helmintos como Monogenea (Santos *et al.*, 2013; Costa *et al.*, 2017) e o acantocéfalo *Neoechinorhynchus buttnerae* (Jerônimo *et al.*, 2017; Pereira e Morey, 2018). Frequentemente estes parasitos alteram a integridade das barreiras físicas dos peixes com suas estruturas de adesão, como ganchos, ventosas e outros, favorecendo a entrada de outros patógenos. Assim, o tambaqui também é altamente susceptível às bacterioses oportunistas, sendo elas responsáveis por grandes perdas econômicas na aquicultura.

Apesar de vários indícios de surtos de bacterioses afetando o tambaqui (Sebastião *et al.*, 2015; Valladão *et al.*, 2018), ao contrário das parasitoses, as informações de identificação de bactérias como patógenos no tambaqui são escassas na literatura. Suspeita-se que as bactérias *Flavobacterium columnare* e *Aeromonas hydrophila*, causadoras da 'doença da sela' e 'septicemia hemorrágica' respectivamente, sejam patogênicas e as mais comuns para a espécie. Porém, é de nosso conhecimento que, até o momento, não há estudos publicados confirmando a ocorrência destas doenças. As poucas informações disponíveis de bactérias que afetam o tambaqui foram isoladas de surtos, mas não foram certificadas se a doença se replicava em animais sadios, portanto, há possibilidade de se tratar de contaminações. Para confirmação da patogenicidade de um agente, as bactérias isoladas de peixes em surtos de mortalidade, devem ser identificadas e cultivadas em meio microbiológico e então re-inoculadas em animais saudáveis para confirmação da reprodução dos sinais clínicos e reisolamento do patógeno. No meio científico, estas etapas são conhecidas como o Postulado de Koch e são necessárias para confirmação da doença e auxiliam na descrição da relação patógeno-hospedeiro.

1.3. *Aeromonas hydrophila*: etiologia, sinais clínicos, diagnóstico e tratamento

Bactérias do gênero *Aeromonas* são ubiquitárias, podendo estar presentes em qualquer ambiente aquático. As doenças causadas por *Aeromonas* móveis são conhecidas como septicemias hemorrágicas e afetam, de forma mais contundente, os peixes nas Américas do Norte e Sul, além da Ásia e Europa (Plumb *et al.*, 2018). Surtos de mortalidade causados por *A. hydrophila* são considerados uma grande preocupação para a aquicultura mundial (Pang *et al.*, 2015), sendo responsáveis por grandes prejuízos econômicos em diversas regiões (Abdelhamed *et al.*, 2017).

Normalmente, *A. hydrophila* é considerada um agente secundário à fatores ambientais e estressores (Baumgartner *et al.*, 2017), ou secundário a outros patógenos, como parasitos (Xu *et al.*, 2012) e até mesmo outras bactérias (Peatman *et al.*, 2018). Esta doença destaca-se, principalmente, em peixes produzidos em viveiros, como os siluriformes (Barcellos *et al*., 2008; Baumgartner *et al.* 2017; Shoemaker *et al.*, 2018; Peatman *et al.*, 2018), caracídeos (Biller-Takahashi *et al.*, 2016; Valladão *et al.*, 2018), ciclídeos (Hamid *et al.*, 2017) e ciprinídeos (Plumb *et al.*, 2018), que compreendem espécies de peixes amplamente produzidos nos continentes Americanos. Apesar de afetar principalmente estas espécies, é importante enfatizar que até o momento, nenhum organismo é considerado totalmente imune ou resistente às infecções por *Aeromonas* (Plumb *et al.*, 2018).

Os sinais clínicos de peixes afetados pelo grupo das *Aeromonas* móveis são variáveis e inespecíficos, incluindo além de anemia e hiperemia de toda a superfície corporal, lesões ulcerativas, corrosão e hemorragia das nadadeiras, opacidade ocular, distensão da cavidade celomática, protrusão e vermelhidão na região anal, e perda de equilíbrio (Austin e Austin, 2016; Plumb *et al.*, 2018). Porém, como a virulência entre as cepas é muito variável, não é incomum os animais apresentarem outros sinais de infecção.

O diagnóstico das *Aeromonas* comumente utilizados, baseado apenas em evidências hemorrágicas é inadequado, pois diferentes patógenos podem causar hemorragia em peixes. Desta forma, como discutido anteriormente, o isolamento e identificação da bactéria em surtos de animais doentes, além da inoculação deste antígeno isolado em animais saudáveis, seguidos da manifestação dos mesmos sinais clínicos e reisolamento do mesmo antígeno é a forma mais adequada para descrever novas doenças em animais aquáticos. Há descrição de alguns relatos de septicemia hemorrágica em tambaqui (Sebastião *et al.* 2015; Valladão *et al.* 2018), surubins (Sebastião *et al.* 2015; Valladão *et al.* 2018) e jundiá (Barcellos *et al*., 2008) com isolamento de bactérias do gênero *Aeromonas* no Brasil, mas nenhum registro de

certificação de que o agente isolado é o patógeno causador da doença. O primeiro trabalho desenvolvido nesta tese foi a comprovação da patogenicidade de um isolado de *A. hydrophila* do tambaqui por meio de um estudo de infecção experimental e reisolamento da bactéria, em conformidade com os Postulados de Koch, seguido da descrição dos sinais clínicos, seguido pelo estudo de letalidade.

Uma vez diagnosticada no Brasil, o tratamento da *A. hydrophila* pode ser feito com o uso do florfenicol, que é o único antimicrobiano registrado para o tratamento deste agente no país (Sindan, 2018). Todavia, o medicamento é indicado somente para tratar tilápia (Sindan, 2018). Portanto, seu uso não é regulamentado para peixes nativos. Com toda a problemática sanitária, a qual é limitante para a criação do tambaqui, fomos motivados a avaliar o efeito de alguns potenciais desinfetantes (cloro, permanganato de potássio, verde malaquita, sulfato de cobre, sal), antimicrobianos (oxitetraciclina, florfenicol, tianfenicol) e produtos derivados de plantas, com promissora atividade medicamentosa e desinfectante (alho, canela, citronela, cravo, laranja-doce, limão, manjericão, melaleuca, menta, palmarosa e tomilho), a fim de prover dados para o controle de *Aeromonas* isolada da principal espécie nativa de peixe produzida no país.

1.4. Sistema imune inato dos peixes: principais células e moléculas envolvidas no reconhecimento e destruição do patógeno

O sistema imune é uma coleção de tecidos, células e moléculas que protegem o organismo de numerosos agente patogênicos e toxinas no ambiente. O sistema imunológico age na proteção do organismo através da identificação e eliminação de eventuais patógenos/antígenos. Assim, como em muitas outras espécies animais, o sistema imune dos peixes pode ser dividido quanto a sua resposta, na qual pode-se ter característica inata ou adquirida. O sistema de defesa imune inato, como o nome sugere, apresenta-se ativo já na

fase inicial de vida do animal, e para os peixes constitui uma poderosa arma de defesa contra patógenos (Hernández *et al.*, 2016). Esse sistema corresponde a um conjunto de respostas que compõe o primeiro mecanismo de defesa capaz de proteger o peixe contra infecções, enquanto a resposta adaptativa dos peixes é mais tardia (em peixes, aproximadamente 7 dias após estímulo patogênico já é possível detectar anticorpos contra o agente). Os mecanismos imunes envolvidos nesta resposta são essenciais para a imunidade duradoura e um fator-chave para o sucesso de vacinações (Magnadóttir, 2010).

Na resposta imune inata, caso o organismo invasor seja uma bactéria ou um parasito, o resultado será a ativação imediata dos componentes do sistema imune, que culminarão nos processos de fagocitose, inflamação e destruição do antígeno. Podemos dividir o sistema imune inato em três tipos de barreiras principais: barreira física e química (muco, escama, pele, ácidos e outros), barreira celular (células de defesa como monócitos-macrófagos, granulócitos e outras) e barreira humoral (citocinas, enzimas líticas como a lisozima, sistema complemento, peptídeos antimicrobianos, proteases, anticorpos e outras) (Magnadóttir, 2006; Hoseinifar *et al.*, 2015), conforme ilustrado resumidamente na Figura 1.

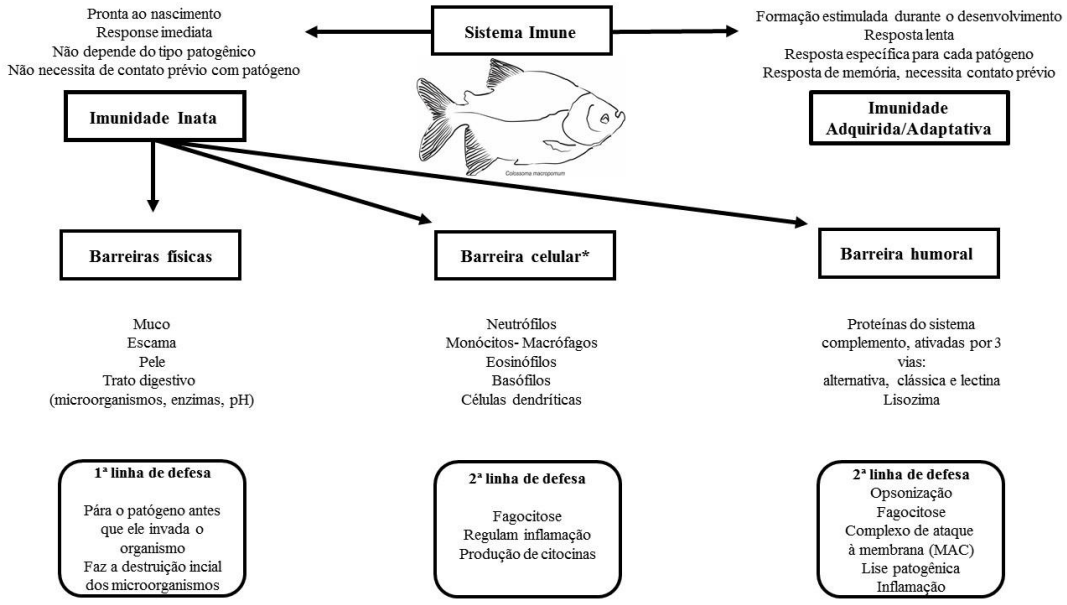

Figura 1. Aspectos gerais do sistema imune do tambaqui: imunidade inata. *Barreira celular: não estão dispostos os linfócitos, envolvidos na imunidade adaptativa e leucócitos teciduais.

1.4.1. Barreiras físicas e químicas

Quando um patógeno entra em contato com o peixe, a primeira barreira componente do sistema inato com a qual ele se depara são as barreiras físicas e químicas. O muco, as escamas, a pele e até mesmo o pH e enzimas do trato digestório são a primeira linha de defesa para evitar a entrada do patógeno (Ellis, 2001; Reverter *et al.*, 2018). Contudo, quando o patógeno consegue ultrapassar estas barreiras, principalmente quando o animal apresenta-se imunossuprimido, ele atinge a circulação, entrando em ação a segunda linha de defesa do sistema imune inato, a fim de neutralizá-lo.

1.4.2. Barreira celular

Os peixes possuem leucócitos especializados em fagocitar e destruir agentes infecciosos, como os neutrófilos polimorfonucleares, monócitos (precursores dos macrófagos), eosinófilos, basófilos, células natural killer e células dendríticas, entre outros, como dispostos resumidamente a seguir na Figura 2, quanto às funções e principais órgãos de atuação da barreira imune celular.

Tipo celular		Principais funções em teleósteos	Localização principal	Referência
	Basófilo	Imunidade contra parasitos. Na degranulação, libera mediadores inflamatórios, como a histamina, que estimula migração de leucócitos para o sítio da infecção/inflamação	Produzido pelo rim, circula nos vasos periféricos e migra para os tecidos	Odaka *et al*., 2018
	Célula Granulocítica Especial ou Leucócito Granular Ácido Periódico de Schiff + (CGE ou LG-PAS)	Degranulação com liberação de histamina e estimula inflamação. Semelhante em função aos mastócitos	Produzido por órgãos hematopoiéticos, é possível encontrar estas células no sangue, rim e baço	Secombes, 1996
	Célula Dendrítica	Célula apresentadora de antígenos (para células T), indutora da resposta imune adaptativa, produtora de citocinas	Localiza-se principalmente na pele e superfície mucosa. Ao encontrar o patógeno, se ativa e migra para tecidos linfóides secundários (baço e gânglios linfáticos), onde apresentam antígeno	Bassity e Clark, 2012
	Eosinófilo	Libera toxinas para combater patógenos. Associado à doenças parasitárias	Produzido pelo rim, circula nos vasos sanguíneos e migra para os tecidos, principalmente, epitélio intestinal	Alvarez-Pellitero, 2008
	Linfócitos B, T e Natural Killer (NK)	Linfócitos B: reconhecimento, fagocitose e apresentação de antígenos para os linfócitos T, produzem anticorpos. Linfócitos T: reconhecem antígeno na presença do complexo principal de histocompatibilidade (MHC), secretam citocinas para atrair leucócitos para o sítio de infecção. Células NK: matam células tumorais e células infectadas por vírus. Uma das células efetoras mais importantes do sistema imune inato	Produzidos pelo timus e rim cefálico (órgãos linfóides primários). Migram e ativam no local onde patógeno se encontra	Abelli, 2016 Biller-Takahashi e Urbinati, 2014
	Macrófago	Fagocita patógenos e apresenta potente atividade de burst respiratório. Estimula resposta das demais células, através de síntese de citocinas, como IL-1b. Também produz proteínas do sistema complemento e lisozima	Produzido principalmente pelo rim cefálico. Derivado do monócito, ele pode ser encontrado em diversos tecidos	Tort *et al*., 2003
	Mastócito	Induz inflamação pela liberação de histamina e heparina. Recruta macrófagos e neutrófilos. Envolvido na defesa contra patógenos e na recuperação de feridas	Origina-se nos órgãos hematopoiéticos, migra para o sítio de maturação, e é encontrado em grande quantidade nos tecidos lesionados	Sfacteria *et al*., 2015
	Monócito	Atua como fagócito. Regula a inflamação. Presente no sangue, micgra para o sítio de infecção. Diferenciam-se em macrófagos e células dendríticas	Produzido principalmente pelo pelo rim cefálico. Estocado no baço, move-se pelos vasos sanguíneos	Geissmann *et al*., 2010 Reite e Evensen, 2006
	Neutrófilo	Primeira célula recrutada no local da infecção. Fagócito que libera toxinas dos grânulos intracelulares. Libera armadilhas para morte do patógeno. Libera citocinas para recrutar mais fagócitos para o local da inflamação	Produzido principalmente pelo rim, circula pelo vaso sanguíneo e migra para tecidos	Havixbeck e Barreda, 2015
	Trombócito	Célula hemostática. Recentemente foi descrito como importante agente fagocítico	Produzido nos órgãos hematopoiéticos, é encontrado principalmente na circulação periférica. Encontrado no baço e rim, sugerindo importante atividade fagocítica em tais órgãos linfóides.	Nagasawa *et al*., 2014

Figura 2: Tipos celulares envolvidos na imunidade de teleósteos: funções e localização principal.

As células brancas estão envolvidas na resposta imune, defendendo o organismo de agentes patogênicos. A fagocitose é o mecanismo central de resposta imune inata, exercido pelos leucócitos, para ingestão e destruição de partículas (Zhao *et al.*, 2016) como debris celulares, microorganismos ou células inteiras. A fagocitose faz a limitação inicial do patógeno, e em teleósteos, as principais células atuantes são os neutrófilos (Figura 3) e os macrófagos, cujo papel dessas células também é de construir e modular a resposta imune inata e adaptativa (Secombes e Fletcher, 1992; Neumann *et al.*, 2001).

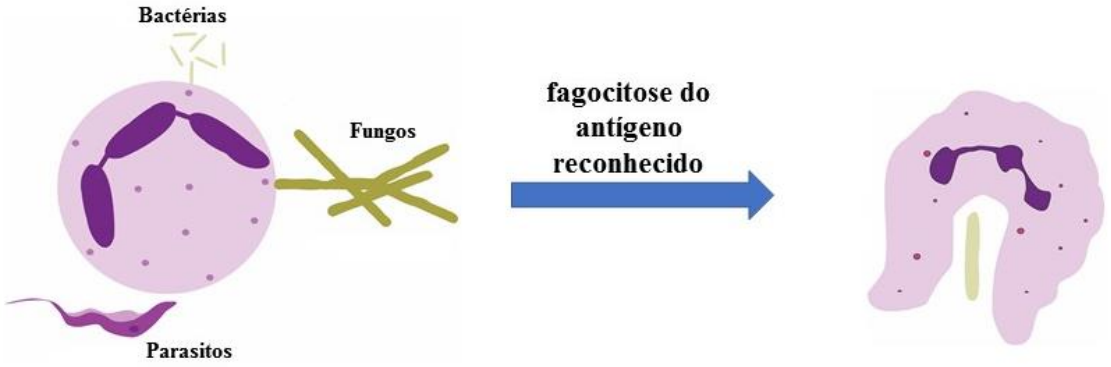

Figura 3: Neutrófilo de teleósteo fagocitando uma bactéria patogênica.

Estas células fagocíticas podem migrar para locais onde são necessárias, através de eventos de sinalização em resposta a um estímulo inflamatório. Desta forma, os leucócitos migram da circulação sanguínea para os tecidos, onde, eficientemente, conseguem matar organismos invasores, como as bactérias, pelo mesmo processo fagocítico, por meio da liberação de enzimas proteolíticas, proteínas antimicrobianas e espécies reativas de oxigênio (Brinkmann *et al.*, 2004; Magnadóttir, 2006), como exemplificado na Figura 4.

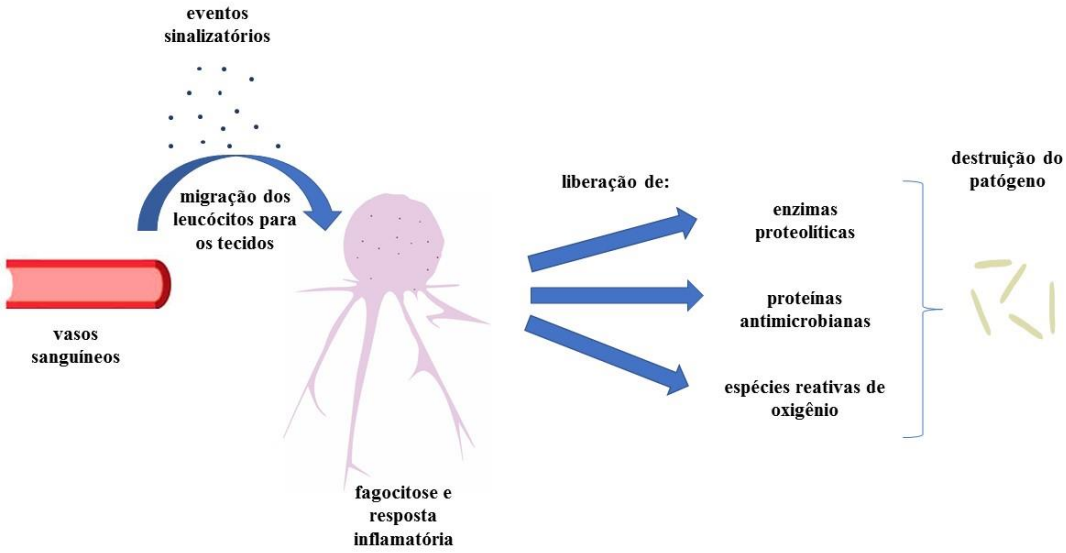

Figura 4: Mecanismo de destruição de patógenos.

Os órgãos que produzem e maturam as células tronco que originam os leucócitos são o rim cefálico e timo, chamados de órgãos linfóides primários. Contudo, o rim cefálico também é reconhecido como órgão linfóide secundário, assim como o baço e as mucosas do tecido linfóide (como intestino, brânquia e pele). Tais órgãos linfoides são responsáveis pela geração do repertório imune nos peixes (Rombout *et al.*, 2005).

1.4.3. Barreira humoral

A imunidade humoral é presente em ambas respostas imunes: inata e adaptativa. No sangue existem diversas proteínas que ajudam a eliminar os patógenos. Alguns componentes humorais respondem de forma específica e tardia, como os anticorpos (imunoglobulinas), que são proteínas produzidas pelos linfócitos B (Tizard, 2014). Estes fazem parte da subdivisão da imunidade adaptativa, que é dividida em imunidade humoral e celular. Alguns estudos de teleósteos indicam que a resposta humoral do sistema imune adaptativo apresenta a cinética similar aquela dos mamíferos, assim como verificado por Kreutz *et al*. (2014) no peixe nativo jundiá *R. quelen*, infectados com *A. hydrophila*, cuja produção de anticorpos

ocorreu de forma rápida e robusta, em resposta à infecção. Contudo, ao contrário dos anticorpos, outros componentes humorais vão responder prontamente à infecção (de forma inespecífica), porém sem desenvolver memória imunológica, como as citocinas, enzimas líticas (ex: lisozima), proteínas componentes do sistema complemento, peptídeos antimicrobianos, proteases e outras. Tais elementos compõem a barreira humoral, subdivisão das barreiras envolvidas na imunidade inata. A seguir, são destacados alguns destes componentes, que, por possuírem funções chave na resposta imune inata dos peixes, serão abordados no decorrer da tese, como o sistema complemento, a lisozima e algumas citocinas pró e anti-inflamatórias.

Sistema complemento

O complemento é um sistema complexo de vigilância imunológica inata, que desempenha papel fundamental na defesa contra potenciais patógenos e na manutenção da homeostase (Boshra *et al.*, 2006; Merle *et al.*, 2015a). Por muitos pesquisadores, o sistema complemento é considerado o principal mecanismo imune dos peixes. Este sistema é composto por um conjunto de proteínas plasmáticas, que podem ser ativadas a partir de três vias: pela deposição do componente C3 na superfície do microorganismo (via alternativa), pela ligação de proteínas que se ligam à manose (via lecitina) ou pela ligação das imunoglobulinas com os antígenos (via clássica) (Kreutz, 2017). A ativação do complemento durante a infecção por patógenos é desencadeada por um componente central do sistema complemento, o C3, o qual está envolvido na cascata destas três vias (Merle *et al.*, 2015a). Após ativação, as proteínas reagem entre elas e se auto-ativam por clivagem proteolítica, resultando numa cascata de ativação sequencial.

Dentre as três vias, a via alternativa é responsável por monitorar a presença de agentes patogênicos, e por isso, fica permanentemente ativa em níveis baixos, para permitir

a identificação imediata dos patógenos (Merle *et al.*, 2015a). A via alternativa é a responsável pela lise de células, e em contraste com mamíferos, a via alternativa nos peixes é de magnitude muito maior (Boshra *et al.*, 2006) e uma das mais avaliadas. Há décadas, Yano (1996) já sugeriu que a via alternativa é mais desenvolvida em peixes do que em mamíferos.

O sistema complemento possui mecanismo fundamental no reconhecimento antigênico para a opsonização e fagocitose. Convenientemente, a palavra "opson" deriva do grego, e significa molho, e o processo de opsonização é o mecanismo em que opsoninas se ligam à superfície antigênica, facilitando a fagocitose, tornando-a mais atrativa (Abbas *et al.*, 2014). Além disso, o sistema complemento também pode acarretar a lise bacteriana através da formação do complexo de ataque à membrana (MAC), que forma aberturas na membrana da bactéria, levando ao influxo de água e íons, e consequente desequilíbrio osmótico, e então, causando lise celular, porém, a maioria dos patógenos conseguem reparar os poros formados, sendo resistentes à lise por MAC (Merle *et al.*, 2015b). O sistema complemento também possui papel crucial na inflamação, através da interação e ativação das células imunes, além de modular a atividade dos linfócitos B e T (Merle *et al.*, 2015b). A degranulação e quimiotaxia de leucócitos (o componente C5a induz degranulação de mastócitos e promove quimiotaxia de neutrófilos) também são eventos verificados na cascata proteica do sistema complemento, dessa forma, induzindo inflamação (Nakao *et al.*, 2011). Outras atividades como regulação da imunidade, angiogênese, remoção de células apoptóticas e coagulação sanguínea também são decorrentes da ativação deste sistema (Tizard, 2014). As principais atividades do sistema complemento para o controle de

patógenos estão representadas na Figura 5.

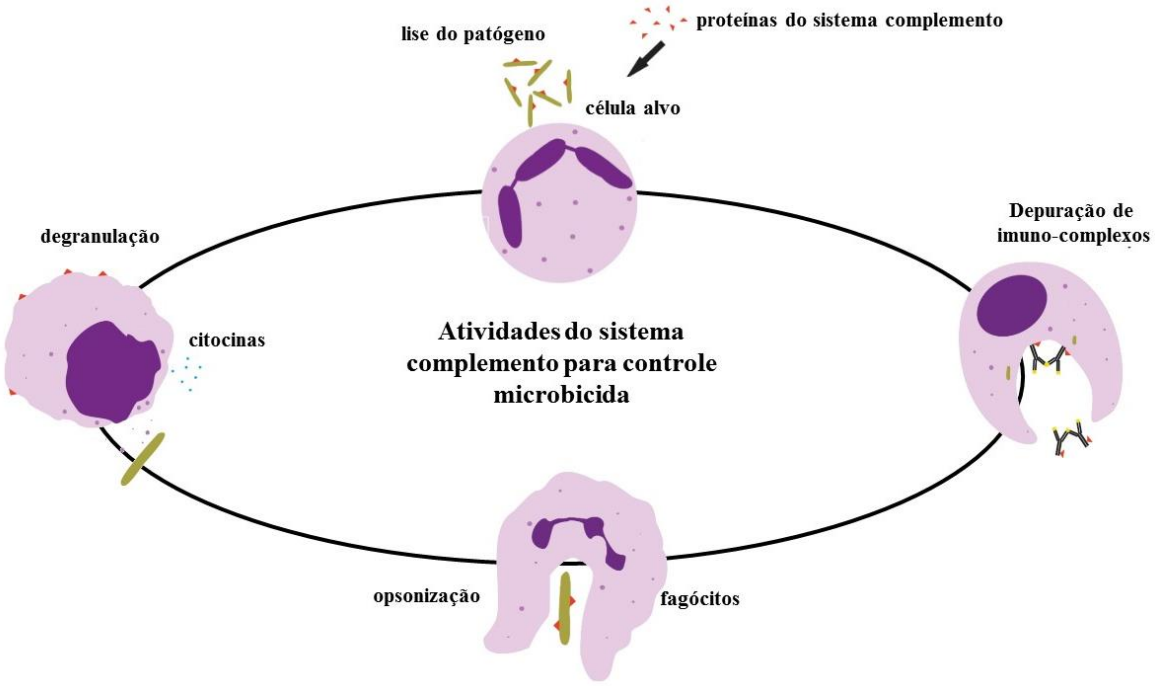

Figura 5. Principais atividades do sistema complemento para o controle de patógenos.

Lisozima

A lisozima é uma enzima encontrada na maioria das secreções biológicas, no muco e em leucócitos (Ogundele, 1998). Possui atividade bactericida, com ação tanto para bactérias gram-positivas quanto negativas (Magnadóttir, 2006). Esta enzima lítica também tem ação opsonizante e ativa o sistema complemento e fagócitos (Magnadóttir, 2006). Em um estudo com diversas espécies de peixes, Lie *et al.* (1989) demonstraram que a atividade desta enzima é maior em tecidos ricos em leucócitos, sendo que o rim, trato digestório e baço foram, respectivamente, os órgãos que apresentaram os maiores níveis enzimáticos, e por esse motivo, são muitas vezes escolhidos para estudos de análise de expressão genética. Esta enzima bacteriolítica também é reportada no muco tegumentar, soro, brânquia, fígado e músculo (Lie *et al.*, 1989; Saurabh e Sahoo, 2008). Uma das descobertas mais interessantes revela que a lisozima possui ação anti-inflamatória, e desta forma, pode ser encontrada em

níveis altos, tanto durante como pós-infecção, isso decorre de sua ação inibitória da atividade hemolítica do complemento sérico de forma dose-dependente (Ogundele, 1998), podendo desta forma, afetar a resposta do sistema complemento. É importante salientar que a atividade da lisozima varia de acordo com o sexo, idade, estação do ano, temperatura e pH da água, infecção, nível de estresse, agentes químicos e imunoestimulantes (Saurabh e Sahoo, 2008).

Citocinas

Citocina é um termo genérico empregado para designar moléculas proteicas, reguladoras do sistema imune (Secombes *et al.,* 2011). Elas são produzidas principalmente pelos leucócitos e liberadas extracelularmente, para atuar em outros leucócitos, coordenando a resposta celular (Abbas *et al.*, 2018), embora sua ação não seja restrita à comunicação de leucócitos, podendo atuar em outras citocinas, por exemplo. A partir de estudos genéticos em peixes, sabemos que a atividade biológica destas proteínas está envolvida no controle da: inflamação, diferenciação celular, ativação de células efetoras, hematopoiese e apoptose (Zou e Secombes, 2016).

As citocinas podem ser enquadradas em diferentes categorias: interferons (IFN), interleucinas (IL), fator estimulador de colônias (CSF), fator de necrose tumoral (TNF), e fator de transformação de crescimento (TGF). Está claro que a maioria das famílias das citocinas está presente nos peixes, associadas à resposta imune inata, como IL-1β, IL-6, IL-12, TNF-α, assim como citocinas associadas à resposta imune adaptiva, como IL-2, IL-4, IL-5, IL-13, TGF-β (Zou e Secombes, 2016).

As interleucinas se destacam como importantes citocinas imunoregulatórias (Asadullah *et al.*, 2003), atuando na supressão e estimulação do sistema imunológico, regulando-o de forma rápida e eficiente, de acordo com a necessidade do organismo. Em

mamíferos, já foram descritas 35 interleucinas, produzidas por uma variedade de tipos celulares (principalmente linfócitos CD4+T helper, macrófagos/monócitos e células endoteliais), assim como ocorre em peixes (Secombes *et al.*, 2011). Porém, o grupo fica ainda maior quando consideramos as subfamílias, tornando os estudos mais complexos. Por exemplo, a família da IL-1 possui 11 membros descritos em mamíferos, agrupados numa subfamília. Muitos deles estão sendo descritos para peixes, destacando-se a IL-1b (Hu *et al.*, 2018; Liao *et al.*, 2018; Wang *et al.*, 2019), com significante atividade pró-inflamatória em peixes. Já a subfamília da IL-10, pertencente à família classe II de citocinas, destaca-se em estudos em peixes com potencial atividade anti-inflamatória (Matsumoto *et al.*, 2018; Tran *et al.*, 2019).

O estudo dessas proteínas progride rapidamente, indo muito além da caracterização genética. Apesar de existirem muitos tipos de interleucinas, cada uma com funções diversas, sua caracterização genética e atuação durante infecções bacterianas em peixes sul-americanos é desconhecida. Por isso, nesta tese, fornecemos a sequência parcial de importantes genes imunes: C3, C4, lisozima, IRAK1, IL-1b e IL-10. Nesta tese também apresentamos a modulação da expressão destes genes e do gene HSP70, já descrito para a espécie, em diversas fases de infecção bacteriana. É importante destacar que outros genes imunes como o TNF, IFN entre outros são relevantes e merecem ser estudados para o tambaqui em novos estudos.

1.5. Genes imunes

Com o rápido avanço da aquicultura, estudos que associam imunidade e genética em resposta a algum estímulo patogênico (Gou *et al.*, 2018; Shahi *et al.*, 2018; Veenstra *et al.*, 2018; Chen *et al.*, 2019; Kumaresan *et al.*, 2019) têm se tornado um dos maiores focos entre os pesquisadores, que almejam obter resultados importantes, de forma precisa

e sensível. Muitos destes estudos são relatados em espécies comercialmente importantes mundialmente, tanto em quantidade produzida quanto em valor comercial, como o salmão *Salmo salar*, a tilápia, a truta *Oncorhynchus mykiss*, a carpa comum *Cyprinus carpio L.* e outras.

Independente do estímulo ambiental/patogênico, alguns genes imunes têm sido destacados em diversos estudos por desempenharem papel chave no entendimento da resposta do hospedeiro frente a qualquer mudança interna ou externa do organismo, como genes associados à produção das seguintes proteínas: da enzima lisozima, de proteínas chaves do sistema complemento (C3 e C4), "heat shock protein 70kb", que é uma proteína do choque térmico (HSP70), da interleucina 1 beta (IL-1b), proteína cinase associada ao receptor de interleucina-1 (IRAK-1) e interleucina 10 (IL-10), abordados nesta tese, e outros importantes , que não serão abordados, como o fator de necrose tumoral (TNF), receptores Toll-like (TLR), interferon (IFN), interleucina 6 (IL-6), interleucina 12 (IL-12).

A citocina TNF-α, produzida principalmente por macrófagos ativados, se destaca em estudos genéticos de teleósteos por estar envolvida em inflamações sistêmicas e na regulação das células imunes (Hong *et al*., 2013). Os receptores Toll-like (TLR) são componentes importantes dos receptores de reconhecimento de padrões (PRRs), que desempenham papéis significativos na imunidade inata, defendendo o peixe contra a invasão de patógenos (Zhang *et al*., 2017). A interleucina 6 (IL-6), é secretada por células T (linfócitos T) e macrófagos, e é destacada nos estudos genéticos de resposta imunidade adquirida dos teleósteos, desempenhando papel importante na regulação, proliferação e diferenciação de células T, e ainda estimulando a produção de imunoglobulinas pelas células B (linfócitos B) (Chen *et al*., 2012). Os genes de interleucina 12 (IL-12) são destacados por desempenhar papel fundamental na resposta imune adquirida, já que IL-

12 estimula a produção de interferons pelas células T e natural killer (linfócitos), aumentando a função citotóxica das células T (Yoshiura *et al*., 2003). Os interferons (IFNs) se destacam nos estudos de peixes por possuírem elevada atividade antiviral (Zou e Secombes, 2011), desta forma, não são foco de estudo no Brasil, mas correspondem a uma das principais citocinas estudadas em teleósteos, principalmente em países de clima frio.

Já com relação aos genes abordados nesta tese, os genes da lisozima se destacam por atuar tanto em infecções bacterianas quanto parasitárias (Carballo *et al.*, 2017). Os estudos de expressões dos genes C3 (Peng *et al.*, 2017) e C4 (Nonaka *et al.*, 2017) estão relacionados à regulação destas proteínas, componentes centrais do sistema complemento, considerado o mecanismo principal contra patógenos em peixes. Os genes que definem HSP70 são importantes porque esta é considerada a principal proteína envolvida no processo de estresse térmico e biológico (Dang *et al.*, 2015). Genes da IL-1β se destacam pois esta proteína atua como principal citocina pró-inflamatória (Jirapongpairoj *et al.*, 2015) e da enzima IRAK-1, também pro-inflammatória, que é enfatizada pelo papel chave na via de sinalização de receptores de interleucina 1 (Shan *et al.*, 2015). Por fim, os genes associados à IL-10 são relevantes por esta ser a principal citocina antiinflamatória, com importante função de limitar a inflamação através da regulação de proliferação, diferenciação e ativação das células imunes (Karan *et al*., 2016).

Para o tambaqui, ainda não há relatos de estudos imunogenéticos da sua resposta imunofisiológica frente aos estímulos patogênicos. Os estudos de tais fatores são urgentes para esta espécie, pois as doenças são atualmente um dos principais entraves na sua produção.

1.6. ETosis: uma nova via de morte celular do mecanismo imune inato

A morte celular via armadilha extracelular, conhecida por ETosis, abreviação em inglês de *extracellular trap cell death*, é um mecanismo microbicida que foi descoberto em 2004 por Brinkmann *et al.* (2004). Primeiramente, descrito por estes autores em neutrófilos de humanos, a liberação destas armadilhas, semelhante a uma teia (Figura 6), ocorre como forma de contenção dos microorganismos, recebendo o nome específico de NETs (que em inglês é uma abreviação para neutrophil extracelullar traps).

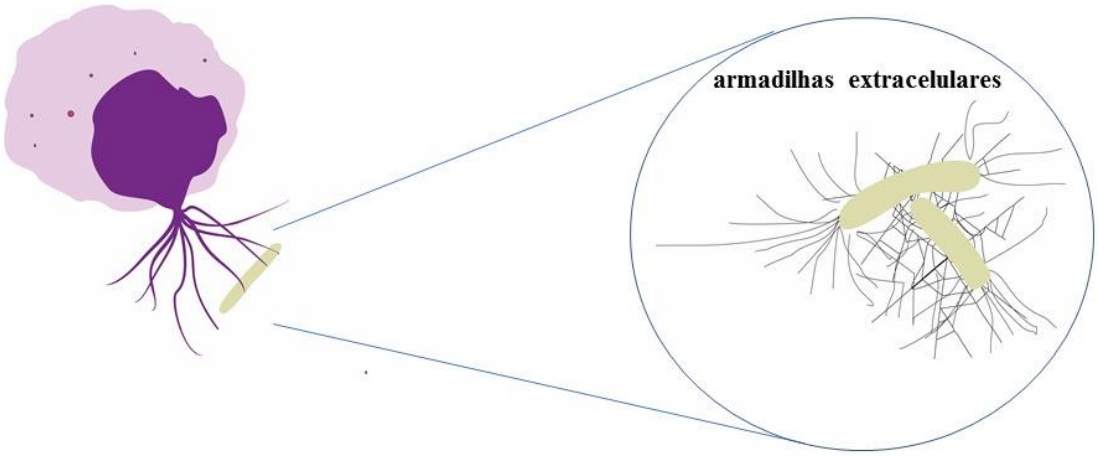

Figura 6: Armadilhas extracelulares liberada por leucócito, com aspecto de teia.

Posteriormente, Wartha e Henriques-Normark (2008) descobriram que esta via de morte celular não é específica dos neutrófilos, podendo ocorrer com outros tipos leucocitários, por isso, recebendo o nome geral de ETosis.

Recentemente, Brinkmann e Zychlinsky (2012) descreverem que a ETosis pode ser considerada como o último mecanismo do sistema imune para controlar uma infecção microbiana. Então, o organismo programa a morte celular dos seus leucócitos, como uma alternativa para tentar conter uma infecção.

Este mecanismo atua extracelularmente da seguinte forma: células de defesa descondensam sua cromatina e liberam-na para o meio extracelular, assim como as proteínas, formando fibras extracelulares que se ligam às bactérias gram-positivas e gram-

negativas (Brinkmann *et al.*, 2004). As estruturas que aprisionam os microorganismos consistem de um esqueleto de DNA de cromatina com peptídeos antimicrobianos e enzimas. Após o aprisionamento dos microorganismos, os leucócitos estimulados vão gerar espécies reativas de oxigênio (EROs), como o peróxido de hidrogênio, e iniciar uma cascata de sinalização, que levam à desintegração das membranas nuclear e celular e à formação de ETosis. A Figura 7 ilustra como ocorre a formação de armadilhas extracelulares por neutrófilos e a Figura 8 mostra imagens de microscopia eletrônica da formação de tais armadilhas em macrófagos.

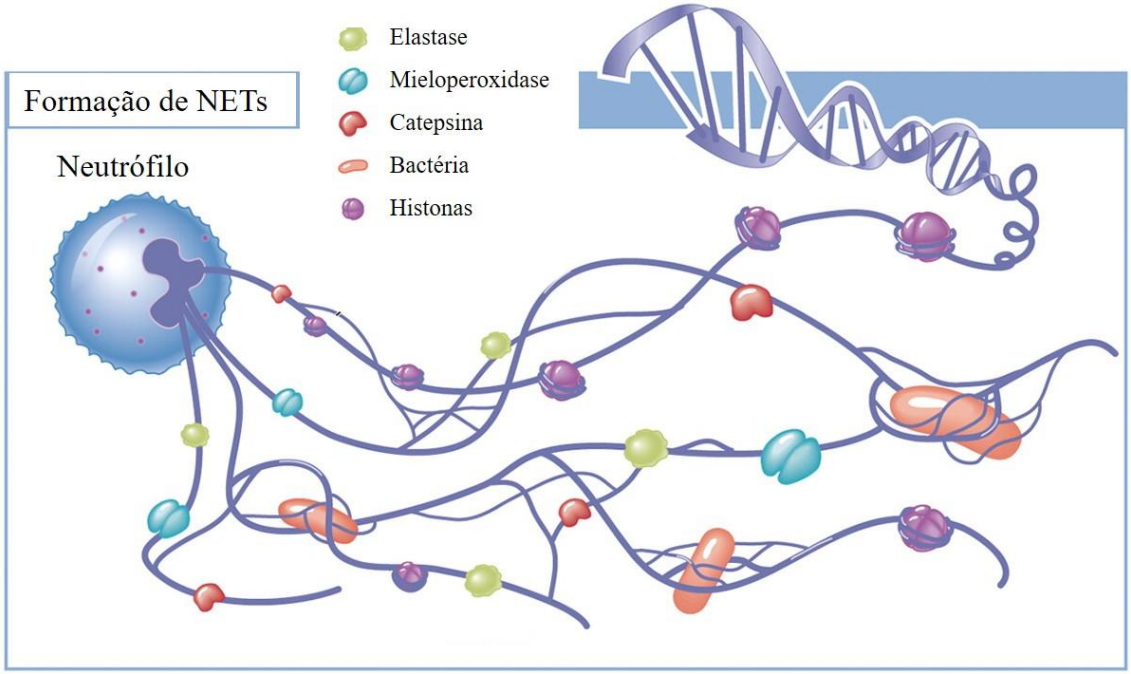

Figura 7. Ilustração sobre a formação de NETs (Adaptada de Miyata e Fan, 2012).

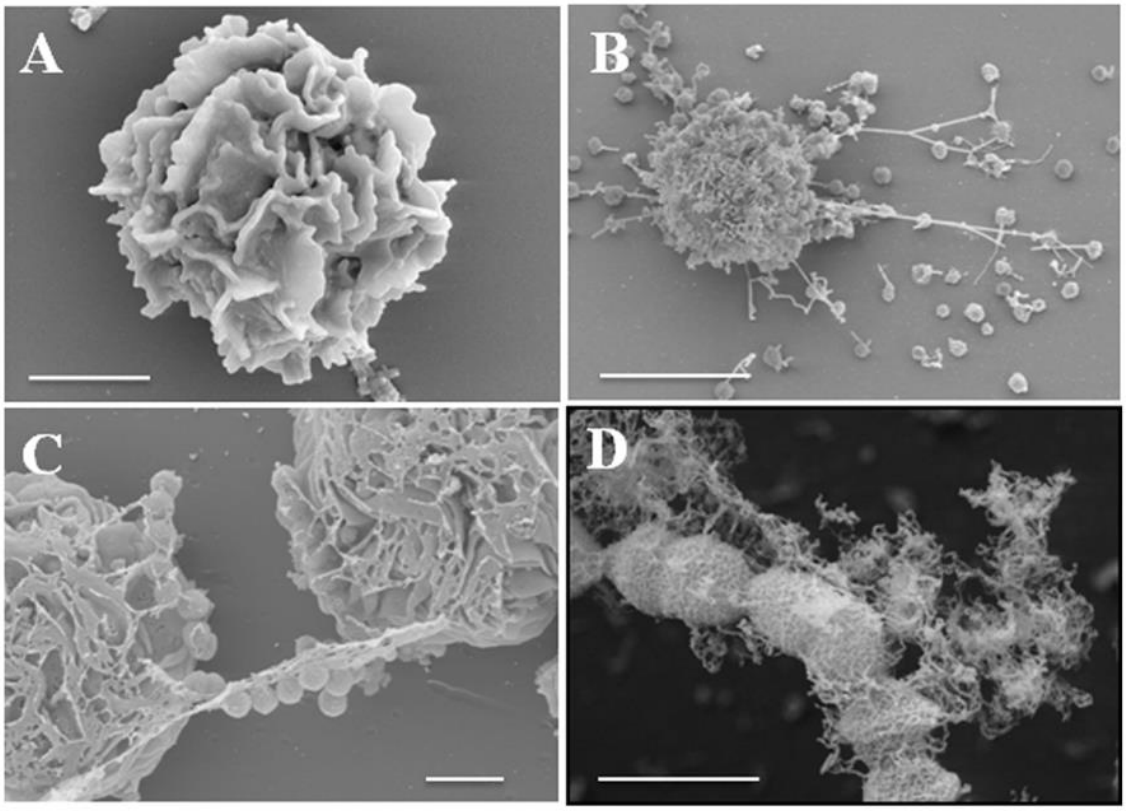

Figura 8. Liberação de armadilhas extracelulares por mastócitos após o encontro com bactérias (adaptada de Goldmann e Medina, 2013). Mastócitos inativados (figura A), mastócitos em processo de liberação de armadilhas extracelulares, em resposta à ativação com bactéria *Staphylococcus pyogenes* (figura B e C) e *S. pyogenes* capturadas nas armadilhas extracelulares (figura D).

A maioria das ETosis são descritas para neutrófilos. De fato, os neutrófilos desempenham função crucial como primeira linha de defesa contra patógenos invasores. Durante uma inflamação, eles são os primeiros a serem recrutados para o sítio inflamatório, num processo orquestrado por citocinas (Guimarães-Costa *et al.*, 2012). No entando, sabe-se que além dos neutrófilos, outras células como os eosinófilos (Simon *et al.*, 2015; Ueki *et al.*, 2016), monócitos (Chow *et al.*, 2010; Webster *et al.*, 2010), macrófagos (Chow *et al.*, 2010), mastócitos (Lin *et al.*, 2011) também executam este mecanismo.

A lista de linhagens celulares que realizam ETosis é crescente, assim como a variedade de microorganismos que podem ativar este mecanismo, como as bactérias gram-

positivas e gram-negativas (Brinkmann *et al.*, 2004), fungos ou leveduras (Urban *et al.*, 2006), protozoários (Reichel *et al.*, 2015), vírus (Papayannopoulos, 2018) ou até mesmo por indução com LPS (Brinkmann *et al.*, 2004), interleucinas como IL-1b (Lin *et al.*, 2011; Keshari *et al.*, 2012), IL-17 (Lin *et al.*, 2011), IL-8, TNF (Keshari *et al.*, 2012) e IFN (Martinelli, *et al.*, 2004).

Apesar da importância deste mecanismo, a ETosis ainda não é descrita em muitas espécies animais. É de nosso conhecimento que em peixes, apenas NETs foram descritas em *Danio rerio* (Palić *et al.*, 2007a), *Pimephales promelas* (Palić *et al.*, 2007b) e *Cyprinus carpio* (Brogden *et al.*, 2012; Pijanowski *et al.*, 2013). Os estímulos para formação das NETs, nestes estudos prévios foram realizados com ionóforo de cálcio (Palić *et al.*, 2007a, b), acetato de miristato de forbol (Palić *et al.*, 2007a; Pijanowski *et al.*, 2013), glucanos (Palić *et al.*, 2007a; Pijanowski *et al.*, 2013; Brogden *et al.*, 2014), lipopolissacarídeo e ácido imunoestimulante (Pijanowski *et al.*, 2013) , e bactéria (Brogden *et al.*, 2012).

Para facilitar a visualização das NETs, Brinkmann *et al.* (2010) publicaram a metodologia para gerar as armadilhas de neutrófilos, e visualizá-las em microscopia eletrônica de varredura (MEV). Através do emprego da MEV, a morfologia da ETosis é facilmente diferenciável de outras estruturas, sendo normalmente caracteriazada como uma estrutura nebulosa (a junção das armadilhas assemelha-se à nuvens), ocupando um volume de 10 a 15 vezes maior do que a célula que a originou (Brinkmann e Zychlinsky, 2012). Nesta tese, realizamos uma adaptação da metodologia para visualização de ETosis após suspeitas da ocorrência deste mecanismo, até então desconhecido, em tambaqui.

2. Objetivo

2.1. Geral

Abordar assuntos básicos, porém, ainda desconhecidos no cultivo do tambaqui, fundamentando a ocorrência da doença causada por *A. hydrophila*, e adicionalmente prover a investigação dos potenciais métodos de controle da cepa. Por fim, efetuar a caracterização dos aspectos imunes do animal em resposta à infecção.

2.2. Específico

Estabelecer a patogenicidade da *A. hydrophila* no tambaqui via Postulado de Koch;

Definir as doses letais e avaliar sinais clínicos de animais experimentalmente infectados;

Avaliar a susceptibilidade da cepa isolada frente a antimicrobianos;

Obter dados promissores de diferentes moléculas (antimicrobianos, fitoterápicos e desinfetantes) com potencial para aplicação na desinfecção e tratamento de *A. hydrophila* isolada de tambaqui;

Caracterizar alterações hemato-imunológicas durante a infecção;

Descrever o mecanismo de morte celular (ETosis) através do desenvolvimento de metodologia prática e de baixo custo;

Descrever as sequências parciais de genes imunes chaves (IL-1b, IRAK1, IL-10, C3, C4, lisozima) do tambaqui;

Gerar primers específicos (b-actina, IL-1b, IRAK1, IL-10, C3, C4, lisozima, HSP70) para o estudo genético na espécie;

Prover os dados de modulação destes genes no decorrer da infecção.

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Capítulo 2

Manuscrito nas normas da revista Microbial Pathogenesis

Aeromonosis in tambaqui *Colossoma macropomum*: pathogenicity, lethality and new insights for control and disinfection in aquaculture

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Abstract

Tambaqui *Colossoma macropomum* is the most produced native fish in South America aquaculture. Besides the lack of knowledge regarding bacterial pathogenicity for this species, it is common the unappropriated using of *off-label* therapies on its farming. In this study, the pathogenicity of *Aeromonas hydrophila* for tambaqui was first established by Koch's Postulate. Lethal dose (LD) experiment was settled for analysis of clinical signs and mortality (intraperitoneal challenge with 4 bacterium concentrations, ranging from 2.9 x 10^7 to 1.1 x 10^8 CFU/mL). The antimicrobial activity was investigated against 11 antibiotics by disk-diffusion test, while 3 antibiotics, 7 disinfectants and 11 herbal medicines were analyzed by broth microdilution methods. LD experiment showed up to 80% of fish mortality, skin darkness, ulcers, hemorrhage, lethargy and hypo/anorexia in all groups, with exception of control. The LD_{10}, LD_{50}, LD_{90} and LD_{99} were simulated and established in 4.1 x 10^7, 8.8 x 10^7, 1.9 x 10^8 e 3.6 x 10^8 CFU/mL, respectively. Regarding the potential of antibiotics, ceftriaxone, florfenicol, oxytetracycline, sulphazotrin and thiamphenicol were considered promising for aeromonosis control. All herbal medicines were classified as bactericides, but clove *Eugenia caryophyllata* and cinnamon *Cinnamomum zeylanicum* were the only with strong inhibition. The settling of disinfectants antimicrobial activity attested that malachite green was the only one that did not present acceptable values, discouraging its use. In conclusion, Koch's postulate was fulfilled and tambaqui entered to the vast list of hosts for *A. hydrophila* and promising results of chemical substances were provided, contributing to aeromonosis control.

Keywords: *Aeromonas hydrophila*; antibiotic; disease; disinfectant; essential oil; hemorrhagic septicemia, native fish.

1. Introduction

Diseases pose a major threat to aquaculture and the outbreaks are usually triggered by intensive farming management. Among bacterium that causes fish diseases, *Aeromonas hydrophila* stands out as one of the greatest causes of mortality [1]. This is considered an opportunistic pathogen for fish [2], causing the infection when a primary factor is able to affect the triad interactions (host–pathogen-environment). Diseases caused by motile *Aeromonas* are known as hemorrhagic septicemia and affect fish worldwide, but more forcefully in America, Asia and Europe [3]. Mortality outbreaks caused by *A. hydrophila* are considered a major concern for world aquaculture [4], and are responsible for large economic losses in several regions [5]. In addition, hemorrhagic septicemia is common in fish cultured in earth ponds, such as Siluriformes [6, 7], Characiformes [8, 9], Cichliformes [10] and Cypriniformes [3].

Tambaqui *Colossoma macropomum* is currently the main native species farmed in South America continental aquaculture, but it is known that there is still a gap to be filled in the field of bacterial diseases of South American fish [9]. Although several indications of bacterial outbreaks in tambaqui [9, 11], the pathogen identification followed by Koch's postulate for this fish are scarce in the literature. It is suspected that *A. hydrophila* is capable of causing hemorrhagic septicemia in tambaqui, but, to our knowledge, to date, there are no published studies confirming the occurrence of this disease through Koch's postulate.

As *A. hydrophila* is understood by multiple strains, its heterogeneity [12] makes it difficult to develop vaccines [13] and also makes prophylactic methods extremely complex, making it sometimes necessary the use veterinary drugs with therapeutic purpose, in order to avoid further productive losses. So, for the purpose of make the therapy more effective, it is important to study the effect of chosen substances against the specific strain that is

affecting the fish. Antimicrobial susceptibility testing can broadly review the best active principle for optimizing bacteriosis treatment.

In this study, we isolated *A. hydrophila* and investigated its pathogenicity of in tambaqui, evaluating the lethal dose, behavior changes and clinical signs of diseased fish. Several compounds widely used *off-label* in aquaculture and promising herbal medicines were screened to identify those with greater activity against *A. hydrophila*, and potential to be used in the treatment of aeromonosis and/or in the cleaning of materials and equipment.

2. Material and methods

For most infectious diseases, the ability to precisely identify the causative pathogen is a critical step before investigate effective measures for microbial control. In order to access the first pathogenicity of *A. hydrophila* in tambaqui and to study potential substances for control of the strain, each criteria of Koch's postulates were followed, using as few experimental fish as possible.

This study was approved in ethical principles established by the Brazilian College of Animal Experimentation (COBEA) and by the Committee on Ethics in Animal Use (CEUA) of the Faculty of Agrarian and Veterinary Sciences, UNESP, Campus Jaboticabal under protocol 009072/17.

2.1. Isolation and identification of *Aeromonas hydrophila* strain from tambaqui

Moribund tambaqui juveniles detected with lethargy and hemorrhage were euthanized in benzocaine (Sigma Aldrich, Brazil) for bacterial isolation. Bacteriological analysis was carried out on head kidney. Samples were streakned onto trypticase soy agar (TSA, Himedia, India) plates and incubated at 24h at 28°C. Pure colonies were DNA extracted according to Sebastião *et al.* [11].

PCR was performed with extracted DNAs using the following mix (ThermoFisher Scientific, Brazil): 1.2 μL of the 10X buffer (10mM Tris-HCl, 50mM KCl); 1.0 μL of dNTP (1.25 mM); 0.75 μL $MgCl_2$ (50 mM); 0.05 μL of taq polymerase (5 U/μL) and 0.40 μL of each primer (27F-AGAGTTTGATCCTGGCTCAG and 1942R-GGTTACCTTGTTACGACTT) (Exxtend®, Brazil) at 10 mM, for bacterial 16S rRNA genes; 0.5 μL of the DNA and 8.2 μL of H_2O ultra-pure (Sigma Aldrich, Brazil), to a final volume of 12.5 μL. The mix were initially denatured at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s and extension at 72°C for 1 min. The annealing temperature was 54°C. The resulting PCR products were analyzed with 1.5% of agarose gel stained with Nancy® (ThermoFisher Scientific, Brazil) and purified with PureLink® Quick Gel Extraction & PCR Purification Combo Kit (ThermoFisher Scientific, Brazil) for sequencing. The identified strain was stored at -80°C ultra-freezer in TSA supplemented with 30% sterile glycerol, until use in study of LD.

2.2. Lethal dose (LD) study and Koch's Postulate: clinical signs, behavior changes and mortality

In LD study, the reproductivity of clinical signs in inoculated healthy fish was evaluated, with further reisolation and reidentification (16S rRNA sequencing) of the strain, wherefore, confirming pathogenicity of the strain for tambaqui.

2.2.1. *Fish and experimental conditions*

Juveniles were obtained from breeders molecularly identified as tambaqui according to Hashimoto *et al.* [14]. The sanitary status of the batch was accessed since eclosion. Ninety individuals (175.5 ± 25 g weight) were randomly and equally divided into 15 tanks (140 L), corresponding to 6 fish per tank. Fish were fed *ad libitum* with the same

commercial diet, specific for omnivorous, twice a day. Since hatching, fish were routinely checked for any disease they were carrying for sanitary ascertaining.

The thermostats were programmed to 30°C, giving good conditions for both fish and bacteria and continuous aeration. The quality of water was monitored with Horiba U-50® multiparameter probe (Japan) every week. Values were within normal range and similar between the tanks (average: pH 7.63 ± 0.05, oxidative reduction potential 98.33 ± 6.43 mV, dissolved oxygen 7.15 ± 0.90 mg/L, conductivity 0.18 ± 0 mS/cm, salinity 0.01 ± 0 %, total dissolved solids 0.12 ± 0 g/L and temperature 30.39 ± 0.03 ºC).

2.2.2. Strain and challenge design

The newly isolated *A. hydrophila* was reactivated in 20 mL of TSA. Pure colonies were transferred to tryptone soybean broth (TSB, Himedia, India). The bacterial solutions were incubated (24h at 28°C) and centrifuged after growth to wash the medium (3000*xg*/ 4°C/10 min). The bacteria pellets were suspended in sterilized phosphate buffered saline (PBS, pH ≅ 7.4) and the procedure was repeated twice. The resulting pellet was diluted in sterile PBS, until reaching the desired optical density (OD) reading (2100 Unico Spectrophotometer, Japan) at 600nm absorbance OD_{600}, established in previously pilot experiments.

The chosen concentrations of the inoculum were established in previous experiments as: 0 (PBS control), 0.300, 0.500, 0.700 and 0.900 (OD_{600}), corresponding to 2.9×10^7, 5.2×10^7, 9.3×10^7 and 1.2×10^8 colony forming units (CFU)/mL. Bacterial concentrations were determined by planting serially diluted stock solutions in triplicate. To confirm sterility of PBS, the solution was plated in same conditions.

For challenge test, fish were anesthetized in 100 mg/L of benzocaine (Sigma-Aldrich, Brazil) and then inoculated with 0.1 mL of the respective solution per 10 g of

fish weight. Fish were analyzed for clinical signs, behavioral changes and record of mortalities twice a day, during 14 days post-challenge.

Freshly dead fish were sampled for microbiological analyses. The head kidney was used to reisolate the colonies in TSA (24h at 28ºC). For confirmation of the bacteria identity, the bacterial DNA extraction and the PCR followed by sequencing was performed as described in item 2.1.

To calculate LD_{10} (10% mortality), LD_{50} (50% mortality), LD_{90} (90% mortality) and LD_{99} (99% mortality), probit-log (dose) regression models established by Lei and Sun [15] were used (95% confidence level).

2.3. Molecules with potential antimicrobial activity for treatment and disinfection of Aeromonas hydrophila: research for antibiotics, disinfectants and herbal medicines

2.3.1. *Agar disk-diffusion susceptibility test*

Disk diffusion susceptibility test was performed according to Kirby-Bauer disk diffusion susceptibility test protocol [16].

Briefly, Muller-Hinton Agar (MHA; Sigma-Aldrich, Brazil) plates. Filter paper disks containing the test compound at desired concentration were placed on agar surface, intercalating tested substances randomly, until reach three replicates of each antibiotic tested: amoxycillin (10 µg, DME, Brazil), ampicillin (10 µg, DME, Brazil), ceftriaxone (30 µg, DME, Brazil), doxycycline (30 µg, DME, Brazil), florfenicol (30 µg, Oxoid, Brazil), gentamicin (10 µg, DME, Brazil), oxytetracycline (30 µg, Oxoid, Brazil), streptomycin (10 µg, DME, Brazil), penicillin (10 µg, DME, Brazil), sulphazotrin (25 µg, DME, Brazil) and vancomycin (30 µg, DME, Brazil). Incubation was proceeded according to Balouiri *et al.* [17] at 35°C for 18h and diameters of inhibition growth zones were measured. Results were

interpreted qualitatively as sensitive (diameter ≥ 20 mm), intermediate (15 mm ≤ diameter ≥ 19 mm) and resistance (diameter ≤ 14 mm) based on zone size interpretative chart of Clinical and Laboratory Standards Institute [18].

2.3.2. Minimum inhibitory and bactericidal concentrations (MIC and MBC)

The MIC and MBC of 3 antibiotics, 7 disinfectants and 11 herbal medicines were determined by broth microdilution susceptibility method as reviewed by Balouiri *et al.* [17]. Briefly, the tested compounds were pippeted in an Elisa plate (96 wells) containing Muller-Hinton broth (MHB, Sigma – Aldrich, Brazil). All substances were tested in triplicate. Each well was pipetted with standardized solution of bacteria, reaching a final concentration of 5 x 10^5 CFU/mL. Appropriate control groups (bacteria stock solution, culture medium and solvents) were tested to guarantee the test quality. Finally, plates were incubated at 28ºC for 24h.

The MIC data corresponded to the lowest concentration in which no bacterial growth was observed. To obtain the MBC, the three lowest concentrations in which no bacterial growth were observed were spread on TSA (Sigma – Aldrich, Brazil). The agar plates were incubated (24h at 28°C) in triplicate. The lowest concentration at which no bacterial growth was certified, was the MBC.

The classification of substances as a bactericidal or bacteriostatic agent was performed based on the respective MBC/MIC ratio. Results between 1-4 are classified as bactericidal and results ≥ 8 are classified as bacteriostatic [19].

Tree antibiotics tested were: florfenicol, oxytetracycline and thiamphenicol (Sigma Aldrich). Ethanol 95% (Sigma–Aldrich) was used as a solvent to prepare thiamphenicol and sterile distilled water as solvent to prepare florfenicol and oxytetracycline. *Escherichia coli* ATCC® 25922™ was used as quality control (QC) for antibiotics.

As highlighted by Assane *et al.* [20], *A. hydrophila* was considered susceptible or resistant for thiamphenicol if the value of MIC (μg/mL) was ≤ 8 or > 8, respectively; for florfenicol, *A. hydrophila* was considered susceptible, intermediately susceptible or resistant if the value of MIC (μg/mL) was ≤ 4, 8 or > 8, respectively; for oxytetracycline, *A. hydrophila* was considered susceptible, intermediately susceptible or resistant if the value of MIC (μg/mL) was ≤ 1, 2 or > 2, respectively.

The seven disinfectants tested were: malachite green (Synth, Brazil), copper sulphate (Synth, Brazil), potassium permanganate (Cinética Química, Brazil), sodium hypochlorite 5% (Audax, Brazil), sodium chloride (Dinamica, Brazil), hydrogen peroxide (Dinamica, Brazil) and formaldehyde (Synth, Brazil). All disinfectants were dissolved or diluted in distilled water before use in the test.

The herbal medicines (11 plant essential oils) were purchased from a commercial company (Phytoterapica, Brazil): garlic *Allium sativum*, lemongrass *Cymbopogon citratus*, palmarosa *Cymbopogon martinii*, citronella *Cymbopogon nardus*, cinnamon *Cinnamomum zeylanicum*, orange *Citrus aurantium linne*, clove *Eugenia caryophyllata*, tea tree *Melaleuca alternifolia*, peppermint *Mentha piperita*, basil *Ocimum basilicum* and thyme *Thymus vulgaris*. All EOs were subjected to Gas Chromatograph – mass spectrometry (GC-MS) QP2010 Ultra (Shimadzu) according to described by Osorio *et al.* [21] for identification and evaluation of the constituents (Supplementary Table 1). For MIC and MBC tests, all EOs were prepared by solubilization in proportion of 1:2 in dimethyl sulphoxide (Sigma–Aldrich, Brazil). The herbal medicines were classified based on MIC results as follows: strong inhibitors – MIC up to 0.5 mg/mL; moderate inhibitors – MIC between 0.6 and 1.5 mg/mL; weak inhibitors – MIC above 1.6 mg/mL [22].

3. Results

3.1. Isolation and identification of Aeromonas hydrophila strain from tambaqui

The sequence of nucleotides from the first sample collected from hemorrhagic tambaqui corresponded to 100% in identity and 100% in query cover with *A. hydrophila* in bacterial (16S rRNA sequences) data bases (Ribossomal Database Project- RDP and National Center for Biotechnology Information- NCBI).

3.2. LD_{50} for analysis of clinical signs, behavior changes and mortality

Healthy fish inoculated with *A. hydrophila* strain, in the LD test developed the same clinical signs of those fish used for the first bacterium isolation. All clinical signs observed were described in detail below and highlighted in Figure 1.

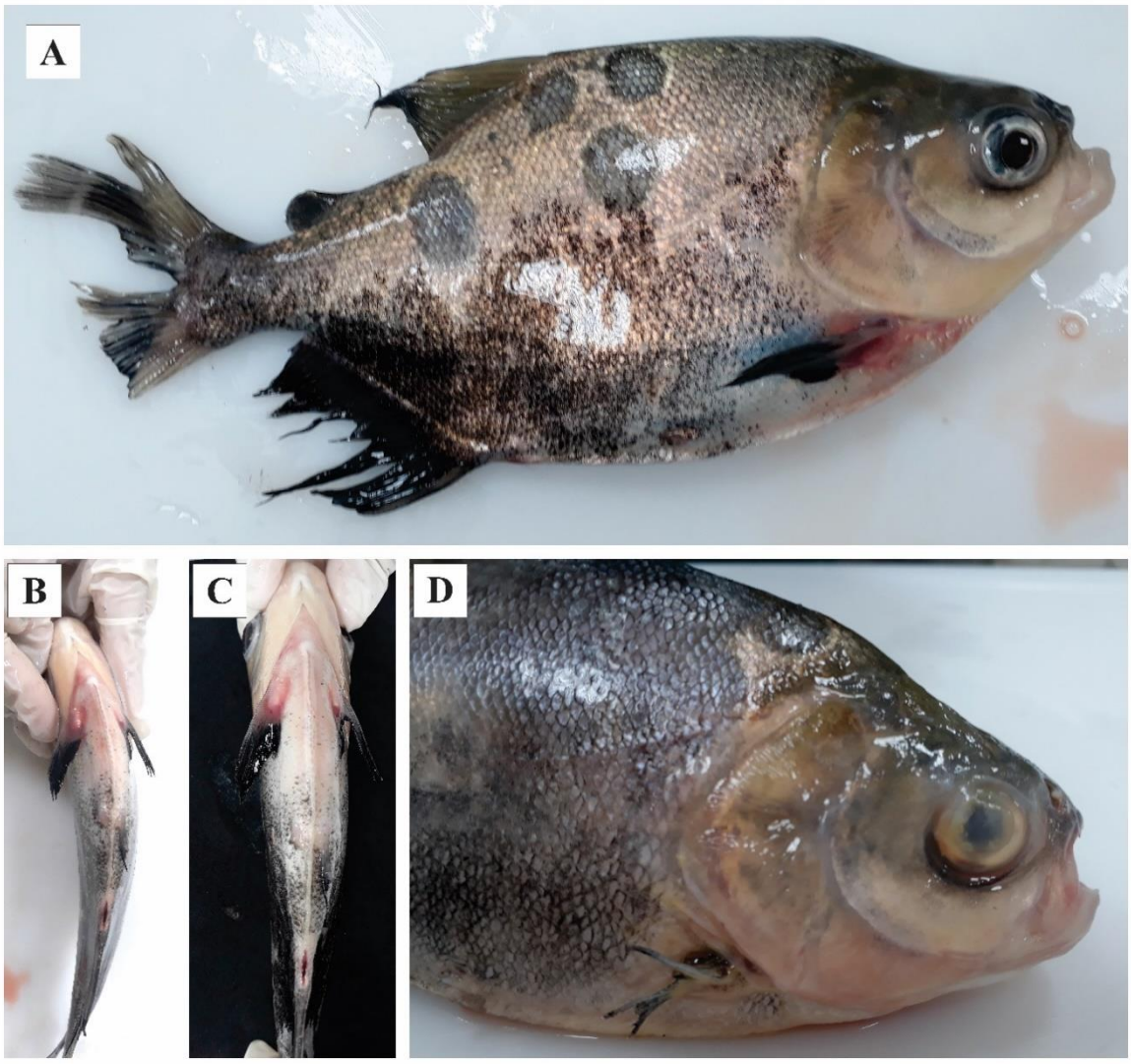

Figure 1. Clinical signs of tambaqui *Colossoma macropomum* experimentally infected by *Aeromonas hydrophila*. Diffuse circular lesions of the integument and hemorrhage at the base of the pectoral fin (A); hemorrhage in the ventral portion and fins corrosions, swelling and bleeding in the anal region (B, C); ocular opacity (D).

Healthy fish (PBS i.p.) presented the skin with dark gray coloration and the ventral region to the lateral line with black coloration, typical of the species. This was the only group that showed no behavior changing, feeding and swimming normally after challenging. No mortality was recorded in this group.

Fish inoculated with 2.9 x 10^7 CFU/mL of *A. hydrophila* (0.300 OD_{600}) presented mild infection. The fish swim normally, however, remained without feeding for a few days after challenge. The characteristic stain of the integument in the species could be seen normally, except for a slight darkening. Some ulcerative lesions in different regions of the skin could be seen from day 2 post-challenge. From day 2 it also could be seen the beginning of fins corrosion injuries. Just 1/ 18 fish died at day 6.

Fish inoculated with 5.2 x 10^7 CFU/mL of *A. hydrophila* (0.500 OD_{600}) presented mild-moderate infection. Since day 1, the fish did not fed. At day 2 fish showed first signs of ulcerative lesions and changing of skin and fin coloration, with darker and lighter spots. Five days post challenge, most of the fish from this group start feeding normally. Mortality was recorded on day 3 and 4 (3/ 18 fish).

Fish inoculated with 9.3 x 10^7 CFU/mL of *A. hydrophila* (0.700 OD_{600}) presented moderate infection. Half of the fish started feeding at 6^{th} day post challenge. The other half remain without feeding until the last day of the experiment. From day 2 post-challenge, the fish began to present: grouping at the edge of the tank, slow swimming, and multiple ulcerative lesions, many in a circular format on the back, skin darkening and

fins corrosion. Some animals had anal and labial hemorrhage protrusion. Mortality starts at day 1 until day 5 (8/ 18 fish).

Fish inoculated with 1.2 x 10^8 CFU/mL of *A. hydrophila* (0.900 OD_{600}) presented severe infection did not feed during the 14 days of the experiment. At day 1 post challenge, fish still feature the characteristic coloring of the integument, but some begin to present the skin turning to light gray. At day 1 post-challenging it was possible to verify skin lesions, mainly in the inoculated area and fins. Ulcerative lesions presented irregular edges and some circular ones in different regions. Some animals showed extreme letargy and lack of swimming on the surface of the water, while others presented slowly lateral swimming. Fish presented hemorrhagic areas in the ventral region and peduncle of the fins. Fish from this group presented the highest mortalities rate (14/ 18 fish), recorded from day 2 to day 4.

The Figure 2 shows the cumulative mortality of tambaqui infected by *A. hydrophila*. From day 6, mortality ceased at all concentrations tested.

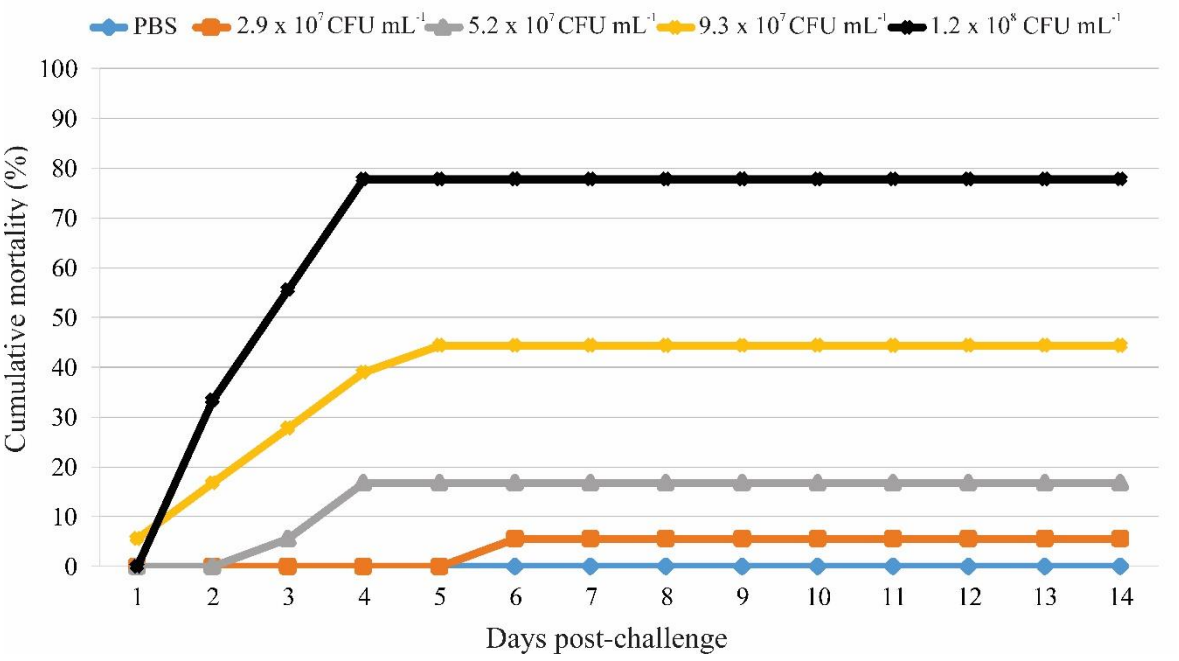

Figure 2. Cumulative mortality of tambaqui *Colossoma macropomum* challenged with *Aeromonas hydrophila*.

Lower and upper values used for LD average estimative (in CFU/mL) were: 1.83 x 10^7 to 8.99 x 10^7 (LD_{10}), 5.53 10^7 to 1.41 x 10^8 (LD_{50}), 6.98 x 10^7 to 5.25 x 10^8 (LD_{90}) and 7.25 10^7 to 1.79 10^9 (LD_{99}). The average was estimated in: LD10 as 4.06 x 10^7, LD50 as 8.82 x 10^7, LD90 as 1.91 x 10^8 and LD_{99} as 3.60 x 10^8 CFU/mL.

The colonies isolated from challenged fish in LD test show the same identity and query cover (corresponded to 100% *A. hydrophila*) from those first isolated. With all this information, Koch's postulate was concluded and *A. hydrophila* was considered pathogenic for tambaqui.

3.3. Molecules with potential antimicrobial activity for treatment and disinfection of Aeromonas hydrophila: research for antibiotics, disinfectants and herbal medicines

The control of pathogens in facilities and equipment through the use of disinfectants to reduce the risks of contamination and the treatment of diseases caused by these agents in fish are highly desirable. Therefore, in this study, promising molecule searches were performed.

3.3.1. Agar disk-diffusion susceptibility test

At agar disk-diffusion susceptibility test, *A. hydrophila* showed to be resistant against ampicillin, amoxicillin, penicillin, streptomycin and vancomycin; intermediate against gentamicin and doxycycline and susceptible against, ceftriaxone, florfenicol,

oxytetracycline and sulphazotrin. Results of the average of the diameters measured in disk diffusion susceptibility test are disposed in Table 1.

Table 1. Diameter of inhibition of 11 antibiotics against *Aeromonas hydrophila.*

Antibiotics	Halo diameter (mm)	Strain susceptibility[1]
Ampicillin	0	R
Amoxicillin	0	R
Ceftriaxone	27.5	S
Doxycycline	22.3	S
Streptomycin	13.6	R
Florfenicol	26.3	S
Gentamicin	18	I
Oxytetracycline	27.6	S
Penicillin	0	R
Sulphazotrin	22	S
Vancomycin	0	R

[1]S (susceptible, diameter ≥ 20 mm); I (intermediate, diameter between 15-19 mm); R (resistant, diameter ≤ 14 mm).

3.3.2. Minimum inhibitory and bactericidal concentrations (MIC and MBC)

Based on the susceptibility classification adopted for MIC, the *A. hydrophila* strain isolated in this study was susceptible to the 3 antibiotics (florfenicol, oxytetracycline and thiamphenicol) tested, as the highest MIC value observed was 0.25 µg/mL. The results of MBC/MIC ratio showed that all antibiotics tested (florfenicol, oxytetracycline and

thiamphenicol) did not present bactericidal activity, but showed bacteriostatic effect against the *A. hydrophila* strain (Table 2).

According to MBC/MIC ratio results (Table 2), formaldehyde, copper sulphate, sodium hypochlorite 5%, sodium chloride and hydrogen peroxide could be classified as bactericidal disinfectants for *A. hydrophila* strain from tambaqui. The malachite green was the only disinfectant tested that was classified as bacteriostatic agent for the strain studied. Potassium permanganate could not be classified because the result of MBC was not defined.

Among the herbal medicine tested, the essential oils of basil, citronella, lemongrass, palmarosa, peppermint, orange, tea tree and thyme were classified as weak inhibitors. The garlic was considered moderate inhibitor and, both, cinnamon and clove were considered strong inhibitors of *A. hydrophila* (Table 2). Based on the result of the MBC/MIC ratio, all EOs tested were classified with bactericidal activity (Table 2).

Table 2. Minimum inhibitory and bactericidal concentrations of three groups of substances (antibiotics, disinfectants and herbal medicines) against *Aeromonas hydrophila*.

Substances	MIC	MBC	MBC/MIC
Antibiotics (µg/mL)			
Florfenicol	0.25	> 2	> 8
Oxytetracycline	0.125	> 1	>8
Thiamphenicol	0.25	> 2	>8
Disinfectants			
Commercial chlorine (mL/L)	7.5	7.5	1
Copper sulphate (g/L)	0.9375	0.9375	1
Formaldehyde (mL/L)	0.0586	0.2344	4
Hydrogen peroxide (mL/L)	7.5	7.5	1

Malachite green (g/L)	0.0146	0.1172	8
Potassium permanganate (g/L)	1.8750	> 3.75	> 2
Sodium chloride (g/L)	75.0	75.0	1
Herbal medicines (mg/mL)			
Garlic *Allium sativum*	0.9375	1.875	2
Cinnamon *Cinnamomum zeylanicum*	0.235	0.235	1
Orange *Citrus aurantium linne*	7.5	7.5	1
Lemongrass *Cymbopogon citratus*	1.875	1.875	1
Palmarosa *Cymbopogon Martini*	3.75	3.75	1
Citronela *Cymbopogon nardus*	3.75	3.75	1
Clove *Eugenia caryophyllata*	0.235	0.470	2
Tea tree *Melaleuca alternifolia*	7.5	15	2
Peppermint *Mentha piperita*	3.75	3.75	1
Basil *Ocimum basilicum*	7.5	7.5	1
Thyme *Thymus vulgaris*	3.75	7.5	2

4. Discussion

The farming of tambaqui achieved a milestone, comprehending the main native species produced in continental aquaculture of South America [9], and to our knowledge, although there is evidence of bacterial infections in this fish, no confirmations were found through the establishment of the Koch's Postulate. This study ascertained the pathogenicity of *A. hydrophila* in tambaqui, completing the Koch's Postulate. This is an initial step to encourage studies of therapeutic substances, creating new and exciting opportunities to mitigate a problem, which is now confirmed as a tambaqui pathogen.

The lesions caused by *Aeromonas* are non-specific such as ulcers and hemorrhages [6], the same clinical signs observed in this study, besides others general signs such as lethargy, skin darkness, erratic swim, fins corrosions and hypo/anorexia. In tambaqui farming, the treatment of fish with hemorrhage became a common practice even without a conclusive diagnosis. Despite *A. hydrophila* causes hemorrhagic septicemia, because of the variability and non-specificity of clinical signs, the diagnosis of this disease based just on the lesion and behavior aspects are highly questionable and can be economically disastrous for fish producers [23]. Thus, inoculation of healthy fish with a natural strain, with reproduction of clinical signs and confirmation of mortality with ascertainment of the pathogen identity was required.

The LD_{50} (8.8×10^7 CFU/mL) observed in this study was lower than LD_{50} of *A. hydrophila* strain isolated from snake head fish *Channa striata* (4.1×10^8 CFU/mL) [24], yet, higher than LD_{50} from a strain isolated from channel catfish *Ictalurus punctatus* (3.2×10^6 CFU/mL) [25]. Recently, other bacteria from *Aeromonas* genre (*A. dhakensis*) was considered pathogenic for other South-American fish (pacu *Piaractus mesopotamicus*) and the LD_{50} of this bacterium was even lower (1.14×10^5 CFU/mL) [26]. Despite studies of LD_{50} depending on many variables evolved in environment-host-pathogen interactions, the results obtained are good indicators to classify the virulence of the strain, and in general the lower DL50 represents the bacterium's greater potential to cause severe disease.

The main way to prevent bacteriosis in aquaculture is through vaccines, however, since bacteria of the *Aeromonas* genus show a great heterogeneity, makes it difficult their development [13]. Therefore, biosecurity and good management strategies stands out as affordable methods, with easy applicability and also effective results. By these means, the use of disinfectants is a good alternative to mitigate the impact of bacteriosis in aquaculture [27], such as *Aeromonas*. A wide range of disinfectants are used in aquaculture, especially

to disinfect equipment, the facilities and to maintain hygiene throughout the production cycle [28]. Formaldehyde [28, 29]; potassium permanganate [30]; chlorine-containing compounds, such as calcium hypochlorite [28, 31, 32] and sodium chloride [28, 31], malachite green [33], copper sulphate [34], hydrogen peroxide and alcohols are amongst the most used disinfectants in aquaculture [35]. Studies on MIC and MBC can be of great help for selecting the best substances and classify antibacterial agents. Substances are usually regarded as bactericidal if the MBC is no more than four times the MIC. By these means, malachite green did not present good activity for control of *Aeromonas*. Besides the abscence of activity against *A. hydrophila* strain, the malachite green is a banned substance in several countries, being considered as a certainly carcinogenic substance by Food and Drug Administration (FDA). Despite toxicity studies comparing chemical compounds be absent for tambaqui until this date, it is of common agreement that regardless the fish species, chloride compounds are known to be less harmful than most of other chemical compounds such as malachite green, formaldehyde and potassium permanganate. Therefore, since chloride compounds showed good antibacterial activity, their use is strongly recommended as potential for cleaning the fish farming equipments.

Preventive health maintenance is very important in helping to control fish diseases, but prophylaxis is not enough to avoid some outbreaks of diseases [3] and the treatment become necessary and urgent. Regarding the treatment of Aeromonosis in tambaqui, different promising molecules were highlighted in the results, either synthetic antibiotics or herbal medicines. Among the synthetic substances tested, the strain showed resistance against ampicillin, amoxicillin, penicillin, streptomycin and vancomycin, corroborating with previous reports [36], which in a review of *A. hydrophila* susceptibility, 100% of the strains isolated from several fish species showed resistance against the same antibiotics. On the other hand, in this study, we found that *A. hydrophila* strain was susceptible to ceftriaxone,

florfenicol, oxytetracycline, sulphazotrin and thiamphenicol. The ceftriaxone, sulphazotrin and thiamphenicol are antibiotics that deserve to be investigated in greater detail, as they may yield new products for aquaculture, while florfenicol and oxytetracycline are substances already known and registered in several countries for use in some species of fish. Both, however, are not yet registered for use in South American fish and these results can provide subsidies to increase the list of species that can be treated with these molecules.

The cinnamon and clove EOs were evidenced with the strongest bactericidal activity against *A. hydrophila* in tambaqui, being classified as promising alternative treatment. The classification of these substances as bactericides is mainly due to the mechanism of action of the essential oils on the microorganisms that is characterized by permeabilization of the membrane culminating in swelling and lysis of the bacterial cell [37]. In previous studies, cinnamon showed antagonistic antibacterial activity to *A. hydrophila* infection in Nile tilapia [38] and clove was found to inhibit the growth of all *Aeromonas* sp. isolates (n = 19) from cyprinids (*Cyprinus carpio* and *Labeo rohita*) [39]. Thus, both plants are considered promising for use in preventive diets and/or for the treatment of *Aeromonas* infections via feed or water in tambaqui production, opening the door to further research in this area. It is worth mentioning that both plants are known anesthetics in fish [40, 41], so they may have a double beneficial effect as an anti-stressant and antibacterial during a more intensive management. However, due to its antibacterial effect, the use of clove and cinnamon EOs may leads to false negative diagnosis of diseased fish, and thus, we do not recommend to anesthetize fish when the aim is to isolate pathogens.

To our knowledge, this is the first report of Koch's Postulate of a strain of *A. hydrophila* isolated from tambaqui. It is important that fish farmers, technicians and researchers are attentive to the possibility of outbreaks of this disease and to the appearance of new pathogens in aquaculture. In addition, relevant data were provided for the future

formulation of treatments or control strategies for greater biosafety in the production of this important South American fish.

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Capítulo 3

Manuscrito nas normas da revista Fish & Shellfish Immunology

ETosis in tambaqui infected with *Aeromonas hydrophila*: a new cell death pathway of the innate immune mechanism and approach of leukocytes immune response

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Abstract

Tambaqui *Colossoma macropomum* is the most cultivated native fish in South America and *Aeromonas hydrophila* is one of the main bacteria of tropical fish. Despite the economic importance of this round fish, investigations on hematological changes in tambaqui are lacking. Detailed blood analyses (0h, 6h, 24h, 7d and 14d) following intraperitoneal challenge with *A. hydrophila* were performed. After analyzing the results, there was a suspicion of a novel cell death by means of extracellular traps (ETosis) in tambaqui. Search for ETosis was based in differential interference contrast (DIC) microscopy and scanning electron microscopy (SEM) assay, beyond development of a low-cost and practical protocol, applying co-incubation of leukocytes with *A. hydrophila*. The cells were investigated at: 0h (control), 4h and 7h after incubation. The complete hemogram profile showed an uncommon severe leukopenia in early phases of infection (6h, $p<0.001$ and ≤ 0.05) due to significantly decreases of the three main leukocytes: lymphocytes (6h, $p\leq 0.001$), monocytes (6h, $p\leq 0.05$) and neutrophils (6h and 24h, $p\leq 0.01$ and $p\leq 0.05$). Leukocytosis and lymphocytosis ($p\leq 0.01$) were ascertained only 7 days pos-infection. Through DIC and SEM it was certified that leukocytes suicide exposes the nuclear contents between 4 and 7 h after stimuli with bacteria. The leukogram profile associated to DIC and SEM analyses suggests that tambaqui leukocytes presented a programed death (ETosis) in order to expose its chromatin and granule proteins as a trap to bind and then kill bacteria, preventing *A. hydrophila* from spreading, resulting in leukopenia during early phase of bacterial infection. In this paper, we presume that ETosis is one of the last resources for tambaqui to contain the infection, and after this leukocyte strategy, the fish returns to produce and release a high number of phagocytic cells on peripheral circulation.

Key-words: Aeromonosis, aquaculture, extracellular trap, hemorrhagic septicemia, NETosis, native fish.

1. Introduction

Tambaqui *Colossoma macropomum* is a continental freshwater fish, native to South America, whose farming has been growing for consumption in several countries of the continent, such as Colombia, Peru, Venezuela, Bolivia, Ecuador and Brazil [1], being the last one, the largest producer. The cultivation of this round fish has a great social, cultural and economic importance for South American countries and efforts have been made to increase its production. Meantime, this fish has been suffering from diseases outbreaks [2-4]. Despite these considerations, studies on tambaqui immunity and physiological responses to infections are unknown.

Microorganisms of the genus *Aeromonas* are gram-negative bacteria and are considered opportunistic pathogens in aquaculture [5]. Among the bacteria of this genus, *Aeromonas hydrophila* stands out as being one of the main etiological agents of fish diseases worldwide [6], causing significant economic losses in the culture environment [7]. Recently, the pathogenicity of *A. hydrophila* to tambaqui [8] has been confirmed, however, knowledge about the hemato-immunological aspects of the disease in this host are still unknown. Studying the effects of pathogens on the immune system of the host can bring relevant information for prevention and control of the pathogen.

The innate immune response (denominated natural or non-specific) is mediated by molecules and cells [9]. Among the cells, leukocytes are one of the main components interfering with the infection, mainly by triggering phagocytic mechanisms and releasing of antimicrobial substances, besides orchestrating inflammation. These cells interact with non-specific pathogens, secreting substances with antimicrobial activity [10]. Nevertheless, several mechanisms are still unknown even for cell components as leukocytes. One of the mechanisms is the programmed process of cellular death for formation of extracellular traps, called ETosis [11]. This type of cell death represents a form of genetically controlled

cellular explosion [12] and is important because release granule proteins and chromatin that together form extracellular fibers that bind bacteria, degrade virulence factors and then, kill the microorganism [13].

First described in humans, Brinkmann and colleagues [13] elucidate the ETosis in neutrophils, calling it specifically as NETosis (neutrophil extracellular traps). Nowadays, it is known that upon activation, other leukocytes such as eosinophils, mast cells and macrophages also present this mechanism [14]. In addition, researchers reported the occurrence of ETosis in different mammals [15, 16] and a decade ago, Palić *et al*., [17] described the occurrence of extracellular traps in fish. There are currently few reports of the occurrence of this type of cellular death in fish such as carp [18], zebra fish [19] and turbot [20] and, to date, this mechanism is unknown in any species of South American fish.

In this study, hematological changes in tambaqui infected with maximum dose of *A. hydrophila* that did not cause mortality were investigated. In addition, the first occurrence of ETosis in South American fish was performed along with a modified methodology description.

2. Material and methods

This study was approved in ethical principles established by the Brazilian College of Animal Experimentation (COBEA) and by the Committee on Ethics in Animal Use (CEUA) of the Faculty of Agrarian and Veterinary Sciences, UNESP, Campus Jaboticabal under protocol 009072/17.

2.1. In vivo study: blood parameters changes in tambaqui infected with Aeromonas hydrophila

2.1.1. Fish and experimental conditions

Tambaqui were obtained from molecularly identified breeders for species certification according to the protocol of Hashimoto and colleagues [21]. After hatching the eggs in incubators, fish larvae were kept in tanks and then transferred to close bioterium for maintenance. When fish become juvenile, they were transferred to tanks with recirculation systems. Sixty fish (185 ± 23 g) were divided in 2 groups: control (healthy) and infected group (each group comprehended by 6 tanks, 5 fish per tank).

In experimental conditions, fish were fed twice a day, until satiety. The thermostats were programmed to maintain the temperature in both systems at 30°C, which matches a good temperature for both fish and the development of the infection. Water quality was monitored the entire experiment with Horiba U-50® multiparameter probe (Japan) and the values were similar in both groups (infected and no infected fish). The averages of the water parameters were: (6.98 ± 0.05 pH, 203.5 ± 20.17 mV oxidative reduction potential, 3.56 ± 0.09 dissolved oxygen, 0.56 ± 0.26 mS cm^{-1} conductivity, 0.03 ± 0.01% salinity, 0.36 ± 0.17 g L^{-1} total dissolved solids and 30.6 ± 0.33ºC temperature). All animals were fed according to required conditions for the age.

2.1.2. Aeromonas hydrophila: cultivation and experimental infection

A pathogenic strain of *A. hydrophila*, identified by sequencing the 16S rRNA gene was isolated from diseased tambaqui. The strain was cultivated on tryptone soybean agar (TSA vegitone, Sigma-Aldrich, India), incubated at 28°C for 24h, transferred to tryptone broth soybean (TSB, Fluka, Sigma-Aldrich, India) and incubated at the same condition. The resulting bacterial culture was centrifuged at 3000 *x*g at 4°C for 10 min. and the pellet suspended in sterilized phosphate buffered saline (PBS, pH~7.4). The procedure was repeated twice. After washing, the concentration of the bacterial solution was suspended in PBS until reaching the desired optical density reading (2100 Unico Spectrophotometer,

Japan) at 600 nm (OD_{600}). The chosen concentration of bacteria for hematological, biochemical and immunological was 0.300 OD, corresponding to 2.9 x 10^7 CFU mL^{-1}, the maximum dose capable of triggering the immune response without causing fish mortalities (based in a previous lethal dose experiment).

For triggering of the host response against the pathogen, fish were individually weighted and intracoelomatic inoculated (0.1 mL of bacteria solution for each 10 g of fish weight). Thus, the same dose of *A. hydrophila* was guarantee in each fish from infected group. Fish from control group were inoculated by the same method with sterile PBS.

2.1.3. Blood and serum analysis during different phases of infection

One fish per tank was randomly blood sampled at 0 h (control), 6 h, 1 d, 7 d and 14 d pos-infection (n=6 per group for each time period). Fish were anesthetized in benzocaine (Sigma-Aldrich, China, ref. E1501) 100 mg L^{-1} for collect of 2.5 mL of total blood. An aliquot (0.5 mL) was diluted in 10 µl of heparin 5000 IU (Homeoderma®, Brazil) for analysis of hemogram, leukogram and leukocyte respiratory activity (burst). For biochemical and immunological analysis, the remaining blood (2 mL, without heparin) was used for obtainment of serum by centrifugation at 1400 *x*g during 10 min at 4°C.

Hematocrit data were expressed in % according to Goldenfarb and colleagues [22] and hemoglobin obtained in g dL^{-1} with commercial kit (Labtest Diagnostic SA, Brazil, ref 43). Counting of red blood cells (RBC) were measured at 10^6 cells mm^{3-1}, using the heparinized total blood diluted 1:200 in formalin citrate solution. The counting was performed according to Hesser [23] in the Neubauer chamber. With these data, the erythrocyte indices corresponding to the mean corpuscular volume (MCV = hematocrit/RBC*10 in femtoliters), mean corpuscular hemoglobin (HCM =

hemoglobin/RBC*10 in picograms) and concentration of mean corpuscular hemoglobin (CHCM hemoglobin/hematocrit in %) were calculated.

The blood extensions were stained with May Grünwald-Giemsa-Wright (MGGW). For complete blood cell data, 2000 erythrocytes were counted for quantification of white blood cells (WBC) and thrombocytes. For differential blood cell counts, 200 white blood cells were examined and categorized as lymphocytes, monocytes, neutrophils, eosinophils or LG-PAS special granulocytic cells. The burst was measured at 540 nm optical density, according to Biller-Takahashi and colleagues [24] (Sigma-Aldrich, CA, USA, ref. N6876) according to the production of reactive oxygen species (ROS).

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), protein, albumin and globulin analysis, were measured in serum, following the manufacturer's instructions. ALT and AST were performed with commercial kit (Labtest Diagnostic SA, ref 108 and 109, respectively). Values were reported in IU L^{-1}. Total protein and albumin concentrations were determined on serum with commercial kit (Labtest Diagnostic SA, ref. 19-1/250). The globulin was calculated by subtracting the albumin value from protein. Values were expressed in g dL^{-1}.

2.2. ETosis assessment: adapted in vitro protocol for analysis by differential interference contrast (DIC) microscopy and scanning electron microscopy (SEM)

After analysis of the hemogram, there was suspicion of the occurrence of ETosis in tambaqui. To evaluate the occurrence of ETosis, an adapted protocol from Brinkmann and colleagues [25] was developed following the detailed sequence described below.

2.2.1. Materials to prepare before starting the procedure

First, *A. hydrophila* strain, previously identified by sequencing of the 16S rRNA gene and stored at -80°C ultra-freezer in TSA supplemented with 30% sterile glycerol was reactivated on TSA (Sigma-Aldrich, India). After striated inoculation of bacteria with platinum loop on the surface of agar plate, bacteria were incubated at 28°C for approximately 18h (Figure 1, Step 1).

Glass coverslips were sterilized in autoclave (121°C for 15 min). After sterilizing, smears were made in all blades, using the reagent 2 (containing 3.8 g dL^{-1} bovine albumin and 0.1% sodium azide) from albumin test used in biochemical analysis (Labtest, Brazil). This step will enable the cells to settle and adhere onto the glass, during incubation procedure. Smears were allowed to dry at room temperature for approximately 18h sterile plates (Figure 1, Step 2).

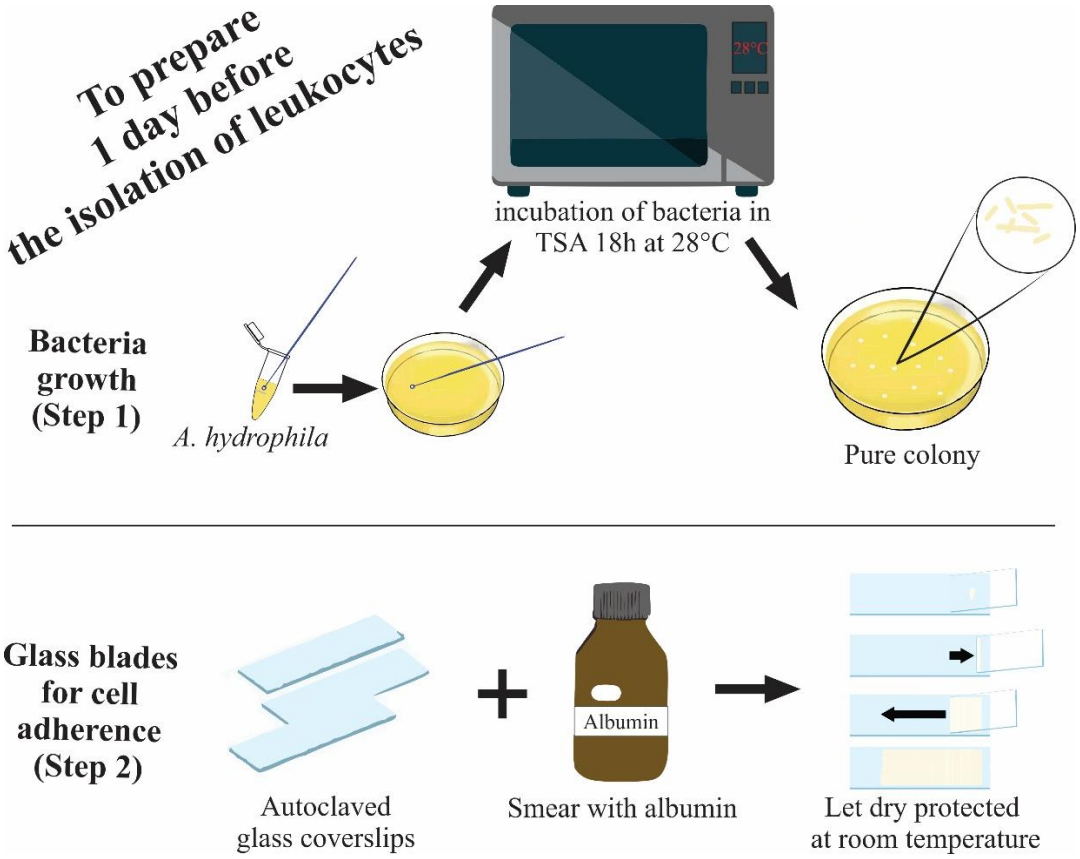

Figure 1. Steps prior to co-incubation of leukocytes with *Aeromonas hydrophila*.

2.2.2. Leukocytes isolation from head kidney

Isolation of leukocytes was performed according Figure 2. In detail, two healthy fish from maintenance tank were euthanized with 0.1 g L^{-1} benzocaine (Sigma Aldrich, Brazil) and rupture of bone marrow. Fish were bled through caudal vessel punction in order to avoid contamination with erythrocyte and head kidney were collected aseptically (Figure 2, Step 1). Over ice, the head kidneys were placed into sterile agar plates with RPMI-1640 containing heparin at 40 U mL^{-1} (Figure 2, Step 2). The organ and solution were gently passed through a 100-μm sterile nylon mesh with sterile syringe plunger and assembled into sterile silicon-coated falcon tubes (ThermoFisher, Brazil) (Figure 2, Step 3). The RPMI-1640 was added in the filter when necessary (approximately 25 mL of the medium). Falcon tubes (one for each fish) were centrifuged at 300 xg for 7 min 4°C without brake (Figure 2, Step 4). After centrifugation, supernatants were discarded and the pellets (cells) were carefully resuspended in 3 mL of RPMI 1640. The cell suspensions were layered onto Percoll gradient and centrifuged at 800 *x*g for 25 min at 4°C without brake. Leukocytes were collected at the interface between 1.0838 and 1.020 g mL^{-1} Percoll (Figure 2, Step 5).

At a final step, the cell layer intake was transferred into fresh Falcon tubes and filled up with RPMI 1640 (ThermoFisher, Brazil) for wash, centrifuging the solution at 600xg for 8 min at 4°C. The procedure was repeated twice, discarding supernatant and resuspending the cells in 2 mL of RPMI 1640. Finally, cells were counted in Neubauer chamber, despising the erythrocytes and dead cells (stain with trypan blue the count-selected solution will color dead cells in blue). Cells were diluted in RPMI 1640 until reach 3 x 10^6 leukocytes mL^{-1} (Figure 2, Step 6).

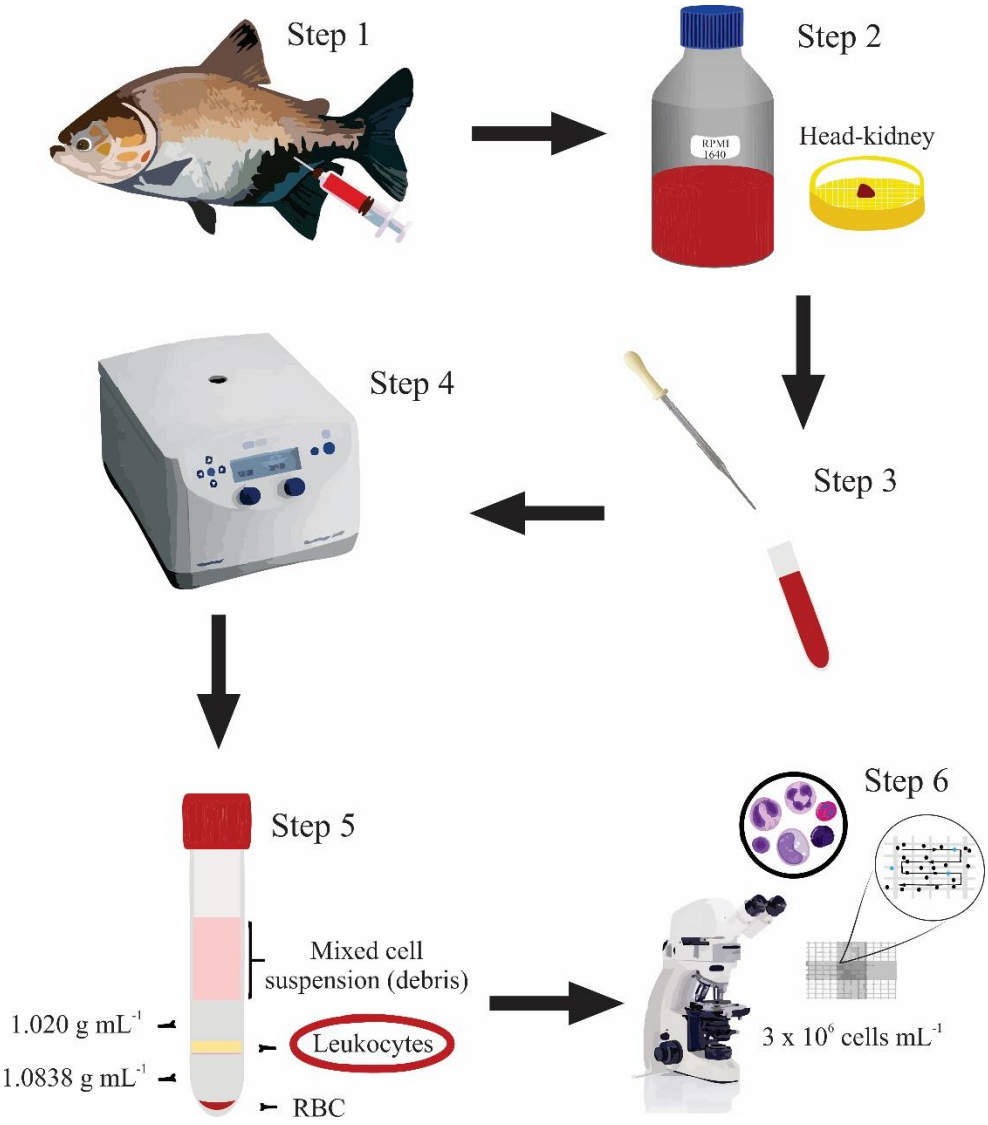

Figure 2. Steps of preparation and isolation of the leukocytes of tambaqui *Colossoma macropomum* for ready co-incubation with *Aeromonas hydrophila*.

2.2.3. Co-incubation: activating leukocytes with Aeromonas hydrophila

The leukocytes were activated according to Figure 3. Categorically, to stimulate the traps formation, head-kidney derived leukocytes at 3 x 10^6 cells mL^{-1} were co-incubated with live bacteria (Figure 3, Step 2). As leukocytes, bacteria were also counted as living cells in Neubauer chamber until reach 3 x 10^6 mL^{-1}. Both solution (1 mL each, at 1:1 v/v) were pipetted in coverslips previously treated with albumin. Then, cells were allowed to incubate at 22°C. Meanwhile, samples were visualized in differential

interference contrast (DIC) microscopy to establish the appropriated periods when the cells should be taken off from co-incubation and preserved in buffered glutaraldehyde 2.5 % (pH 7.4) (1:10 v/v) for 24h.

2.2.4. SEM processing

Scanning electron microscopy (SEM) were performed in order to confirm the formation of ETosis for *A. hydrophila* in each established time (0, 4 and 7h) (Figure 3, Step 3).

First, samples were dehydrated in serial concentrations of alcohol at 30, 70, 80, 90 and 100%. Then, samples at glass blades were carefully cracked into small pieces to fit inside the equipment, ascertaining that biological material were on the top of the blades. After critical-point dried, samples were mounted on aluminum stubs and gold-coated. Samples were photographed at Zeiss EVO MA-10 at 15 kV (Figure 3, Step 3).

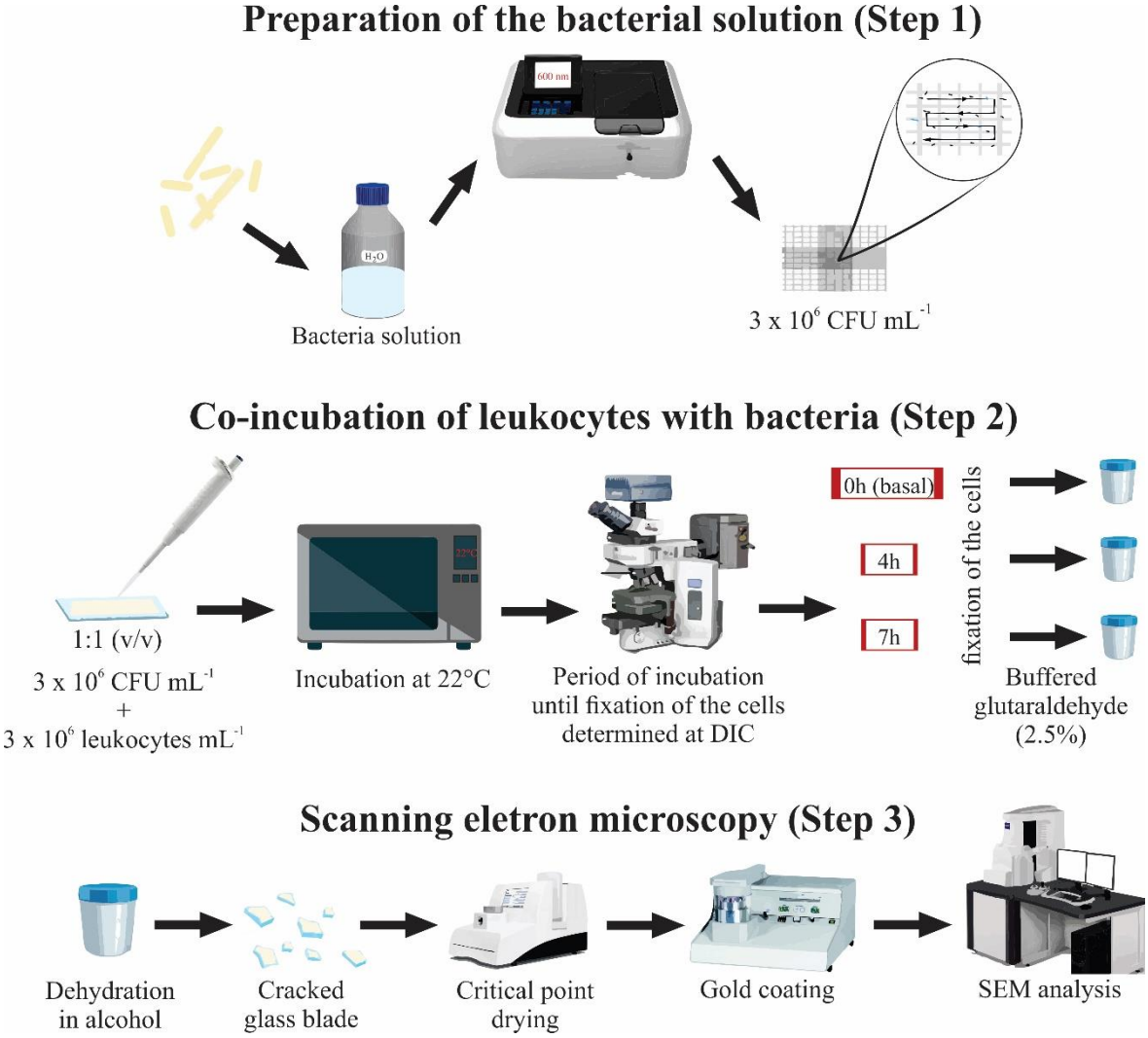

Figure 3. Steps of co-incubation of leukocytes with *Aeromonas hydrophila* and evaluation in differential interference contrast (DIC) microscopy and scanning electron microscopy (SEM).

2.3. Statistical analysis

Data from blood and serum analysis were expressed as mean ± standard deviation, subjected to tests for homoscedasticity (Levene) and normality (Cramer Von Mises), and analyzed with Software R using the unidirectional analysis of variance (ANOVA) procedure. When ANOVA-test was significant, least significant difference was calculated using Tukey test for the comparison among means. Differences with a probability value less than or equal to 0.05, 0.01 and 0.001 (represented in the graphics by *, ** and ***, respectively) were considered significant. ETosis data assessment were addressed descriptively.

3. Results

3.1. Blood and serum analysis in different phases of infection

In early phase of infection, hematological parameters of hematocrit (from 6 h), hemoglobin (from 24 h) (Figure 4) and RBC (from 24 h) decreased ($p<0.05$) (Figure 2) in infected animals. All these parameters were lower ($p<0.05$) in the infected group up to 14 days after infection. MCV, MCH and MCHC did not change with the presence of infection (Figure 4).

In Figure 5, the cellular changes of tambaqui during *Aeromonas* infection are evidenced. Leukocytes and lymphocytes presented the same patterns, decreasing drastically at 6 h and enhancing at 7 d ($p<0.05$). Monocytes and neutrophils decreased at 6 h, but neutropenia remained until 24 h ($p<0.05$), returning to normality after this period. At 24 h, granulocyte special cells (GEC) enhanced in infected fish ($p<0.05$). Eosinophils did not change due to bacteria. Thrombocytes decreased in peripheral blood at day 7 in infected ($p<0.05$).

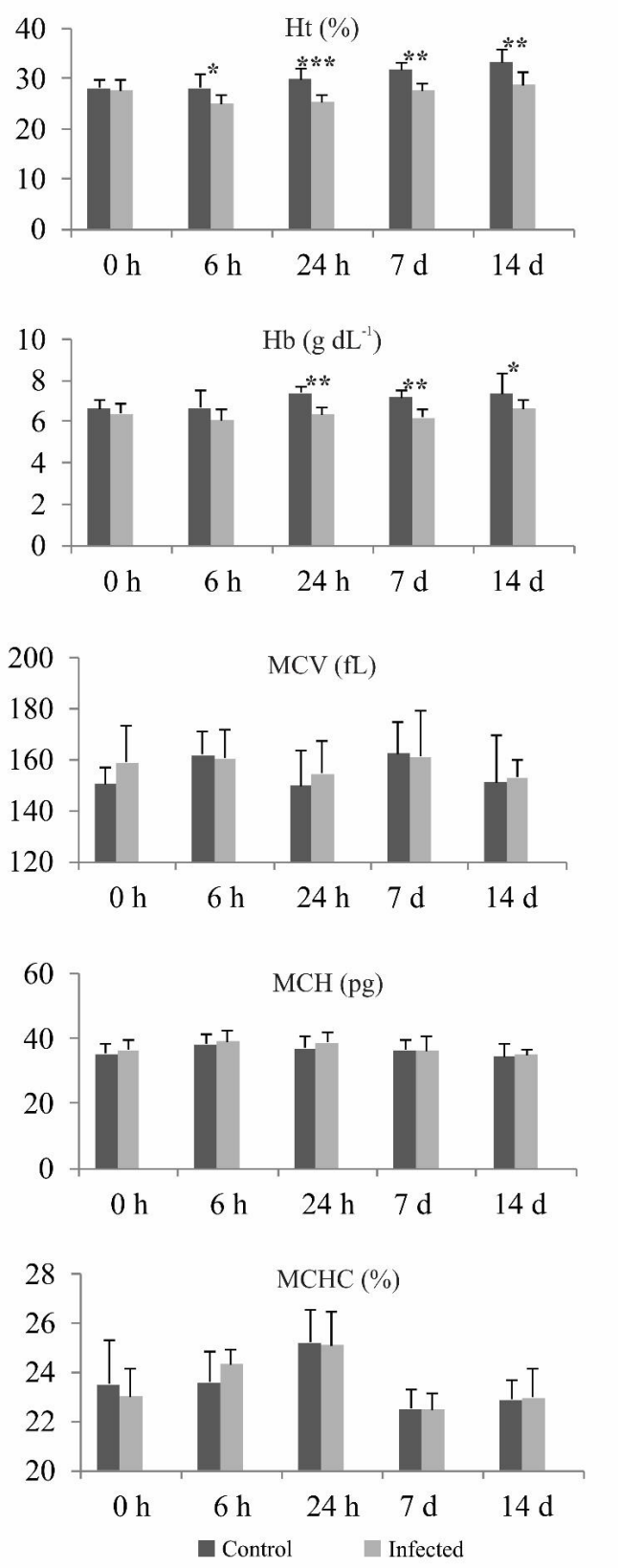

Figure 4. Characterization of erythrocytic lineage of healthy and *Aeromonas*-infected tambaqui *Colossoma macropomum* during 14 days after inoculation with PBS and bacteria, respectivelly. [1]Hematocrit (Ht), hemoglobin (Hg), mean corpuscular volume (MCV), mean

corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC). [2]Values within different superscript *, ** and *** means p≤0.05, 0.01 and 0.001.

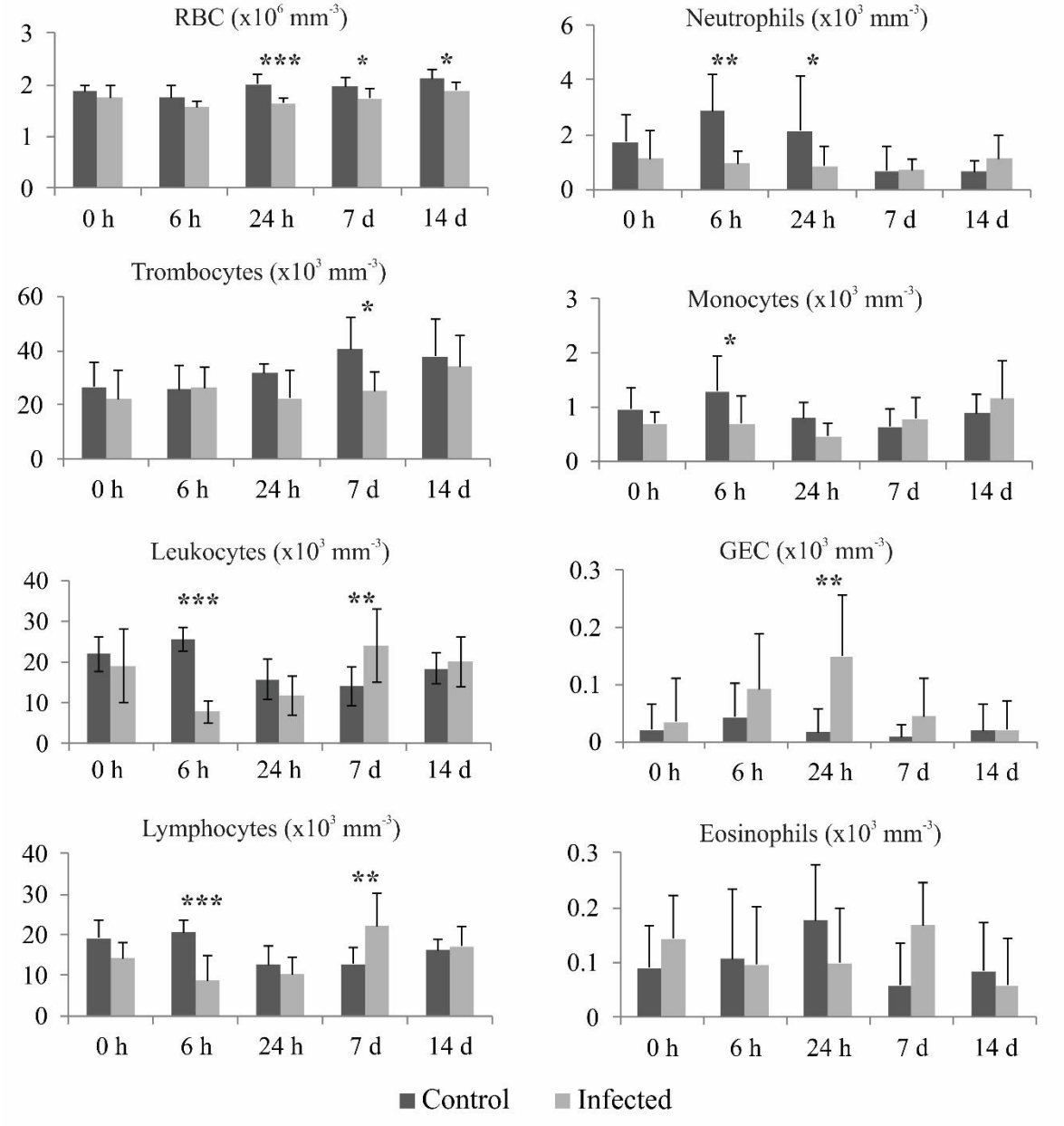

Figure 5. Blood cells profile of healthy and *Aeromonas*-infected tambaqui *Colossoma macropomum* during 14 days after inoculation with PBS and bacteria, respectivelly. [1]Red blood cells (RBC) and granulocyte special cells (GEC). [2]Values within different superscript *, ** and *** means p≤0.05, 0.01 and 0.001.

Albumin decreased after 24 h in infected animals and the alteration remain until 14 d ($p<0.05$). Protein was not affected, but globulin decreased at 6h ($p<0.05$). ALT was not affected, but AST enhanced at 24 h in diseased fish ($p<0.05$). Burst was not affected by bacteriosis (Figure 6).

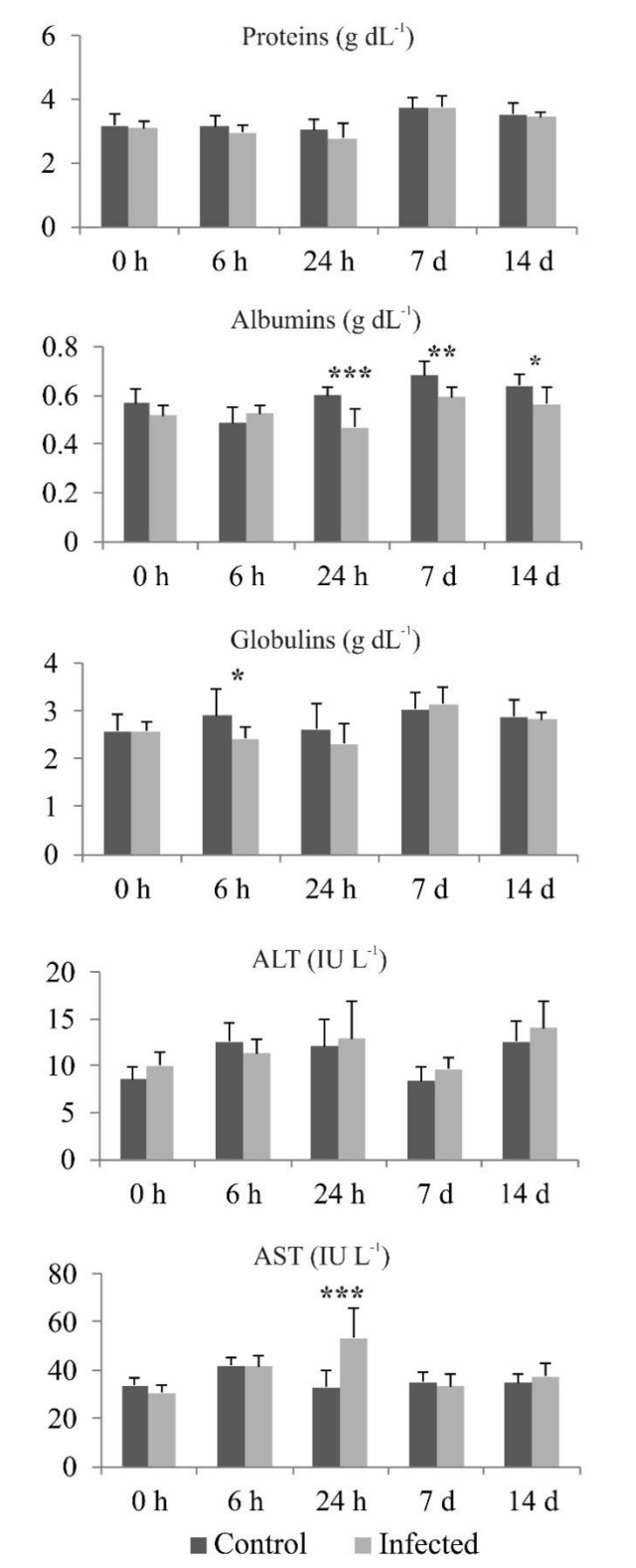

Figure 6. Serum biochemical profile of proteins (total protein, albumin and globulin in g dL^{-1}) and hepatic enzymes (alanine aminotransferase and aspartate aminotransferase in IU L^{-1}) of healthy and *Aeromonas*-infected tambaqui *Colossoma macropomum* during 14 days after

inoculation with PBS and bacteria, respectivelly. [1]Values within different superscript *, ** and *** means p≤0.05, 0.01 and 0.001.

3.2. ETosis assessment: adapted protocol for analysis by DIC and SEM

Photomicrographs in DIC microscopy (Figure 7a) and SEM (Figure 7b-d) confirmed the integrity of leukocytes from control group before co-incubation with *A. hydrophila*, which shows that the method of fixation and evaluation were adequate.

After 4h of co-incubation (Figure 7e, h), most of the cells from both bacteria and fish were integrated (Figure 7e). However, at Figure 7f it is possible to visualize the phagolysosome formation from a granular leukocyte, which is engulfing bacteria to destruct *A. hydrophila* throw phagocytosis, while in Figure 7h lysed cells with exposure of their components were observed.

After 7h of co-incubation (Figure 7i-l), the intact leukocytes could not be identified at DIC (Figure 7i), indicating that filaments were nets formation released from nuclei of the suicided leukocytes. Field emission scanning electron microscopy photograph shows advanced stage of ETosis (Figure 7j-l) with cloudy aspect of the traps. Figures 7k and 7l show typical images of the formation of extracellular traps, being identical to previous studies in different animals.

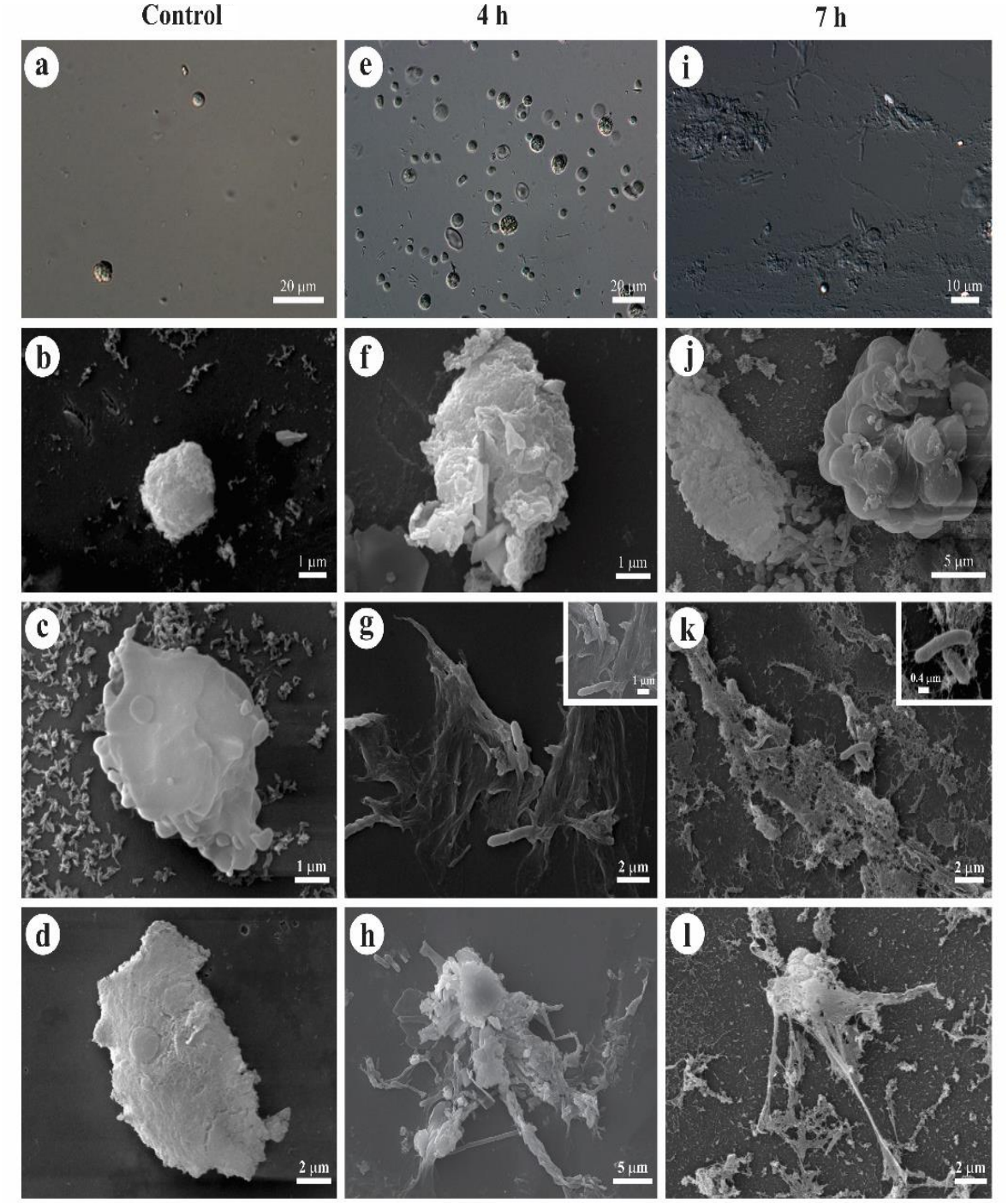

Figure 7. ETosis visualization in tambaqui *Colossoma macropomum* leukocytes: control (a-d), 4h pos-infection (e-h) and 7h post infection (i-l). Photomicrographs in differential interference contrast (DIC) microscopy showing: intact cells (a), part of intact cells associated with *Aeromonas* (e) and absence of intact cell (i). Scanning photomicrographs showing intact cells (b-d), cell phagocytizing the *Aeromonas* (f) and dead cells with exposure of their content attached to the bacteria (h, k, l).

4. Discussion

Tambaqui is the most produced native species in South America but a deeper knowledge of its immune system is still lacking and this might hamper disease control. To our knowledge, this is the first description of pathophysiological aspects during the acute and late phase of *A. hydrophila* infection in tambaqui. This study also reports a novel type of cell death for tambaqui during infection, resulting in severe leukopenia.

Based on the results of RBC, Ht, Hb, MCV, MCH and MCHC, infected tambaqui showed normocytic and normochromic anemia, consistent with hemorrhagic septicemia caused by the bacterium *A. hydrophila*. The most significant cellular alterations at the beginning of the infection were the drastic reduction of neutrophils (from 6 to 24h post infection), monocytes (6h after infection), lymphocytes (6h after infection), and consequently of total leukocytes. When fighting the antigen at the higher inoculated doses (maximum concentration of inoculated bacteria, capable of activating its immune system, but without killing the host), the leukogram did not follow the paradigm that leukocytes increase during the inflammatory response against a bacterium. Based on this unusual result, the occurrence of death of these cells was investigated. In relation to the lymphocyte depletion at the beginning of the infection, Garcia and Moraes [26] pointed out that this also occurred in pacu *Piaractus mesopotamicus* infected by *Aeromonas* and that this is due to the migration of these cells to the inflamed tissues. However, we strongly recommend that findings of leukocyte depletion after challenging to be investigated for ETosis, besides the possibility of cells migration. After 7 days of infection, the level of these cells in the blood has risen drastically which is a reflection of their immense immuno-cellular and immunohumoral (in the formation of antibodies) importance. Regarding the biochemical analyzes, the most relevant result was the reduction of albumin from 24h, which was maintained up to 14 days after infection. The decline of the albumin was also the most

significant result described in *A. hydrophila* infection in trout [27] and, according to this author, the low level of albumin may be the result of lesions, increased catabolism in acute inflammation or reduced synthesis due to hepatopathy, or may be related to a renal damage. Punctually, serum globulin levels decreased significantly only 6h after infection, which was similar to data obtained by Garcia and Moraes [26] in *A. hydrophila*-infected pacu and this may be related to the greater contribution of proteins to damaged tissues in inflammatory process and/or as a result of losses of these proteins due to hemorrhages. Furthermore, AST levels increased 24h post infection, which was a similar result to that observed in goldfish after infection (12 to 36h) by *A. hydrophila* [28]. This enzyme, at high levels indicates possible liver damage, which is a common occurrence caused by bacteria in fish and which also resulted in lower albumin synthesis.

The observed leukogram during *Aeromonas* infection, allowed new suspicions about the role of leukocytes in tambaqui. In this way, the programmed death of the defense cells occurs as one of the last mechanisms of the immune system, in an attempt to control *A. hydrophila*, thus avoiding the death of the host. ETosis appear to be a form of innate response that binds microorganisms, prevents them from spreading, and ensures a high local concentration of antimicrobial agents to degrade virulence factors and kill bacteria [13]. This is an unusual mechanism of the host to control and destruct a pathogen, which leads to leukopenia in the cell count. Among the leukocytes, the neutrophil and monocytes were the cells that showed significant decrease in blood during infection. The occurrence of ETosis of both cells are the well-documented in humans [29-31], terrestrial animals [15, 16] and aquatic organisms [18, 19]. Based on leukogram, DIC and SEM results, there is great suspicion that much of tambaqui's cellular suicide was related to neutrophils and monocytes. However, it is well known that other cell types, such as eosinophils [32] and mast cells [33] also perform this mechanism of death to combat infections. The dose of infection used in

this study was the maximum in which no mortality occurred, so the occurrence of this mechanism of cell death was shown to be highly important and efficient. Despite drastic cellular changes at the onset of infection, the fish survived and tended to normal after 2 weeks of infection. Cellular suicide appears to have been crucial in combating the high initial bacterial concentration.

Moreover, this study provides a new methodology, fast and low-cost, for visualization of ETosis. Usually, to confirm ETosis formation, their generation is induced *in vitro* for visualization using correlative microscopy combining: transmission electron microscopy (TEM), scanning electron microscopy (SEM), immunofluorescence and live cell imaging techniques [25]. This study provides a protocol to visualize the ETosis in SEM. The provided methodology made the protocol more feasible (at least 1.100 dollars less costly, disregarding the values of required equipments and only taking account the reagents quoting). The method does not require cell-culture plates, replacing by glass cover slips coated with albumin and since co-incubation of leukocytes with bacteria were performed in a very short time, CO_2 incubators were not mandatory for success of methodology. Furthermore, the protocol was adjusted for bacteria, thus, PMA solution was not needed for activation of cells. Since this reagent is frequently expensive for researches that do not make use of it frequently, the technique using bacteria is more appropriated. At SEM procedure, the use of osmium tetroxide (OsO_4) and tannic acid were also removed, making the procedure even more feasible. The simplified and low-cost methodology implemented in this study, which was characterized by leukocyte co-incubation with *A. hydrophila* (1: 1 ratio) in albumin-coated glass coverslips, was sufficient to confirm the occurrence of ETosis in leukocytes of tambaqui. Isolating the different leukocytes from tambaqui was not the target of this study and is a methodology that deserves to be standardized for this species of

fish, which can bring more news about each cell capable of committing suicide to fight infection.

5. Conclusion

All results suggest that the innate system was highly modulated during *Aeromonas* infection to guarantee protection for tambaqui. This study also describes for the first time the occurrence of ETosis for this important South American fish.

The occurrence of ETosis was confirmed in tambaqui throw development of a modified (fast and low-cost) protocol for DIC microscopy and SEM. Results suggest that tambaqui leukocytes expose its chromatin and granule proteins to bind and kill *A. hydrophila* as a last resource to fight infection and it has been concluded that was highly efficient.

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Capítulo 4

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Patterns of innate immune response of tambaqui *Colossoma macropomum*: modulation of gene expression in hemorrhagic septicemia

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Abstract

The production of tambaqui *Colossoma macropomum*, reached a milestone, being considered the main native species produced in South America continental waters. Although the importance of this fish, its immunity is not well understood. In this study we stablished some patterns of innate immunity for the species in two experiments. Both studies evaluated the fish due to the absence (intraperitoneal saline) or presence (intraperitoneal, 3 x 10^7 CFU ml^{-1} of *A. hydrophila* at 0.1 ml 10 g^{-1} of living weight) of infection during time-course of 5 periods (0h, 6h, 24h, 7d, 14d). In the first experiment, the partial gene sequences and the gene expression of the IL-1β, IRAK-1, C3, C4, lysozyme, IL-10, HSP70 and β-actin were determined in the main secondary lymphoid organs of fish: spleen and head-kidney. The second study was performed for analysis of the alternative complement pathway ACH50 in serum for support on C3 gene-expression elucidation. Results of gene expressions show a tendency of up-regulation of these immune genes in infected fish in early phase of infection (mostly around 6h and 24h) and chronic phase (7d and 14d) with exception of HSP70 which shows down-regulation in infected fish. Results of this study suggest that lysozyme is evolved in both pro and anti-inflammatory activities. The genes of the complement system demonstrate that C4 regulation follow the tendency of pro-inflammatory genes while C3 gene was surprisingly not expressed in most fish and this corroborated with the results of the complement system activity in serum that did not show activity in most fish as well. Possible reasons for regulation of gene expressions and association with fish disease are addressed in this paper.

Key-words: black pacu, cachama, aquaculture, *Aeromonas hydrophila*, complement system, interleukins, lysozyme.

1. Introduction

The tambaqui *Colossoma macropomum*, also known as black pacu (in English) or cachama (in Spanish), is the most produced native fish in South America [1, 2, 3]. Since its production reached a milestone, diseases have been one of the main obstacles to its farming development [3]. It is from our knowledge that up to date, this tropical round fish has no genome description and none genetic studies regarding expression of immune-related genes, which leads to delay of knowledge, thus, compromising the development of vaccines, immunostimulants, and other forms to prevent diseases. Describing some of these genes may be of great help in the immunology research of this species.

Studies of immune genes of aquatic organisms have been disseminated quickly, since they play a key role in determining the resistance factors against environmental condition that leads to stresses [4] and also infections [5,6]. Some immune molecules like lysozyme (Lys) [7], interleukin 1 beta (IL-1β), interleukin associated kinase 1 (IRAK 1), interleukin 10 (IL-10) [8,9] and proteins from the complement system such as C3 and C4 [10] are among the most studied in immune-genetic, for playing a central role in control of the infection and inflammation. It is known that different fish species present different amount of these antimicrobial proteins in tissues and fluids [11], and thus, important to characterize their gene expressions and modulations during infection, in order to understand particularities of the species immune response. However, to date, the gene sequence of these molecules and proteins, and their respective expressions in the tissue, are unknown in tambaqui. Knowledge of how fish respond to infections is essential for the application of appropriated sanitary management and treatment measures.

Infectious diseases are considered the biggest constraint that aquaculture currently faces to reach its maximum potential, demanding urgency in the development of basic and applied health studies and hemorrhagic septicemia caused by *Aeromonas hydrophila* is

pointed as one of the main causes of fish mortalities, thus, several studies have been focused on evaluate the immune-related genes expression during *Aeromonas* infection of important farmed fish species [12 - 17]. Although the literature evidences massive mortality outbreaks by hemorrhagic septicemia in tambaqui [3, 18], only recently *A. hydrophila* was confirmed as pathogenic through the establishment of Koch postulate [19], then, immunology studies are needed to understand the course of this disease in this species.

The aim of this study was to characterize for the first time some key immune genes (C3, C4, IRAK 1, IL-1β, IL-10, lysozyme and HSP70) in tambaqui, to describe primers and to evaluate a time-course study of genes expression during 2 weeks of *A. hydrophila* infection.

2. Material and methods

2.1. Fish, experimental conditions and sampling

Breeders from Aquaculture Center of São Paulo State University (Caunesp, São Paulo, Brazil) were molecularly identified as tambaqui according to Hashimoto [20] for offspring obtainment. The sanitary status of the batch was accessed since then, until the final of the experimental period.

Fish (170 ± 20 g) were transferred from maintenance tanks (500 L, with continuous water flow and aeration) to experimental tanks (140L, with water recirculation system), divided randonmly in two groups: control (healthy, PBS-inoculated) and infected group (*A. hydrophila*-inoculated), as following described in item 2.2.. Each group was comprehended by 3 tanks and 5 fish per tank). One fish per tank from both groups (healthy and infected) was sampled for RNA extraction of lymphoid organs at 5 time-points, comprehending triplicates at each time period: 0h (gene basal expression, prior to inoculation), 6 h, 24 h, 7 days and 14 days. The comparison was performed in fold-change.

All animals were fed twice a day, until satiety. The thermostats were programmed to maintain the temperature in both systems at 30°C, corresponding to a good temperature both for the fish and for the development of the infection. The water parameters were periodically evaluated using the Horiba U-50® multiparameter probe (Japan). The averages of the water parameters were: 7.00 ± 0.01 pH, 144.2 ± 4.09 mV oxidative reduction potential, 3.18 ± 0.48 mg L^{-1}dissolved oxygen, 0.24 ± 0 mS cm^{-1} conductivity, 0.15 ± 0 g L^{-1} total dissolved solids and 29.73 ± 0.02ºC temperature).

Fish were anesthetized in benzocaine solution (0.1 g L^{-1}) (Sigma Aldrich, ref E1501) before blood sampling, extraction of lymphoid organs or intraperitoneal (i.p.) challenging with bacteria.

The experimental procedures were approved in ethical principles established by the Brazilian College of Animal Experimentation (COBEA) and by the Committee on Ethics in Animal Use (CEUA) of the Faculty of Agrarian and Veterinary Sciences, UNESP, Campus Jaboticabal under protocol 009072/17.

2.2. Experimental disease

A pathogenic strain of *A. hydrophila*, isolated from diseased tambaqui (stock culture from laboratory bank, stored in glycerol 30% vol./vol. at -80°C) was reactivated in tryptone soybean agar (TSA, Himedia, India) and incubated at 28°C for 24 hours for bacterial growth. One colony was transferred to tryptone broth soybean (TSB, Himedia, India) and incubated at 28°C for 24 hours. After growth, the broth medium was washed by centrifugation at 3000*xg* at 4°C for 10 min. and the bacteria pellet suspended in sterilized phosphate buffered saline (PBS, pH ≅ 7.4). This procedure was repeated twice. Then, the bacterial solution was resuspended in sterile PBS until reaching the desired optical density reading (2100 Unico Spectrophotometer, Japan) at 600nm OD_{600}. The

chosen absorbance in both experiments was 0.300 OD, the maximum concentration of bacteria capable of triggering the immune response without causing fish mortalities, determined by pilot experiment. Serial dilutions of homogenates at 0.300 OD were plated in TSA. Plates were incubated at 28° for 24 hours. Bacteria concentration at homogenate corresponded to 2.9 x 10^7 CFU ml^{-1}, ascertained in both experiments.

This bacterial solution was used for triggering of the innate immune response. The doses of inoculum for i.p. challenge was determined according to weight, corresponding to 0.1ml $10g^{-1}$ (v/w) of suspension of *A. hydrophila* in the infected group and 0.1ml $10g^{-1}$ (v/w) of sterile PBS in the control group.

Extra fish inoculated from infected and control group were euthanized (benzocaine overdose) for reisolation of bacteria from head kidney and bacteria sequencing to confirm infection by *A. hydrophila*. The DNA of the bacterium was extracted according to Sebastião *et al*. [18] from the bacterial colonies. The identity of bacteria was ascertained in NCBI database with sequencing of 16S rRNA gene.

2.3. Primers design

Fasta sequence of Mexican tetra *Astyanax mexicanus* and piranha *Pygocentrus nattereri* (the two most closely species to tambaqui provided by genome) were obtained from nucleotides data bank (NCBI). The complete genes sequence (IL-1β, IRAK-1, C3, C4, lysozyme and IL-10) of both species was aligned in Bioedit software using ClustalW for multiple sequence alignment. The conserved regions were obtained visually by analyzing the consensus sequence created by the software and then the primers were designed. The b-actin (GenBank access number: MF370934.1) and HSP70 (GenBank access number: KX444555.1) primers were designed based on FASTA sequence of tambaqui disponible at NCBI database. The design (specific and degenerate) and quality

of the primers were performed, respectively, with the PrimerQuest and OligoAnalyzer tools from Integrated DNA Technologies (IDT) company. Custom primers (Table 1) were purchased from ThermoFisher Scientific.

Table 1. List of primers designed for conventional PCR and qPCR.

Gene target	Primer designation	Degenerated and non-degenerated primers sequence (5'-3') for PCR	TM (°C)	Specific primers sequence (5'-3') for qPCR	TM (°C)
Reference gene					
β-actin	F	-	-	ATG AAG ATC CTG ACC GAG AGA	60
(KX444555.1, NCBI)	R	-	-	TCG AAG TCA AGG GCA ACA TAG	60
Cytokines					
IRAK 1	F	TCT CCT GGT CAC AGC GTG	50	TCA CAG GCA GAA GAG CAT TAG	60
	R	GGA TCC AGA TGC TTC TTC CAG AT	50	CTT CCC ATC ATC CTC AAC TTC T	60
IL-1B	F	TGG AYT GCT CTG ATC CTT TGG CC	50	CAA GTT CAA GCA CAC TCA GAAA T	60
	R	GGA ARC GYT CCA TGC CAT CAT T	50	ACT CTC TTC AAT TAT GCT CTC CAA	60
IL10	F	CAC TTC TTC AAG TTC CTC	50	TCA AGA GGG ACC TGA TCA AAT G	60
	R	CTT TGA TGC CAG ATA CT	50	TTG TAC AGC CCT TTC TTC TTC A	60
Anti-bacterial peptides					
Lysozyme	F	ATG GSA CCT CAG GTG ATC	50	GTC ACT CAG GGA ACT GAG ATT T	60
	R	CTC RTA TGT GCG RAC GTT	50	CCA GCG TTG TAG GCT GAT ATT	60
C3	F	ACG CAC TAC CTG GAC AC	50	AGA TGT GCG AGG AAA GGA TG	60
	R	CTG TTG AGC CAG TGC ACG	50	TGG AAG ACT AGG GAC TGA CTT A	60
C4	F	TTC CCA GTT TCR GTG TGT CCA TC	50	CAG AAC ACG GTC ACA CTA CAT	60
	R	CCG TCC ACT GTT ACC TCC A	50	TGT TGG ATA CGT CAG TCT TCA C	60
HSP70	F	-	-	CCT GTC CAA GGA AGA CAT TGA	60
(MF370934.1, NCBI)	R	-	-	GTT CTT GGC TGT GAC CTT CT	60

For testing the customized primers (Table 1), standard PCR was performed using the mix: 1.2 µl of the 10X buffer; 1.0 µl of dNTP; 0.75 µl $MgCl_2$; 0.05 µl of taq polymerase and 0.40 µl of each primer (F and R) at 10 mM; 0.5 µl of the cDNA and 8.2 µl of H_2O ultra-pure (Sigma Aldrich, Brazil), to a final volume of 12.5 µl. The mix was initially denatured at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s and extension at 72°C for 1 min. The annealing temperature for all primers was 50°C. The resulting PCR product was analyzed with 2% of agarose gel stained with Nancy® (ThermoFisher Scientific, Brazil). When a single band was obtained, it was purified (PureLink Kit, ThermoFisher, Brazil) and sent for sequencing with Sanger ABI 3730 xl DNA Analyzer (Applied Biosystems, Foster City, California, CA).

When more than one band was obtained, DNA fragments were separated according to manufacturer's instructions with E-gel® Electrophoresis System (ThermoFisher, Brazil) or with PureLink® Kit (ThermoFisher, Brazil).

When no band was identified or in the presence of weak bands, two strategies were adopted to obtain an initial gene sequence of tambaqui: nested PCR and changes in the melting temperature (MT). Both strategies were sufficient to obtain the partial sequence of all aimed genes.

The identity of each tambaqui amplicon was attested by comparison with the known sequence from others fish in NCBI data base.

2.4. RNA extraction for relative qPCR

In each sample period (0, 6h, 1 day, 7 days and 14 days post-infection), 3 fish from each group (infected and control) were euthanized in benzocaine (100 mg L^{-1}). The main secondary lymphoid organs (head kidney and spleen) were immediately collected in clean and cooled room and stored in RNase, DNase and pyrogen free tubes in 1:10 volumes of tissue in RNA later (Sigma Aldrich, ref. MFCD03453003). Samples were stored at -80°C until the extraction of the genomic RNA.

Total RNA was extracted using TRIzol® (Invitrogen, CA, USA) following manufacturer's instructions, eluted in DEPC® treated water (Invitrogen, CA, USA) and stored in -80°C. The quantity and purity of samples were ascertained in Nanodrop One/One (ThermoFisher, CA, USA). The integrity of genetic material was certified in Bioanalyzer System using the Agilent RNA 6000 Nano Kit® (Agilent Genomics, CA, USA, ref. 5067-1511), as manufacturer's instruction. Samples with RNA Integrity Number above 7 were selected for treatment with AmbionDnaseI® (ThermoFisher, CA, USA, ref. AM2222) and RNase OUT Recombinant Ribonuclease Inhibitor® (ThermoFisher, CA, USA, 10777019) according to manufacturer's instructions and transformed into complementary DNA (cDNA) with SuperScript™ IV Reverse Transcriptase (ThermoFisher, CA, USA, ref 18090010). The cDNA samples were then diluted in nuclease-free water prior to qPCR analysis.

2.5. Real time PCR (qPCR)- relative quantification of immune genes

Samples of spleen and kidney standardized in amount of 100 ng μl^{-1} cDNA were used for qPCR. The qPCR was performed into 96-well plates (MicroAmp Fast Optical Reaction Plate Applied Biosystems, USA) in a final volume of 10 µL. The mix consists of 0.4 µL of each primer, 5 µL of SYBR Green Select Master Mix (Applied Biosystems, USA) and 1 µL

of cDNA (diluted 1:10), completing with ultra-pure H_2O. The plates were sealed (Applied Biosystems adhesive film) and the reaction performed on Step One equipment (Applied Biosystems, USA) under the following conditions: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 30 seconds for denaturation and 72°C for 30 seconds for primer extension. The annealing temperature for qPCR was 60°C.

2.6. Complementary study: serum complement system analysis

To provide data for discussion of C3 gene results, data from an experiment analyzing the serum complement activity (ACH50) of tambaqui was provided. For this experiment, sixty fish (average weight 185 ± 23 g) were blood sampled for serum obtention, comprehended six replicates for each treatment at the same conditions of infection. One fish per tank was blood sampled (n=6) in each time period: 0 h (control), 6 h, 1 day, 7 days and 14 days post-infection. Total blood was sampled from caudal vein of fish anesthetized in 0.1 g L^{-1} of benzocaine (Sigma-Aldrich, China, ref. E1501). The blood was poured on Eppendorf's tube and left to clot. After 2 hours, the blood was centrifuged for serum obtainment (3000xg/10min/4°C) and immediately storage at -80°C ultra-freezer, until complement system analysis. The complement system activity (alternative pathway) in serum was estimated according to Zanuzzo [21] in minutes required for 50% hemolysis of the rabbit erythrocytes.

2.7. Data statistics and analysis

The relative quantifications of the target genes transcripts (C3, C4, IRAK 1, IL-1β, IL-10, lysozyme and HSP70) were calculated with a chosen reference gene transcript (b-actin) using the $2^{-\Delta\Delta CT}$ method that not require a calibration curve, and computes an expression ratio based on real-time PCR efficiency and the crossing point deviation of the

samples [22]. We measured the PCR efficiency by constructing a standard curve using a serial dilution of cDNA. Two independent qPCR replicates of each sample were used for calculation. The calibration curve and efficiency of the qPCR were performed for each gene according to Biorad protocol (http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_5279.pdf) and analysis showed high amplification efficiency (90–105%), guarantying consistency across replicate reactions.

Data were subjected to one-way analysis of variance (ANOVA) for statistical comparison and expressed as fold change. All the data were examined for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Levene median test). Significant results ($p \leq 0.05$) on ANOVA were tested with the Tukey multiple comparison test of means using a significance level of 0.05. Significant gene expression ($p \leq 0.05$) were expressed with asterisks above the histogram bars.

3. Results

3.1. Gene sequences of tambaqui

The identity of immune gene amplicons was highly similar to other fish species, especially with South American fish, such as piranha *P. nattereri* and Mexican tetra *A. mexicanus*, both used previously for the design of degenerated and non-degenerated primers for conventional PCR. For all of the immune genes studied in tambaqui, the homology with piranha was greater than 90%, whereas for the Mexican tetra, the homology was greater than 75%. In addition, the sequences obtained showed high homology (72 to 92%) with species from other continents, including channel catfish *Ictalurus punctatus* and pangasius *Pangasianodon hypophthalmus*. Complete data are shown in Table 2.

Table 2. Amplicon sequencing used to design conventional and qPCR primers of selective immune-related genes of tambaqui *Colossoma macropomum*.

Gene target	Amplicon sequencing (5'-3')	Fragment size (bp)	Query cover (%)	Homology to other fish species (%)
Reference gene				
β-actin (KX444555.1, NCBI access number) (Nascimento *et al.*, 2016)	GCTGCGTGTTGCCCCTGAGGAGCACCCCGTCCTGCTTACTGAGGCTCC CCTGAACCCCAAAGCCAACAGGGAAAAGATGACACAGATCATGTTT GAGACCTTCAACACCCCAGCCATGTACGTTGCCATCCAGGCTGTGCT GTCCCTGTACGCCTCTGGTCGTACCACTGGTATCGTGATGGACTCTGG TGATGGTGTTACCCACACTGTGCCCATCTACGAAGGTTACGCCCTGCC CCATGCCATCCTCCGTCTGGACCTGGCTGGTCGTGACCTGACTGACTA CCTCATGAAGATCCTGACCGAGAGAGGCTACAGCTTCACCACCACAG CCGAGAGGGAAATTGTCCGTGACATCAAGGAGAAGCTCTGCTATGTT GCCCTTGACTTCGAGCAGGAGATGGGCACTGCTGCTTCCTCCTCCTCC CTGGAGAAGAGCTATGAGCTGCCTGACGGGCAGGTCATCACCATTGG CAATGAGAGGTTCAGGTGCCCTGAGGCTCTCTTCCAGCCATCTTTCCT GGGTAAGAACATTGTGCAGCAGTCTACAGATAGCTTACAGCTTCCTG TGAATCCACGARACCACCTTCAACTCATCATGAAGTGTGATGTGGAC ATCCGTAAGGATCTGTATGCCAACACTGTATTGTCTGGTGGTACCACC	840	100%	95% *Pygocentrus nattereri* 93% *Astyanax mexicanus* 93% *Ictalurus punctatus* 92% *Rhamdia quelen* 92% *Pangasius hypophthalmus* 92% *Tachysurus fulvidraco* 92% *Mylopharyngodon piceus* 91% *Danio rerio*

	ATGTACCCTGGCATTGCAGACAGAATGCAAAAGGAAATCACATCCCT GGCCCCTAGCACAATGAAAATTAAGATTATTGCCCCACCTGAGCGTA AATACTCTGTCTGGATCGGAGGCTCCATCCTGGCCTCCCTGTCCACCT TCCAGCAGATGTGGATCAGCAAGCAGGAGTACGA			
Cytokines				
IRAK 1	CTGCCTGATGAATACCTGAAAGATGGCCAGCTGGGCATGGAGATCGA CATCTACAGTTTCGGAGTGGTGTTGTTGGAGGTCCTCACAGGCAGAA GAGCATTAGAAACTGACAGCAAGTCAAAGACTGTCTACCTGAAAGA CCTGGTGTCAGAAGTTGAGGATGATGGGAAGAGCTTCAGTAAAGGG AGGAATTCTAGGGAGCTGGCCTTTTCCCAGGCAGCTGAAAACATCTG GAAGAAGCATCTGGATCC	251	100%	96% *P. nattereri* 87% *A. mexicanus* 83% *P. hypophthalmus* 82% *I. punctatus* 82% *Electrophorus electricus*
IL 1 β	ACAAGTTCAAGCACACTCAGAAATTCCGTGTCCACAGAATTCACCGA CCATGAGCTATTCAACATCATCTTGGAGAGCATAATTGAAGAGAGTG		100% 89% 89% 84% 67% 64%	91% *P. nattereri* 80% *P. Hypophthalmus* 76% *T. fulvidraco* 76% *I. punctatus* 83% *Hemibagrus macropterus* 82% *A. mexicanus*
IL10		374	99%	94% *P. nattereri*

	AGACTACTATGAGGAGAAAGATGAGCTTGACAGTGCACTTTTTAACA			79% *A. mexicanus*
	AGACAGTCCTTCAGAAATCCTTCAATAGCCCTTACGGCTGCCATGCG			77% *E. electricus*
	ATGAACGACGTTTTGCATTTCTATCTGGACATCGTGCTGCCCACGGCG			74% *P. hypophthalmus*
	ATTACTGAGGACGGGAAGAGCTTCAAAACGCCAATAGACGAAATTG		98%	76% *T. fulvidraco*
	GAAACATCTTCCACGAGCTCAAGAGGGACCTGATCAAATGCAGGAAT			75% *I. punctatus*
	TACTTTGGATGCCAGAAGCCCTTTGAAATTTCCAGCATCAGGGACTC		81%	74% *Sinocyclocheilus*
	ATATCAACAAATGAAGAAGAAAGGGCTGTACAAAGCCATGGGGGAG			*Grahami*
	CTGGATATGCTGTTCAACTACATTGAAAAGTATCTGGCATCAAAGA			73% *Ciprinus carpio*
Anti-bacterial peptides				
Lysozyme g-like	TTGACAAGCGCCACCACATTCCAAGGGGTGCCTGGAACAGTGAGGA	175	100%	97% *P. nattereri*
	GCATGTCACTCAGGGAACTGAGATTTTGATTGATTCCATCAAAGCAA			86% *I. punctatus*
	TTCAACGGAAATTCCCCAAATGGCCAAAGGAGCACCAGTTTAAAGGA			84% *P. hypophthalmus*
	GGAATATCAGCCTACAACGCTGGTGTTGGAAACGT			84% *Ictalurus furcatus*
				82% *Anguilla japonica*
				81% *E. electricus*
				78% *T. fulvidraco*
			98%	80% *A. mexicanus*
			89%	84% *Clarias gariepinus*

C3	GTTCTCTGCAGCGCTCTTAAGTGGCTGGTTCTGAACGGACAGATGCC AGATGGCATGTTTAAAGAAAATGCACCTGTGGTTCATGGGGAGATGG TCGGAGATGTGCGAGGAAAGGATGCAGACGCATCTCTGACAGCATTT GTCCTGATTGCCATGCAAGAAGGAAGTGAACTTTGTGCTAAGTCAGT CCCTAGTCTTCCAGAGAGTATGAAGAAGGCCATTGATTATCTGGAAC GTAGAATTCCCACATTGACCAATCCTTATGCTGTTGCTATGACGTCCT ATGCCATGGCCAACGCTGGCAAATTCGACAAAGATTTCCTGATGAAG TATTCCTCTGGAGACGGTGCTTTTTGGCAAGTCCCTAGAGGACACCA CTTCTCCCTGGAAGCCACAGCCTATGCCCTGCTGGCTCTGGTAAAGGT GAAGCAGTTTGACGAAGCAGGGAAAGCCGTGC	457	100% 99% 	94% *P. nattereri* 83% *A. mexicanus* 83% *E. electricus* 82% *P. hypophthalmus* 79% *I. punctatus* 79% *Clarias* *macrocephalus* 78% *T. fulvidraco*
C4	CACTGTCGGTTTGGGGTCAGAACCGGGTCAGATGACATCACATTCAT AAAGGGTCTGGAGAAAACTGGACCAATACGAGACGGCAAAGCCGAC GTGACTCTGAGTCTGTCAGACGTCCGGCAGAAGCTCCAGAACACAAC ACTGCAGCGCCTGGCAGAGGGCGGAGCTCGGTTCTACATCAGCGTCA CTGTCACAGACAAAATCAGTGGTGAGGTACAGGAGACAGAAATGTT CCTTCCTATCGTATCCCAGCCGTACCTGGTGGACCTGTCCAGAACACG GTCACACTACATCCCCCAGATGCCACTGGACGTGGTGGTTGTGGTGC GCACTCCAAACGGACTGCCAGCTAAAGGCGTCCCAGTGAAGACTGAC GTATCCAACACTGTGGAGAAATCCTTCGTTAAAAACACAGACGATGA	476	99% 94% 84% 79% 	92% *P. nattereri* 75% *A. mexicanus* 72% *P. hypophthalmus* 72% *I. punctatus* 70% *E. electricus*

	GGGGATCGCAACACACCCATTCAATCTTGTGCAAAGGCCTTCGTCCA TAACTGT			
HSP70 (MF370934.1, NCBI access number) (Nahum *et al.*, 2017)	AGGACGCCTGTCCAAGGAAGACATTGAGCGCATGGTGCAGGAGGCT GACAGGTACCGGGCAGAGGATGAAGCCCAGAGAGAGAAGGTCACAG CCAAGAACACTTTGGAGTCCCTGGCCTTCAACATGAAGAGCACAGTG GAGGATGAGAAGCTGAAGGACAAAATCAGCGCTGATGACAAGAAGA CAATTGTGGACAAGTG	201	100%	97% *P. nattereri* 90% *E. electricus* 89% *P. hypophthalmus* 88% *A. mexicanus* 86% *I. punctatus* 85% *T. fulvidraco* 85% *Carebara semilaevis* 85% *Oncorhynchus mykiss*

3.2. Real time PCR (qPCR)- relative quantification of immune genes

3.2.1. Spleen

The expressions of immune-regulatory genes are illustrated in Figure 1 as fold-change, according to period of infection. All significant expressions of immune genes were ascertained in infected fish, as up-regulation.

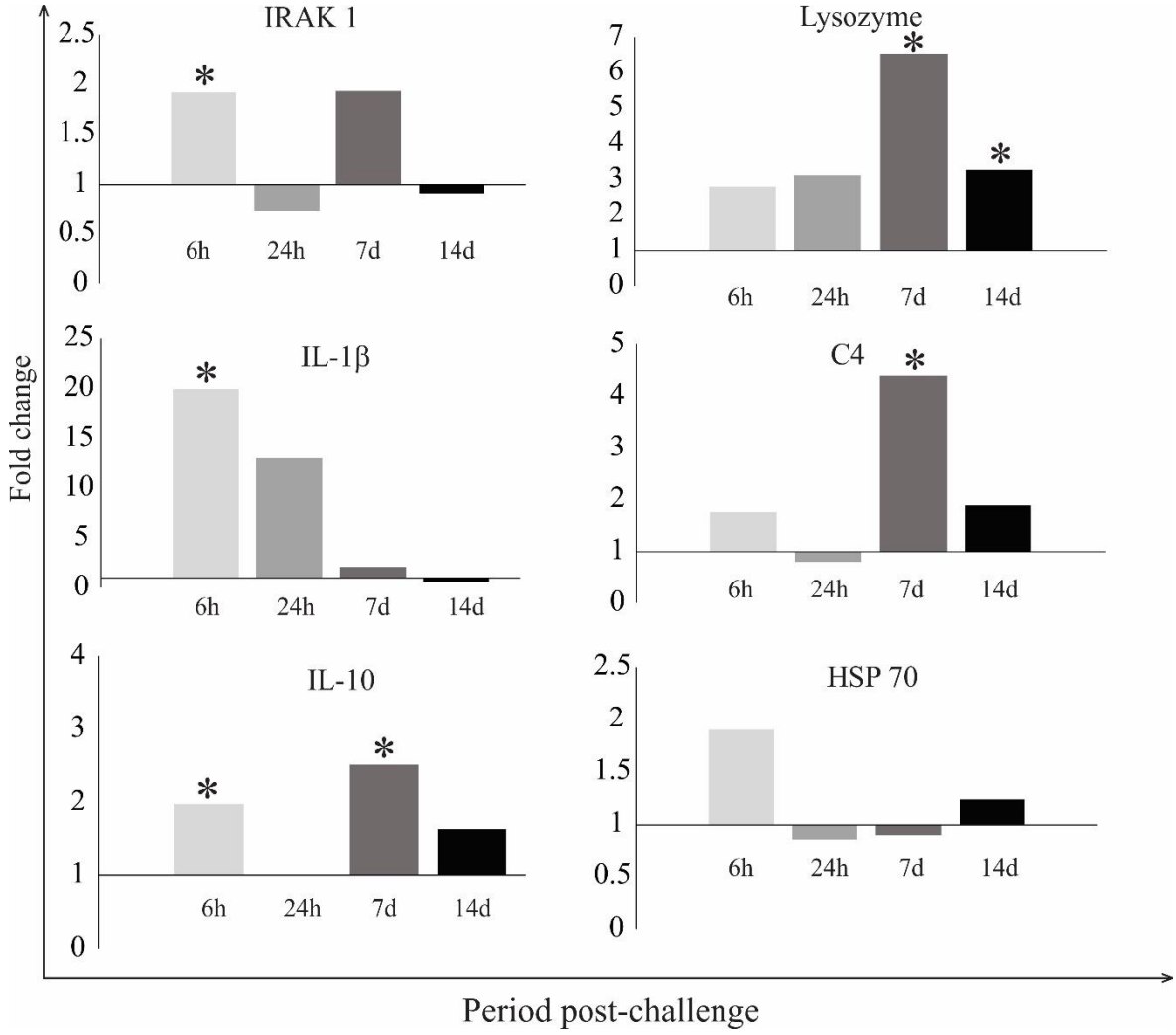

Figure 1. Real time PCR amplification of cytokines and antimicrobial peptides gene expression in spleen samples from infected and uninfected (control) fish at 6h, 24h, 7 and 14 days post-inoculation (p.i.). Statistically significant up-regulation in target gene expression relative to the uninfected control at the same time p.i. ($p \leq 0.05$) are marked with asterisks.

The expression of IRAK 1 and IL-1β genes was higher ($p \leq 0.05$) during acute phase of infection (6h), returning to basal conditions after this period. Additional up-regulation of IRAK 1 was observed 7 days post-infection but it was not statistically significant in relation to healthy fish. Meantime, IL-10 gene was up regulated from 6h to 7d post infection ($p \leq 0.05$), returning to basal conditions at day 14.

The expression of lysozyme gene was significantly up-regulated 7 days post infection ($p \leq 0.05$), while heat shock protein (HSP70) gene expression was not affected. The C3 gene was not expressed sufficient for obtainment of CT value in several samples or low expressed in others, making statically impossible to compare the groups. C4 gene was upregulated ($p \leq 0.05$) on day 7 after challenge, returning to basal conditions on day 14.

3.2.2. Head kidney

Immune modulation of cytokines and antimicrobial peptides genes from head-kidney of healthy and *A. hydrophila*-infected fish are illustrated in Figure 2 as fold-change, according to period of infection. All significant expressions of immune genes were ascertained in infected fish.

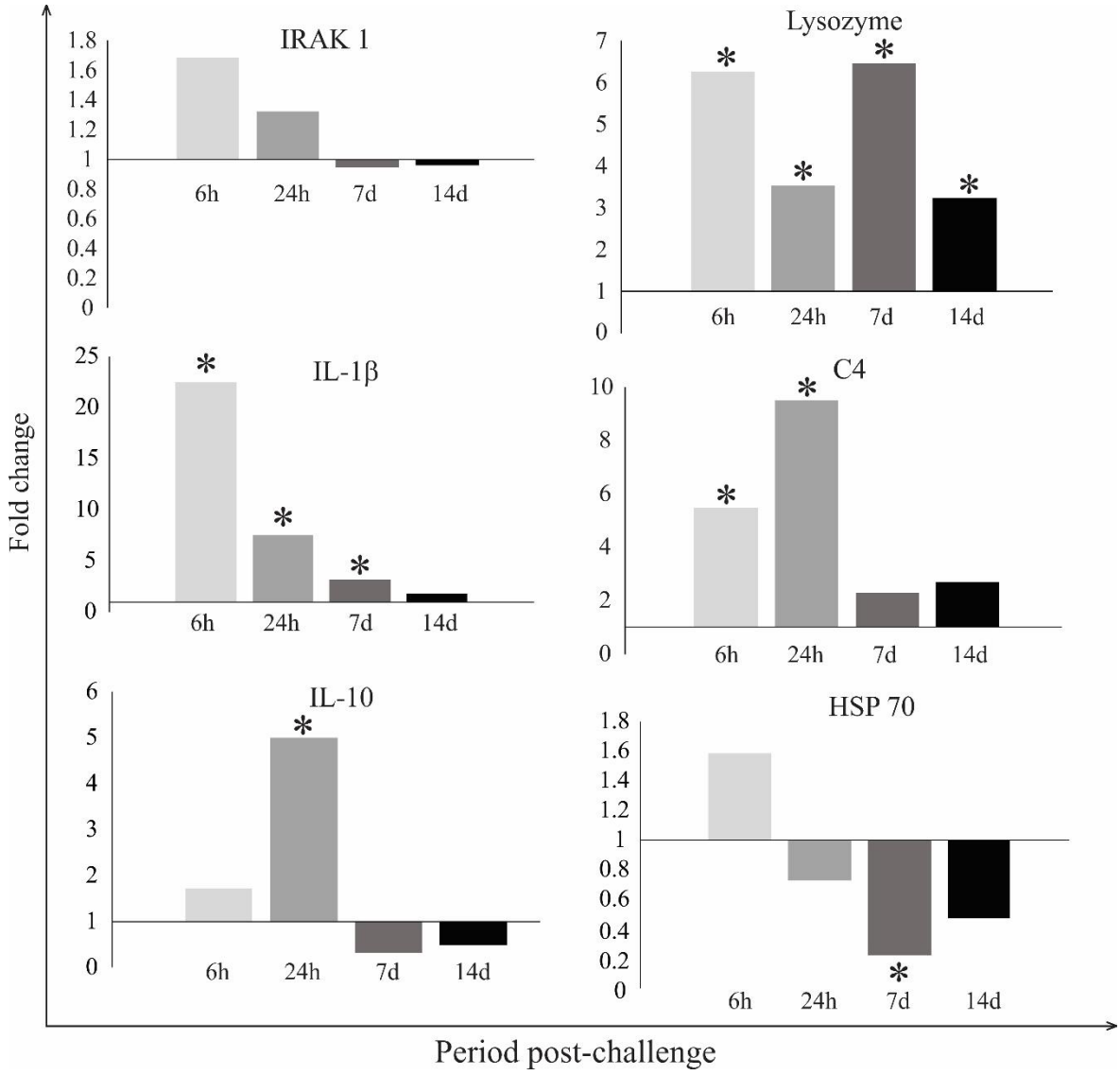

Figure 2. Real time PCR amplification of cytokines and antimicrobial peptides gene expression in head-kidney samples from infected and uninfected (control) fish at 6h, 24h, 7 and 14 days post-inoculation. Statistically significant up-regulation in target gene expression relative to the uninfected control at the same time p.i. ($p \leq 0.05$) are marked with asterisks.

IRAK 1 did not differ statically between groups during the infection, while IL-1β was ascertained as up-regulated ($p \leq 0.05$) right after infection, until day 7. IL-10 showed up-regulation ($p \leq 0.05$) at 24h.

Concerning antimicrobial peptides, lysozyme was up regulated ($p \leq 0.05$) at all periods after the infection, while the heat shock protein (HSP70) was the only gene that

showed significant down-regulation ($p \leq 0.05$), which occurred at day 7 post infection, returning to the same conditions as control group after this period. As the analysis of C3 expression on spleen, this gene was not expressed sufficiently to obtain CT values on head kidney. C4 was upregulated ($p \leq 0.05$) at 6 h and 24 h post-challenge, returning to basal conditions on day 7.

3.3. Complement system analysis

The ACH50 did not show hemolytic activity in rabbit's blood. Despite some individual attending 50% of hemolysis between 5 and 30 minutes of the assay, the statistical analyzes of the data obtained were inaccessible because most of the samples did not show activity, making non-viable values.

4. Discussion

Overall, the reference gene, cytokines and anti-microbial peptides showed highest similarity to other South American fish, specially *P. nattereri* and *A. mexicanus* (used to design the conserved and degenerated primers for conventional PCR, for obtainment of long amplicons of the immune genes), suggesting that for majority of immune genes, Characiforms species are great models to study immunity of tambaqui. Furthermore, similarity to *R. quelen* and *E. electricus*, indicates that alignment of known sequences between South American species is possibly a good alternative for those species with lacking of knowledge regarding immune genes. Besides South American species, the immune genes also present high similarity to Siluriform or other catfish species, such as *I. punctatus, I. furcatus, C. gariepinus, T. fulvidraco* and *P. hypophthalmus*, as well as Cyprinids, such as *C. carpio*, which like tambaqui, are intensively reared in fish ponds and are stricken by *Aeromonas* in captive farming.

Genetic studies are advancing fast, granting the comprehension of immune system mechanisms. Since tambaqui has no genome description, the development of its technological package ends up being affected, so immunology studies are important and urgent for this fish. In the present study, we supply the first data from partial sequence of nucleotides of key immune genes and also provide the patterns of these gene expressions and modulation during *Aeromonas* infection. Below, we discuss in detail the cytokines-receptor, pro and anti-inflammatory cytokines (IRAK1, IL-1β, and IL-10) besides antimicrobial peptides (lysozyme, C3, C4 and HSP70) to which mRNA from spleen and head-kidney of infected/uninfected fish showed modulation.

Cytokines receptor, pro and anti-inflammatory cytokines (IRAK1, IL-1β, and IL-10)

Cytokines have been in the focus of scientific interest, advancing the immunological studies the improvement of aquaculture. Analyzing the expression of cytokine genes has enabled a better understanding of the pathogenesis of various diseases [23]. Despite the preeminence of tambaqui, its immune characterization is a basic research that is still lacking.

IRAK-1

IRAK 1 plays a central role in signal transducer for the proinflammatory cytokine IL-1, and its expression followed patterns of IL-1β, but just in spleen. This molecule expression in early phase of infection (6h) normally it implies in activation of other pro-inflammatory molecules, such as for members of the Toll-like receptor (TLR) and in tumor necrosis factor receptor (TNFR) superfamily by inducing signaling pathways as well [24].

IL-1 β

The IL-1β plays a key role in regulating the immune response, acting as the main pro-inflammatory cytokine, highly expressed in the acute phase of the infection [8]. Its expression enables the fish to respond immediately to bacterial disease, inducing the cascade of inflammation. For tambaqui, IL-1β presented early expression (6h) in spleen and head-kidney (6 and 24h), but in head kidney its expression perdure until one week after infection, indicating still the necessity of this cytokine. Not only the long period in which this gene was expressed, but the highest fold-change (up to 20 in both organs), suggest the necessity of this molecule in infected tambaqui. This pro-inflammatory cytokine expression enables the organism to respond promptly to pathogen, orchestrating the inflammation. According to Seppola [25] this occurs through inducing of a cascade of reactions that leads to inflammation. The leukocyte recruitment, mainly neutrophils and monocytes, is a consequence of the inflammatory response, which can be triggered by various mechanisms, including infection diseases [26]. Based on all this information, IL-1β seems to play an important role in the immune response of tambaqui against *A. hydrophila*.

IL-10

The IL-10 modulation indicates not only chronic expression that remains until 7 days in spleen, but also acute expression in both organs (6h in spleen and 24h in kidney). Despite researches have been agreed that the main biological function of IL-10 is to control and terminate the inflammation, it seems that this cytokine also controls regulation of differentiation and proliferation of several immune cells such as granulocytes, T cells, B cells, natural killer cells, mast cells and antigen-presenting cells, also mediating immunostimulatory properties that help to eliminate infectious and noninfectious particles with limited inflammation following acute stress reactions [23], which suggests the early expression of IL-10 in tambaqui.

In addition, since it is considered the main anti-inflammatory cytokine [9] and its gene acts as a regulator with an important immunosuppressive function, limiting the synthesis of cytokines and chemokines by the macrophages [9], this gene expression is usually attributed to chronic phase of infection, as verified on spleen.

Antimicrobial peptides (HSP70, lysozyme, C3, C4)

HSP70

In this study it was found that the down-regulation of HSP70 protein in kidney (7d post-challenge) was influenced due to *A. hydrophila* infection, since fish were exposed to same thermal conditions (30ºC) in both treatments, corresponding an optimal temperature for the fish and also for the bacteria development. The result of HSP70 gene expression suggests that this protein evolved with stress thermic regulation is negatively affected in diseased fish. Furthermore, it leads to inference that head kidney is more sensitive to this protein expression than spleen. In a review of the activity of heat shock proteins in fish and their role in relation to fish healthy, Roberts [27] described that the following gene expression regulation is now recognized that occurs in response to biological stress and not restricted to heat stress.

It is well known that the inhibition of HSP70 gene expression causes thermosensitivity. Despite the few information about thermal sensitivity of tambaqui, it is known that this fish, from Amazon, is intolerant to high variance of temperatures [3, 28]. Usually, the thermal variations affect host immunity, making it susceptible to secondary infections, such as *Flavobacterium columnare* and *Ichthyophthirius multifiliis* [29], which are also important pathogens for tambaqui, responsible to huge losses on its farming [3]. With this study, it is possible to claim that o the opposite may also occur, when the fish is stricken primarily by disease, the organism can become more susceptible

to thermic stress. Once HSP70 is down-regulated by *Aeromonas*, the diseased fish cannot lead to thermic stress, as well as a healthy fish, becoming more susceptible to other pathogens.

Lysozyme

Lysozyme is a protein described in several studies with antimicrobial activity against both gram-positive and gram-negative bacteria [11, 30], such as *A. hydrophila*. Because this enzyme mediate fish protection against microbial invasion, its level or activity determination is an important index of immunity [31], making it one of the main parameters used for in studies. From 5 types of lysozyme, G-type and C-type are reported in fish. At the present study, lysozyme g-like showed significant up-regulation in infected tambaqui, indicating the necessity of the organism to produce more enzymes to fight *Aeromonas*. Its activity was ascertained during all periods of infection on head-kidney cells, and only after 7 d and 14 d of infection in spleen. Sahoo *et al*. [32] also reported genes of lysozyme G up-regulated in kidneys of *A. hydrophila*-infected fish *Puntius sarana*, similar to our findings (up-regulation at the same periods evaluated: 6h, 24h, 7d and 14d), correlating the modulation as a response to degree and time of damage to the organs. Thus, the early expression of its related gene in head-kidney can be explained because this enzyme has activity against pathogen, causing it lysis promptly after contact with the fish, in order to avoid or control the infection. However, the expression in the late phase of infection may occur because lysozyme also play a role in control the inflammation, acting as an immunosuppressant in chronic phase, as pointed by Ogundele *et al*. [33].

Proteins of the complement system: C3 and C4

The C3 factor is necessary in all three pathways of the complement system cascade, mediating the inflammation, promoting the attack of the membrane of pathogen and interacting with numerous serums, cell surface and foreign proteins [34]. The functionally active form of C3 is a product of a multiple genes and the diversity of C3 isoforms in fish can be responsible to their immune diversity [34]. In the present study, despite some individual CT values attending below 30 for C3 gene, it was impossible to statistically analyze the data obtained because most of them, from fish in both groups, were higher than 35 (showing no expression in the majority of fish). These few animals that expressed the C3, were not sufficient to evaluate statistically, but enough to certify that the primers and techniques worked properly (as well as for all other genes). Based on these assertions, and in the fact that serum complement analysis also shows weak or no activation, there is a suspicion that low expression of the C3 gene in tambaqui may result in low serum activity of this species. It is noteworthy that the analysis of serum and complement C3 expression of the gene was performed with different batches of fish, discarding the possibility that a specific problem of a batch of fish. According to Circolo *et al.* [35], genetic C3 deficiencies were described in humans and animals and these result in a vast range of disorders including increased susceptibility to infection and according to Quigg *et al.* [36] there are animals with inherited complete deficiencies of complement components, but most of these animals are clinically normal. Despite the suspicion of deficiency in the activity of the tambaqui complement system, these fish may use of several other mechanisms to compensate. As a recent example, Gallani *et al.* [37] have described the presence of a novel cell death pathway (named ETosis) in tambaqui as a mechanism to combat infection by *Aeromonas*.

Interestingly, although tambaqui is the most commonly grown native fish in South America, there is a gap in studies evaluating its complement system, while for other

species such as pacu *Piaractus mesopotamicus* [21, 38, 39], and matrinxã *Brycon amazonicus* [40] some data on the activity of the complement system were provided. Based on all data presented, a more in-depth study on the complement system in tambaqui deserves to be performed.

Regarding C4 gene expression, it was properly detected and increased in head-kidney (6h and 24h) and spleen (7d) of infected fish. The component C4 plays a pivotal role in the activation of immune defenses and the clearance of immune complexes or apoptotic debris, and according to Li [41] there are two important C4 gene products (C4A and C4B). The C4B-binding protein is best known a potent soluble inhibitor of the classical and lectin pathways of the complement system [42]. It serves as cofactor in factor I–mediated cleavage of C4b and C3b and it accelerates the decay of complement convertases. The main role of C4BP is to prevent overt complement-mediated inflammation [43]. However, even though there was no expression of the C3 gene (including control group) that plays a central role in the activation of the complement system and in the fact that no activity of this system was detected in the serum of most fish (including control group), it is still not possible to conclude the real role of C4 up regulation for tambaqui.

5. Conclusion

In this study, we provide the partial description of the sequences of key immune genes of tambaqui and its primers will allow an increase in studies of fish health and immunology of the main South American native fish for aquaculture. With this, we provide information and tools that serve as basis for the evaluation of immune response to different environmental or infectious challenges and for evaluation of the efficacy of vaccines and immunostimulants, urgent matters to tambaqui farming fast improvement.

Data of real-time PCR show that the molecular patterns of the gene expression provided conclusive information about *Aeromonas* infection in tambaqui, with exception of the C3 protein. This main finding strongly suggests a significant difference or problem in the activation of the complement system cascade in this fish species due to absence of C3 gene expression, besides the lack of ACH50 activity in both healthy and infected fish. We highlight that to evaluate possible deficience of the complement system, several other hypothesis deserves to be investigated in details for this and other South American fish.

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Capítulo 5

Considerações Finais

O sucesso produtivo de uma espécie de peixe, requer o domínio do conhecimento de todos os aspectos sanitários básicos, já que, atualmente, as enfermidades são apontadas por muitos estudiosos como o principal entrave para o desenvolvimento da aquicultura mundial. Por isso, nossos estudos foram baseados na atual necessidade de informações para a criação do tambaqui.

Na tese destacamos que o tambaqui é o peixe nativo mais produzido na América do Sul, e que é comum produtores terem problemas de mortalidade em sua produção. Apesar disto, na literatura, não foram encontrados dados que confirmassem a identidade e patogenicidade da *Aeromonas* neste peixe. O nosso primeiro estudo confirmou, através do cumprimento do Postulado de Koch e estudo de letalidade, que a *Aeromonas hydrophila* causa doença e septicemia hemorrágica em tambaqui. Além disto, baseado na confirmação da presença desta importante doença para o tambaqui, no primeiro estudo realizamos experimentos para encontrar moléculas promissoras para o controle da aeromonose. Como o uso de substâncias *off-label* é comum no tratamento de enfermidades na aquicultura, nesta tese, esclarecemos porque o uso de medicamentos deve ser previamente estudado, e mostramos o potencial dos óleos essenciais de plantas medicinais, antimicrobianos e desinfetantes no controle de *Aeromonas* no tambaqui.

Conforme citado ao longo do trabalho, apesar do sistema imune inato dos peixes ser um dos principais mecanismos de proteção contra patógenos, a resposta, que é intrínseca à cada espécie, não foi caracterizada no tambaqui até o momento. Por isso, no segundo estudo, avaliamos a imunidade inata (atividade antimicrobiana humoral e celular) no tambaqui, e os achados em peixes infectados incluíram principalmente uma leucopenia grave. Isto proporcionou novos estudos devido a suspeita da ocorrência da

morte celular de leucócitos para liberação de armadilhas extracelulares (ETosis), um mecanismo imune do hospedeiro para contenção da infecção. Ao ser investigado, desenvolvemos uma metodologia adaptada para visualização desta formação de armadilhas extracelulares por leucócitos em microscopia de varredura. Com este segundo estudo, descrevemos o mecanismo de ETosis pela primeira vez em um peixe nativo da América do Sul, e presumimos que este é um dos últimos recursos executados pelo tambaqui para conter a infecção. Evidenciamos que após essa estratégia, ele retorna a produção e liberação de um número elevado de células fagocitárias na circulação periférica.

Por fim, no terceiro estudo , estabelecemos os padrões de resposta imune inata do tambaqui, desvendando trechos parciais da sequência de 7 genes imunes chaves, o que poderá auxiliar futuros estudos de expressão gênica para esta espécie. A modulação da expressão gênica foi estabelecida em diferentes fases da infecção por *Aeromonas* pela primeira vez para este peixe nativo.

A partir dos próximos anos, a literatura sobre os peixes nativos será mais robusta e esta tese pode prover subsídios para a formulação de vacinas e tratamentos contra aeromonose na aquicultura, além de prover mais discussão sobre aspectos básicos da imunidade dos peixes sul-americanos abrindo portas para estudos mais aprofundados sobre o suicídio celular e sobre a expressão de genes imunes chaves em diferentes condições.