UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM FARMACOLOGIA

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INFECÇÃO POR *Aeromonas hydrophila* EM JUNDIÁ: ESTRESSE E EFEITO TERAPÊUTICO DA COMBINAÇÃO DE FLORFENICOL COM LINALOL VIA BANHO

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Farmacologia, Área de Concentração em Farmacologia Aplicada à Produção Animal da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Doutor em Farmacologia**

Orientador: Bernardo Baldisserotto
Co-orientadora: Juliana Felipetto Cargnelutti

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This study was financied in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) – Finance Code 001

Bandeira Junior, Guerino
Infecção por Aeromonas hydrophila em jundiá: estresse e efeito terapêutico da combinação de florfenicol com linalol via banho / Guerino Bandeira Junior.- 2022.
126 p.; 30 cm

Orientador: Bernardo Baldisserotto
Coorientadora: Juliana Felipetto Cargnelutti
Tese (doutorado) - Universidade Federal de Santa Maria, Centro de Ciências da Saúde, Programa de Pós Graduação em Farmacologia, RS, 2022

1. Antimicrobianos 2. Estresse oxidativo 3. Expressão gênica 4. Histopatologia 5. Perda evaporativa I. Baldisserotto, Bernardo II. Felipetto Cargnelutti, Juliana III. Título.

Sistema de geração automática de ficha catalográfica da UFSM. Dados fornecidos pelo autor(a). Sob supervisão da Direção da Divisão de Processos Técnicos da Biblioteca Central. Bibliotecária responsável Paula Schoenfeldt Patta CRB 10/1728.

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Aprovado em 11 de janeiro de 2022:

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Santa Maria, RS
2022

AGRADECIMENTOS

Agradeço primeiramente à Deus, por me dar saúde para concluir mais uma etapa da minha vida.

Aos meus pais Guerino Bandeira e Irene Judit Bandeira, e às minhas irmãs Denise Bandeira e Danieli Bandeira, pelo apoio incondicional.

À minha noiva Isabel Cristina Markowski Brusque, pela companhia durante minha caminhada.

Ao meu orientador Bernardo Baldisserotto, pelas ideias e pelos conselhos que só um grande pesquisador poderia compartilhar.

À Universidade Federal de Santa Maria (UFSM), pela estrutura e pelas oportunidades ofertadas.

Às equipes dos Laboratórios de Bacteriologia (LABAC), Biotecnologia e Reprodução Animal (BIOREP), Histologia e Embriologia (LABHISTO), e Extrativos Vegetais (LABEVE) da UFSM, por sempre manter as portas abertas para a realização de pesquisas.

À equipe do Laboratório de Fisiologia de Peixes (LAFIPE) da UFSM, pelo companheirismo durante os anos de convivência.

Enfim, a todas as pessoas que, de alguma forma, contribuíram para a minha formação acadêmica, o meu muito obrigado!

“A educação é a arma mais poderosa que
você pode usar para mudar o mundo.”
(Nelson Mandela)

RESUMO

INFECÇÃO POR *Aeromonas hydrophila* EM JUNDIÁ: ESTRESSE E EFEITO TERAPÊUTICO DA COMBINAÇÃO DE FLORFENICOL COM LINALOL VIA BANHO

AUTOR: GUERINO BANDEIRA JUNIOR
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O primeiro artigo consistiu em um artigo de revisão cujo objetivo foi sintetizar o conhecimento atual a respeito dos efeitos da aeromonose sobre o estado oxidativo dos peixes. O segundo artigo consistiu em um artigo original cujo objetivo foi avaliar o efeito da infecção por *Aeromonas hydrophila* no comportamento individual (teste do tanque novo) e na expressão cerebral de genes relacionados ao estresse (*slc6a2*, *hsp90*, *hspa12a*, *hsd20b*, *hsd11b2*, *crh*) em jundiá (*Rhamdia quelen*). O terceiro artigo consistiu em um artigo original cujo objetivo foi avaliar o efeito da combinação de florfenicol com linalol via banho contra aeromonose em jundiá e suas respostas no equilíbrio redox e na histologia de brânquias, fígado e rim caudal. Em relação ao artigo de revisão, podemos concluir que níveis aumentados de espécies reativas de oxigênio (EROs), malondialdeído (MDA) e proteínas carboniladas (PC) indicam que a infecção por *Aeromonas* geralmente causa estresse oxidativo. Portanto, esses três biomarcadores são excelentes indicadores de estresse oxidativo durante a infecção. Em adição, o aumento na atividade de *burst* respiratório e nos níveis de óxido nítrico indicam que o sistema imune inato dos peixes é geralmente mais ativo durante a infecção. Em relação ao segundo artigo, podemos concluir que a infecção por *A. hydrophila* em jundiá causa hiperlocomoção relacionada ao estresse e alterações no eixo hipotálamo-pituitária-interrenal (HPI), aumentando a expressão cerebral de *hspa12a* e *crh*, os quais são relacionados à proteína de choque térmico e à rota de síntese de cortisol, respectivamente. Por fim, em relação ao terceiro artigo, podemos concluir que o uso combinado de florfenicol com linalol via banho apresentou um efeito benéfico sobre os níveis de substâncias reativas ao ácido tiobarbitúrico (TBARS) e análises histopatológicas em brânquias e fígado de jundiás experimentalmente infectados com *A. hydrophila*. Apesar da considerável perda por evaporação, ainda permaneceu uma concentração satisfatória de linalol um dia após o banho.

Palavras-chave: Antimicrobianos. Estresse oxidativo. Expressão gênica. Histopatologia. Perda evaporativa. Teste do tanque novo.

ABSTRACT

Aeromonas hydrophila INFECTION IN SILVER CATFISH: STRESS AND THERAPEUTIC EFFECT OF THE COMBINATION OF FLORPHENICOL WITH LINALOOL VIA BATH

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The first article consisted of a review article whose objective was to synthesize the current knowledge regarding the effects of aeromonosis on fish oxidative status. The second article consisted of an original article whose objective was to evaluate the effect of *Aeromonas hydrophila* infection on individual behavior (novel tank test) and brain expression of stress-related genes (*slc6a2*, *hsp90*, *hspa12a*, *hsd20b*, *hsd11b2*, *crh*) in silver catfish (*Rhamdia quelen*). The third article consisted of an original article whose objective was to evaluate the effect of the combination of florfenicol with linalool via bath against aeromonosis in silver catfish and their responses in the redox balance and histology of gills, liver and caudal kidney. Regarding the review article, we can conclude that increased levels of reactive oxygen species (ROS), malondialdehyde (MDA) and protein carbonylation (PC) indicate that *Aeromonas* infection generally causes oxidative stress. Therefore, these three biomarkers are excellent indicators of oxidative stress during infection. In addition, the increase in respiratory burst activity (RBA) and nitric oxide (NO) levels indicate that the fish's innate immune system is generally more active during infection. Regarding the second article, we can conclude that *A. hydrophila* infection in silver catfish causes stress-related hyperlocomotion and changes in the hypothalamic-pituitary-interrenal (HPI) axis, increasing the cerebral expression of *hspa12a* and *crh*, which are related to heat shock protein and cortisol synthesis route, respectively. Finally, regarding the third article, we can conclude that the combined use of florfenicol with linalool via bath had a beneficial effect on the levels of thiobarbituric acid reactive substances (TBARS) and histopathological analyzes in gills and liver of silver catfish experimentally infected with *A. hydrophila*. Despite the considerable loss by evaporation, a satisfactory linalool concentration remained one day after the bath.

Keywords: Antimicrobials. Oxidative stress. Gene expression. Histopathology. Evaporative loss. Novel tank test.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

CAT	Catalase
EROs	Espécies Reativas de Oxigênio
GST	Glutationa S-Transferase
HPI	Hipotálamo-Pituitária-Interrenal
HSPs	Proteínas de Choque Térmico
MDA	Malondialdeído
OE	Óleo Essencial
PC	Proteínas Carboniladas
SNC	Sistema Nervoso Central
SOD	Superóxido Dismutase
TBARS	Substâncias Reativas ao Ácido Tiobarbitúrico

SUMÁRIO

1 INTRODUÇÃO

Aquicultura diz respeito ao cultivo de diversos organismos aquáticos, incluídos neste contexto plantas aquáticas, moluscos, crustáceos e peixes, sendo que a intervenção ou manejo adequado do processo de criação é imprescindível para o aumento da produção (REBOUÇAS e GOMES, 2016). Ela representa a atividade agropecuária que mais cresce no Brasil e no mundo, sendo o pescado a proteína animal mais produzida no planeta. Ao longo dos últimos anos, a produção aquícola mundial tem aumentado progressivamente, enquanto que as taxas anuais de pesca de captura estão estagnadas (FAO, 2020).

A intensificação na produção de peixes gera um aumento da densidade de estocagem e, consequentemente, da matéria orgânica e amônia produzidas, e uma diminuição nos níveis de oxigênio dissolvido. Esses fatores, aliados às manipulações e transportes rotineiros, geram estresse nesses animais, fazendo com que aumentem os níveis de hormônios como cortisol e catecolaminas (noradrenalina e adrenalina), os quais provocam impactos negativos no sistema imunológico dos peixes (BAKER; GOBUSH e VYNNE, 2013; BALDISSEROTTO et al., 2014; JØRGENSEN et al., 2017). Como resultado, há a instalação de infecções causadas por bactérias oportunistas, gerando sérios prejuízos econômicos ao produtor.

O aumento na prevalência de infecções bacterianas na piscicultura faz com que os produtores utilizem de forma indiscriminada a antibioticoterapia ou a antibioticoprofilaxia. Pesquisas evidenciaram que o desenvolvimento de resistência bacteriana aos antimicrobianos de uso convencional tem sido particularmente motivado pelo emprego de antimicrobianos como promotores de crescimento ou como meios inespecíficos de prevenção de infecções em práticas veterinárias (ECONOMOU e GOUSIA, 2015; DODDS, 2017). Assim, essas substâncias permanecem no ambiente aquático, exercendo uma pressão seletiva por longos períodos de tempo, resultando no surgimento de bactérias multirresistentes (BELEM-COSTA e CYRINO, 2006; YANG et al., 2018). O uso indiscriminado de antimicrobianos também gera problemas para a saúde pública, pois aumenta a quantidade de seus resíduos em produtos à base de carne de peixe. Além disso, muitos patógenos isolados da carne desses animais apresentam resistência bacteriana a vários antimicrobianos listados pela Organização Mundial de Saúde (CABELLO, 2006; DONE; VENKATESAN e HALDEN, 2015). No Brasil, somente dois antimicrobianos são licenciados para utilização na aquicultura continental, sendo eles o florfenicol e a oxitetraciclina (SINDAN, 2021). Tendo em vista esse cenário, surgiram

pesquisas com enfoque em substâncias naturais com propriedades antimicrobianas, pois são uma fonte alternativa no tratamento e prevenção dessas bacterioses. Diversos óleos essenciais (OEs) de plantas, tais como os de manjericão (*Ocimum basilicum*), pau rosa (*Aniba rosaeodora*) e erva cidreira brasileira (*Lippia alba*) possuem o monoterpeno linalol como um dos componentes majoritários (AVETISYAN et al., 2017; SAMPAIO et al., 2012; SOUZA et al., 2017). O linalol possui propriedades antibacterianas frente à *Aeromonas hydrophila*, além de propriedades sedativas e anestésicas em jundiás (*Rhamdia quelen*) (DORMAN e DEANS, 2000; HELDWEIN et al., 2014; SILVA et al., 2017).

O jundiá é um peixe encontrado do sudeste do México ao centro da Argentina que tem sido alvo de interesse na piscicultura por ter boa aceitação no mercado consumidor, facilidade na indução da reprodução e hábito alimentar onívoro (BALDISSEROTTO, 2004) (Figura 1). Oitenta por cento das enfermidades de jundiás possuem bactérias como agentes causadores, sendo a aeromonose a bacteriose mais frequente (BALDISSEROTTO; RADÜNZ NETO e BARCELLOS, 2005; BALDISSEROTTO, 2004). Neste estudo, foi utilizado o jundiá como modelo animal para a pesquisa sobre a infecção de *A. hydrophila* e a atividade antibacteriana *in vivo* do linalol.

Figura 1 - Espécime de jundiá (*Rhamdia quelen*). Fonte: autor.

Aeromonas hydrophila é um bacilo Gram-negativo oxidase positivo e anaeróbico facultativo amplamente conhecido por causar ulcerações cutâneas e septicemia hemorrágica em peixes, especialmente jundiás (BARCELLOS et al., 2008) (Figura 2). A aeromonose em jundiás leva ao dano oxidativo, aumentando os níveis de substâncias reativas ao ácido tiobarbitúrico (TBARS) e de proteínas carboniladas (PC), e diminuindo a atividade da enzima catalase (CAT) (BALDISSERA et al., 2017a). Além disso, a

infecção por esta bactéria leva a alterações histológicas, principalmente envolvendo o rim, o fígado e as brânquias (ABDELHAMED et al., 2017). Não existem trabalhos analisando a atividade locomotora de jundiás infectados por esta bactéria. Porém, jundiás infectados com *Pseudomonas aeruginosa* apresentaram hiperlocomoção relacionada ao estresse (BALDISSERA et al., 2017b).

Figura 2 - Necrose de nadadeira e úlcera cutânea em jundiá infectado com *Aeromonas hydrophila*. Fonte: autor.

Como a antibioticoterapia, isoladamente, não tem sido eficaz no combate a essa bactéria (SAKA; ADEYEMO e ODESEYE, 2017), estão emergindo pesquisas avaliando a atividade sinérgica de antimicrobianos convencionais com substâncias isoladas de plantas, com a finalidade de aumentar o sucesso terapêutico, vencer os mecanismos de resistência, além de reduzir o uso de antimicrobianos convencionais e, consequentemente, sua acumulação no ambiente aquático e na carne dos peixes (ASSANE; VALLADÃO e PILARSKI, 2021; DEEPIKA et al., 2019; DE SOUZA et al., 2017). Nesta linha, já foi relatado que o fitoquímico linalol possui atividade sinérgica com florfenicol contra *A. hydrophila* em um estudo *in vitro* (BANDEIRA JUNIOR et al., 2018a). Porém, há carência de estudos *in vivo* para confirmar esse achado.

O linalol é um composto volátil, portanto tem-se preocupação a respeito de sua evaporação. Soltanbeigi (2020) relatou que a presença de água e gás atmosféricos leva ao processo de evaporação e conversão desse fitoquímico. Porém, pouco se sabe sobre o grau de perda desse fitoquímico ao ambiente durante seu uso dissolvido na água.

1.1 OBJETIVOS

1.1.1 Objetivo geral

- Avaliar a relação entre a expressão gênica e atividade locomotora em jundiás desafiados com *A. hydrophila*, assim como o efeito *in vivo* da combinação de linalol com florfenicol via banho sobre a infecção e suas respostas no equilíbrio redox e histologia renal, hepática e branquial.

1.1.2 Objetivos específicos

- Realizar uma revisão bibliográfica sobre a influência da aeromonose no equilíbrio redox de peixes;
- Avaliar a atividade locomotora de jundiás infectados com *A. hydrophila*;
- Avaliar a expressão cerebral de genes diretamente relacionados ao estresse em jundiás infectados com *A. hydrophila*;
- Avaliar o efeito da combinação de linalol com florfenicol via banho em jundiás infectados com *A. hydrophila* através de parâmetros de estresse oxidativo no fígado, rim caudal e brânquias;
- Avaliar o efeito da combinação de linalol com florfenicol via banho por meio de análise histológica do fígado, rim caudal e brânquias;
- Avaliar a perda do linalol por evaporação e por metabolismo ou acumulação desse fitoquímico pelo corpo dos peixes, através da análise por cromatografia em fase gasosa com detector por ionização de chama.

1.2 JUSTIFICATIVA

Com a intensificação da produção aquícola e o aumento da densidade nos viveiros, os peixes ficaram mais susceptíveis a infecções bacterianas diversas. Muitas bacterioses são detectadas pelos produtores somente em estágios avançados da doença, quando o tratamento já não é mais eficaz. Porém, caso conseguirmos detectar alterações sutis e precoces nesses animais infectados, tais como modificações na atividade locomotora (manifestadas pela maior permanência na parte superior no tanque de cultivo),

poderíamos antecipar o início do tratamento, aumentando as possibilidades de sucesso terapêutico.

O uso indiscriminado de antimicrobianos na aquicultura gera problemas como resistência bacteriana e presença de resíduos na carne de peixe. Assim, cresce a demanda por fontes naturais de antimicrobianos, por questões ecológicas e de saúde pública. O fitoquímico linalol já foi relatado por possuir atividade sinérgica com florfenicol contra *A. hydrophila* em um estudo *in vitro*. Porém, não há estudos *in vivo* para confirmar esse achado. Os fitoquímicos agem em diferentes locais no microrganismo, se comparados aos antimicrobianos convencionais. Um efeito sinérgico *in vivo* da combinação de linalol com florfenicol é desejável, pois assim seria possível desenvolver protocolos de tratamentos de bacterioses utilizando menores quantidades do antimicrobiano convencional, diminuindo seus níveis no ambiente aquático e, consequentemente, na carne dos peixes, e retardando o processo de resistência bacteriana.

1.3 HIPÓTESE

A infecção por *A. hydrophila* em jundiá altera a atividade locomotora e causa estresse nesses animais, enquanto que o uso combinado de linalol com florfenicol possui efeito terapêutico, atenuando os danos causados pela infecção.

2 REFERENCIAL TEÓRICO

2.1 INFECÇÕES BACTERIANAS NA PISCICULTURA

Dentre as bactérias encontradas no ambiente aquático está *A. hydrophila*, espécie amplamente estudada como causadora de doença em peixes. Esta doença manifesta-se como septicemia hemorrágica, levando também ao aparecimento de úlceras cutâneas (ALYAHYA et al., 2018). Essa bactéria é habitante natural do fundo do viveiro e do trato intestinal dos peixes, mas pode causar infecções secundárias ou até mesmo primárias, dependendo da virulência da cepa e do estado do sistema imunológico do peixe (AOKI, 1998). Bactérias do gênero *Aeromonas* também podem causar doenças em humanos, sendo a gastroenterite a mais comum delas (GUERRA et al., 2007). Infecções bacterianas em peixes podem levar a alterações comportamentais nestes animais, as quais podem ser detectadas através de testes comportamentais específicos, tais como o teste do tanque novo (BALDISSERA et al., 2017b). Essas infecções também podem levar a alterações na expressão de genes relacionados ao estresse, além de levar ao estresse oxidativo e a alterações histológicas nos órgãos internos dos animais afetados (ABDELHAMED et al., 2017; BALDISSERA et al., 2017a; FU et al., 2011).

Diversas cepas de bactérias patogênicas para peixes, especialmente as do gênero *Aeromonas*, apresentam fatores de virulência, sendo que a capacidade de causar hemólise e de produzir biofilme são dois dos mais estudados (MILLEZI et al., 2013; SUTILI et al., 2014). A hemólise é a lise de hemácias causada pela ação de exotoxinas (hemolisinas) produzidas pelas bactérias hemolíticas e que agem induzindo a formação de poros nas membranas das células afetadas (WANG et al., 2003). Já o biofilme constitui uma estrutura ligada a uma superfície e formada por células microbianas coladas entre si e cercadas por uma matriz polimérica extracelular autoproduzida. A formação de biofilme é considerada uma adaptação dos microrganismos a ambientes hostis e confere maior resistência aos sanitizantes, aos antimicrobianos e às defesas do hospedeiro (VASUDEVAN, 2014). Um microrganismo que possua esses fatores de virulência é mais perigoso e mais difícil de combater, tornando-se necessários estudos a fim de identificar novas substâncias capazes de atenuá-los. Em um estudo prévio, demonstramos que fitoquímicos, tais como o linalol, possuem a capacidade de diminuir a formação de biofilme e a atividade hemolítica de uma cepa de *A. hydrophila* (BANDEIRA JUNIOR et al., 2018a).

2.2 O USO DE SUBSTÂNCIAS ISOLADAS DE PLANTAS COMO ANTIMICROBIANOS

As plantas vêm sendo utilizadas na medicina tradicional desde os tempos antigos, sendo atribuídas propriedades farmacológicas aos seus metabólitos secundários, principalmente fenois. Existem vários benefícios do uso desses compostos, principalmente envolvendo atividades antimicrobianas, antioxidantes e nutricionais (LAI e ROY, 2004). Citarasu (2010) destacou os fitoterápicos como importantes alternativas a serem utilizadas na aquicultura como promotores de crescimento, imunoestimulantes, estimuladores de apetite, preventores de estresse, além de serem alternativas muito eficazes aos antimicrobianos. O mesmo autor destaca a atividade antimicrobiana de compostos isolados de plantas contra bactérias, fungos e vírus. Nesta mesma linha de pensamento, Zhu (2020) destacou o uso de medicamentos fitoterápicos no controle de doenças de animais aquáticos, revisando na literatura as atividades antibacteriana, antiviral e antiparasitária de substâncias isoladas de plantas.

O linalol é um monoterpenoide, ou seja, sua origem biosintética deriva da condensação de duas unidades isoprênicas (C5), apresentando dez carbonos em sua estrutura química (BAKKALI et al., 2008) (Figura 3). Esse fitoquímico já foi relatado por possuir propriedades antibacterianas *in vitro* frente à *A. hydrophila* (DORMAN e DEANS, 2000; SILVA et al., 2017). Relatos envolvendo o linalol com equilíbrio redox em peixes são escassos. Yousefi et al. (2019) testaram os efeitos da anestesia com 982 mg/L de linalol sobre o estresse e a resposta antioxidante em carpa comum (*Cyprinus carpio*), e observaram que a exposição ao linalol causou um aumento nos níveis plasmáticos de cortisol e glicose e também levou ao estresse oxidativo devido ao aumento no nível de malondialdeído (MDA) e diminuição da atividade de enzimas antioxidantes no plasma se comparado aos peixes anestesiados com outros fitoquímicos (eugenol e citronelal). Esse estudo não apresentou um grupo controle, sendo feitas apenas comparações estatísticas entre os diferentes anestésicos. Além disso, existe a necessidade de testar o linalol em outras espécies de peixes e em diferentes órgãos, assim como em outras concentrações.

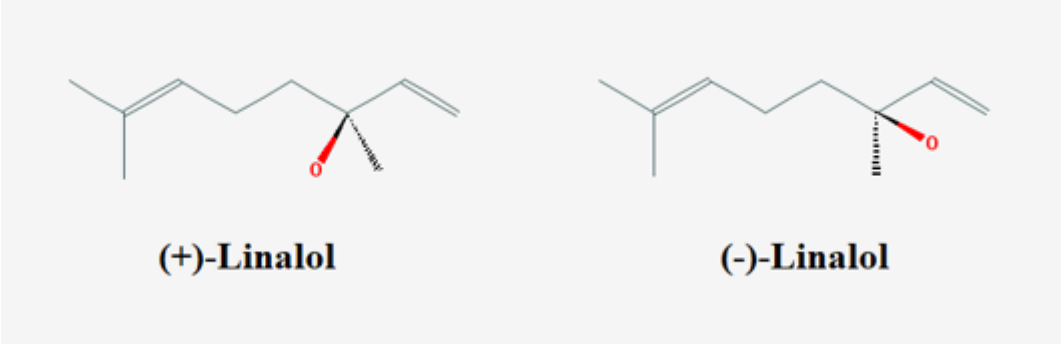

Figura 3 - Estruturas químicas dos enantiômeros de linalol. Fonte: adaptada de NCBI (2021).

2.3 EFEITO DO USO COMBINADO DE FITOTERÁPICOS COM ANTIMICROBIANOS CONVENCIONAIS

Além do uso de terapias alternativas e fitoterápicos no tratamento de infecções causadas por bactérias resistentes, uma estratégia que pode ser empregada para superar os mecanismos de resistência é a terapia combinada, em que duas substâncias com efeito sinérgico agem frente a um microrganismo. Aponta-se que combinações de produtos naturais com antimicrobianos convencionais podem ser benéficas no tratamento de infecções bacterianas (HEMAISWARYA; KRUTHIVENTI e DOBLE, 2008). Além de aumentar o sucesso terapêutico, esse tipo de terapia combinada é positivo do ponto de vista ambiental, visto que diminui a quantidade de resíduos de antimicrobianos na natureza, e também do ponto de vista da saúde pública, pois possibilita diminuir a quantidade destes resíduos na carne dos peixes. Nesta linha de pensamento, sabe-se que a combinação de linalol com florfenicol possui efeito sinérgico *in vitro* contra *A. hydrophila* (BANDEIRA JUNIOR et al., 2018a). Este mesmo estudo destacou o sinergismo da combinação do fitoquímico citral com o antimicrobiano oxitetraciclina. Além disso, destacou o potencial destes fitoquímicos em inibir a formação de biofilme e a atividade hemolítica de cepa de *A. hydrophila*.

2.4 ESTRESSE CAUSADO POR INFECÇÕES BACTERIANAS EM PEIXES

Infecções bacterianas podem induzir estresse nos peixes acometidos e o teste de tanque novo pode ser útil para constatar esse estresse, auxiliando em uma detecção precoce da doença. Esse teste é capaz de detectar um comportamento tipo ansiedade, evidenciado pela maior permanência do peixe na zona inferior do aquário, buscando proteção de possíveis ameaças externas. Além disso, esse teste pode ser útil na detecção do efeito ansiolítico de um fármaco, evidenciado pela maior permanência do peixe na

zona superior do aquário, estando alheio ao ambiente externo e aos perigos que o cercam (BANDEIRA JUNIOR et al., 2018b). Sabe-se que peixes-zebra (*Danio rerio*) infectados com *Edwardsiella tarda* apresentam diminuição da velocidade média e da distância percorrida (LEE et al., 2015). No entanto, jundiás infectados com *P. aeruginosa* apresentaram hiperlocomoção relacionada ao estresse, aumento no número de entradas na zona superior do aquário e maior permanência nessa zona (BALDISSERA et al., 2017b). Essa diferença comportamental observada nas duas espécies pode ser devido ao fato de que o peixe-zebra é social e prefere nadar perto da superfície da água (GERLAI et al., 2000), enquanto que o jundiá não tem um comportamento de cardume marcado e prefere viver perto do fundo sob plantas e rochas (SCHULZ e LEUCHTENBERGER, 2006).

Para fins didáticos, as respostas fisiológicas dos peixes aos estressores podem ser classificadas como primárias, secundárias e terciárias. As respostas fisiológicas primárias envolvem respostas neuroendócrinas iniciais, as quais incluem a liberação de catecolaminas do tecido cromafínico e a estimulação do eixo hipotálamo-hipófise-interrenal (HPI), culminando na liberação de hormônios corticosteroides na circulação sanguínea. As respostas secundárias incluem alterações nos níveis de íons e metabólitos plasmáticos e teciduais, alterações hematológicas e proteínas de choque térmico ou estresse (HSPs), todas relacionadas a ajustes fisiológicos, tais como metabolismo, respiração, equilíbrio acidobásico, equilíbrio hidromineral, sistema imunológico e respostas celulares. Além disso, ocorrem respostas terciárias, relacionadas ao desempenho dos animais como um todo, tais como crescimento, resistência a doenças, *status* metabólico, comportamento e, finalmente, sobrevivência (BALASCH e TORT, 2019; BARTON, 2002).

O estresse dos peixes acometidos por bacterioses pode ser aferido pela expressão de determinados genes. O gene *slc6a2* (transportador de noradrenalina sódio-dependente) codifica uma proteína transmembrana de múltiplas passagens presente no cérebro, que é responsável pela recaptação da noradrenalina nos terminais nervosos pré-sinápticos e é um regulador da homeostase da noradrenalina (GENE CARDS, 2021). A glândula inter-renal em peixes (similar à adrenal dos outros vertebrados) possui células cromafins, as quais produzem catecolaminas (adrenalina e noradrenalina) envolvidas na resposta primária e mais rápida ao estresse agudo. Estímulos estressores, como mudança de ambiente, manuseio e predação, podem promover a liberação de serotonina no sistema nervoso central ou de acetilcolina nas fibras pré-ganglionares do sistema nervoso

simpático, estimulando a liberação de catecolaminas pelas células cromafins (BARTON, 2002). As respostas a esses estímulos preparam o organismo do animal para "luta ou fuga". Algumas alterações fisiológicas nesse sentido são: ativação do sistema respiratório e cardiovascular, aumento da perfusão das lamelas branquiais, aumento da permeabilidade branquial e promoção de glicogenólise no fígado, levando à hiperglicemia a fim de suprir as demandas energéticas das ações de "luta ou fuga" (BALDISSEROTTO, 2013) (Figura 4).

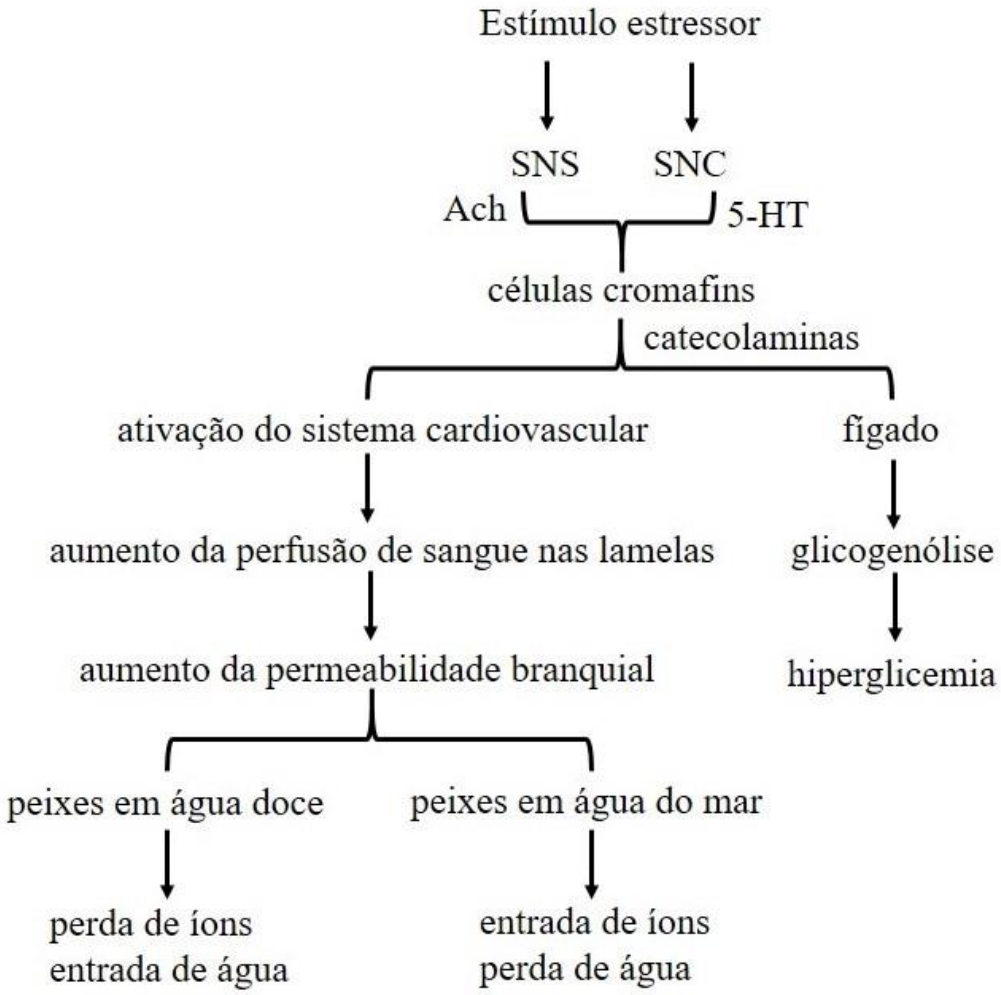

Figura 4 - Esquema simplificado do controle da liberação das catecolaminas e seu efeito nos teleósteos. Ach: acetilcolina, 5-HT: serotonina, SNC: sistema nervoso central e SNS: sistema nervoso simpático. Fonte: adaptada de Baldisserotto (2013).

O gene *crh* (hormônio liberador de corticotrofina) codifica um membro da família dos fatores de liberação de hormônio adrenocorticotrófico (corticotrofina) (GENE CARDS, 2021). Quando há um estímulo estressor, pode haver a liberação de dopamina e serotonina no sistema nervoso central (SNC), estimulando o núcleo paraventricular do

hipotálamo a secretar o hormônio liberador de corticotrofina e a urotensina I, que por sua vez agirão na adenohipófise estimulando a liberação de corticotrofina, a qual vai via hematógena até a glândula inter-renal, onde estimula a síntese e secreção de cortisol pelas células inter-renais (Figura 5). O cortisol distribui-se para todo o corpo através da corrente sanguínea e, por ter natureza lipofílica, penetra no interior das células por difusão passiva através da membrana celular, ligando-se no receptor glicocorticoide intracelular, onde exerce sua ação. O próprio cortisol pode fazer uma retroalimentação negativa nessa via de liberação de cortisol, inibindo a ação do hormônio liberador de corticotrofina e da urotensina I. O hormônio liberador de corticotrofina é um importante regulador da homeostase, mediando as respostas autonômicas, comportamentais e neuroendócrinas ao estresse (BARTON, 2002). O aumento do cortisol plasmático em peixes submetidos a estímulos estressores pode ser rápido (10-60 min após evento estressor) e voltar ao normal após 24 h da remoção do estresse. Porém, no estresse crônico os níveis de cortisol plasmáticos podem permanecer elevados por um maior período, podendo causar vários efeitos maléficos ao organismo, tais como a imunossupressão, a qual predispõe a infecções por microrganismos oportunistas. No entanto, o organismo do animal pode adaptar-se ao estresse crônico reduzindo o número de receptores para esse hormônio, o que leva a uma diminuição de sua ação (BALDISSEROTTO, 2013).

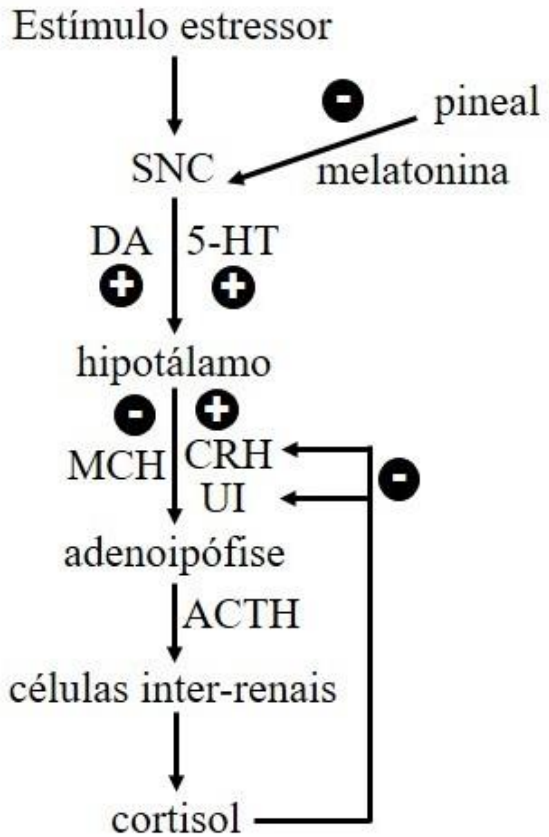

Figura 5 - Representação esquemática do controle da liberação de cortisol pelas células inter-renais. ACTH: hormônio adrenocorticotrófico, CRH: hormônio liberador de corticotrofina, DA: dopamina, MCH: hormônio concentrador de melanóforos, SNC: sistema nervoso central, UI: urotensina I, 5-HT: serotonina, (+) estimulação, (-) inibição. Fonte: adaptada de Baldisserotto (2013).

O gene *hsd11b2* (11β-hidroxiesteroide desidrogenase tipo 2) codifica uma enzima que possui a função de catalisar a conversão do cortisol para cortisona metabolizada inativa. Ela modula os níveis intracelulares de glicocorticoides, protegendo assim o receptor mineralocorticoide não seletivo da ocupação pelos glicocorticoides (GENE CARDS, 2021). Por fim, o gene *hsd20b* (20β-hidroxiesteroide desidrogenase) codifica uma enzima que também é envolvida na inativação do cortisol. Portanto, essas duas enzimas estão envolvidas na resposta ao estresse em peixes (SOUZA et al., 2018).

O gene *hspa12a* (proteína de choque térmico família A, membro 12A) codifica um membro da proteína de choque térmico família A e relaciona-se à resposta celular ao estresse térmico e à senescência. Outro gene que codifica um membro da proteína de choque térmico é o *hsp90* (proteína de choque térmico 90, α 1). Estas proteínas estão envolvidas na transdução de sinal, dobramento e degradação de proteínas e evolução morfológica (GENE CARDS, 2021). A *hsp90* é uma chaperona multifuncional que desempenha um papel essencial no metabolismo celular e na resposta ao estresse, protegendo a célula contra danos e estímulos estressantes e podendo ser regulada por uma variedade de estressores ambientais, tais como choque térmico, metais pesados, estresse osmótico e desafio bacteriano (FU et al., 2011). Assim, pode-se concluir que a expressão dos genes supracitados está diretamente relacionada ao estresse, sendo uma ferramenta útil para avaliar a resposta dos peixes a eventos estressores, tais como infecções bacterianas.

2.5 INFECCIÕES BACTERIANAS EM PEIXES E ESTRESSE OXIDATIVO

O estresse oxidativo é um componente inevitável da vida aeróbica. No organismo aeróbico saudável, existe um equilíbrio entre a produção de espécies reativas de oxigênio (EROs) e o sistema para proteger as células das EROs. O aumento da produção EROs resulta em defeitos que podem causar danos às células ou até mesmo a morte delas. Esse

desequilíbrio é chamado de estresse oxidativo. As EROs causam efeitos deletérios que incluem a oxidação de proteínas, DNA e componentes esteroides, bem como a peroxidação de lipídios insaturados nas membranas celulares. Isso produz hidroperóxidos lipídicos instáveis, cujos produtos, ao se decomporem, são altamente reativos, ameaçando a integridade da célula. Além disso, esses produtos podem se decompor em radicais livres que podem perpetuar o ciclo destrutivo das reações em cadeia da lipoperoxidação (SVOBODOVA et al., 2009).

Doenças bacterianas em peixes também podem levar ao estresse oxidativo, configurado por uma geração excessiva ou menor remoção de radicais livres, levando à perda da função biológica, ao dano tecidual e ao desequilíbrio homeostático (MARTÍNEZ-ÁLVAREZ; MORALES e SANZ, 2005). A fim de neutralizar os radicais livres que estão em excesso, o organismo pode aumentar a atividade de enzimas antioxidantes, tais como a superóxido dismutase (SOD) e a CAT (MORENO et al., 2005). Além disso, a glutationa S-transferase (GST) é uma enzima importante que atua no processo de biotransformação e catalisa a conjugação de uma variedade de metabólitos (incluindo produtos xenobióticos), transformando o composto tóxico em outro mais facilmente excretado (LUSHCHAK et al., 2009). A peroxidação lipídica pode ser avaliada através da formação de TBARS, que são subprodutos aldeídos, destacando-se o MDA, o qual é utilizado como marcador de peroxidação lipídica nos testes laboratoriais (ALMROTH et al., 2005). Sabe-se que a infecção por *A. hydrophila* em jundiás leva ao dano oxidativo, aumentando os níveis de TBARS e diminuindo a atividade da CAT (BALDISSERA et al., 2017a). Portanto, pode-se utilizar esses testes a fim de determinar a ação de fármacos na prevenção ou reversão do estresse oxidativo causado por essa bactéria em jundiás.

2.6 INFECÇÕES BACTERIANAS EM PEIXES E ALTERAÇÕES HISTOLÓGICAS

Em relação à histopatologia, essa infecção pode levar à hemorragia, infiltração de leucócitos e degeneração no rim e no fígado (GUPTA et al., 2008). Além disso, as brânquias podem apresentar fusão lamelar, secreção excessiva de muco e hipertrofia das células epiteliais (SAHARIA et al., 2018). Assim, pode-se constatar que esses órgãos são alvos da infecção causada por essa bactéria e conclui-se que a histopatologia é uma ferramenta útil para avaliar se os tratamentos previnem ou reduzem as alterações teciduais causadas pela infecção.

3 DESENVOLVIMENTO

3.1 ARTIGO DE REVISÃO

A revisão bibliográfica intitulada “Fish infections associated with the genus *Aeromonas*: a review of the effects on oxidative status” foi publicada na revista científica “Journal of Applied Microbiology” (fator de impacto 3.772). Endereço de acesso: https://doi.org/10.1111/jam.14986 (BANDEIRA JUNIOR e BALDISSEROTTO, 2020).

Fish infections associated with the genus *Aeromonas*: a review of the effects on oxidative status

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Running title: Fish aeromonosis and redox balance

Abstract

The aim of this review was to summarize the current knowledge regarding the effects of aeromonosis on fish oxidative status. The bibliographic survey was carried out on the research platforms Scopus and Science Direct. The keywords "*Aeromonas,*" "fish," and "oxidative status" (or "oxidative stress," "oxidative damage," and similar terms) were used. Scientific papers and short communications were considered. Studies involving fish aeromonosis and enzymatic or non-enzymatic markers of oxidative status were selected. The results of antioxidant enzymes activities/expressions after infection lack consistency, suggesting that these findings should be interpreted with caution. Most of the analyzed studies pointed to an increase in reactive oxygen species, malondialdehyde and protein carbonylation levels, indicating possible oxidative damage caused by the infection. Thus, these three biomarkers are excellent indicators of oxidative stress during infection. Regarding respiratory burst activity, several studies have indicated increased activity, but other studies have indicated unchanged activity after infection. Nitric oxide levels also increased after infection in most studies. Therefore, it is suggested that the fish's immune system tries to fight a bacterial infection by releasing reactive oxygen and nitrogen species.

Keywords: Aquaculture, Diseases, Fish (live), Infection, Oxidation.

Introduction

Bacteria of the genus *Aeromonas* are oxidase-positive, facultatively anaerobic, Gram-negative rods that grow readily on basic laboratory media, such as heart infusion agar (Janda and Abbott 2010). These bacteria are known to cause hemorrhagic septicemia, cutaneous ulcerations, and enteritis in aquatic organisms (Beaz-Hidalgo and Figueras 2013). Outbreaks are associated with high economic losses in fish farms due to increased mortality rate, decreased growth rate, and drug expenses for prophylaxis and treatment of bacteriosis (Tavares-Dias and Martins 2017). In humans, *Aeromonas* infections are mostly acquired due to exposure to freshwater or from eating raw fish, and is a common cause of enteritis and clinically manifested by watery diarrhea (Kishimoto and Watari 2018). To cause these deleterious effects on the host, most strains of *Aeromonas* have several virulence factors, such as flagella, adhesins, hemolysins, proteases, lipases, DNases, enterotoxins, aerolysin, iron acquisition mechanisms, and quorum sensing (Beaz-Hidalgo and Figueras 2013; Qin *et al.* 2014). In this sense, species of *Aeromonas* pathogenic to fish are described as multidrug-resistant, hemolytic, and biofilm-forming (Fernández-Bravo and Figueras 2020).

Fish bacterial diseases can lead to oxidative stress, caused by excessive generation or impaired removal of free radicals, leading to loss of biological function, tissue damage, and homeostatic imbalance (Baldissera *et al.* 2018). The excess of reactive oxygen species (ROS) cause deleterious effects that include the oxidation of proteins, DNA, and steroidal components, as well as the peroxidation of unsaturated lipids in cell membranes (Figure 1). This produces unstable lipid hydroperoxides, whose products are highly reactive and threaten the integrity of the cell when decomposed. In addition, these products can decompose into free radicals that can perpetuate the destructive cycle of lipoperoxidation chain reactions. In order to neutralize excess free radicals, the fish body can modulate antioxidant defense mechanisms by increasing the activity of specific enzymes (Martínez-Álvarez *et al.* 2005).

This review aimed to summarize the current knowledge regarding the effects of aeromonosis on fish oxidative status. We searched for scientific papers and short communications in the research platforms Scopus and Science Direct using the following keywords: "*Aeromonas*," "fish," and "oxidative status" (or "oxidative stress," "oxidative damage," and similar terms). Finally, we pre-selected several files and performed a careful reading in order to select studies involving fish aeromonosis and enzymatic or non-enzymatic markers of oxidative status.

Among the analyzed studies, the most studied *Aeromonas* species was *A. hydrophila*. This is possibly because *A. hydrophila* is one of the most prevalent species of *Aeromonas* in fish (Aberoum and Jooyandeh 2010) and also because this bacterium is widely recognized as a human pathogen, causing mainly diarrhea (von Graevenitz 2007). This bacteria species is well known for causing hemorrhagic septicemia in freshwater fish (Ghatak *et al.* 2016). The second most studied *Aeromonas* species was *A. salmonicida*. This bacteria species is widely reported to cause furunculosis in salmonids, although it is also related to diseases in other fish groups and even in humans (Menanteau-Ledouble *et al.* 2016).

Effect of fish aeromonosis on enzyme activity related to the oxidative status

Among the antioxidant enzymes, we highlight superoxide dismutase (SOD), catalase (CAT), peroxiredoxin (Prx), glutathione peroxidase (GPx), glutathione S-transferase (GST), and glutathione reductase (GR). SODs can prevent oxidative stress by catalyzing the dismutation reaction of superoxide anion ($O_2^{\bullet-}$) to oxygen and hydrogen peroxide (H_2O_2) in living organisms. In animals, SODs are classified into copper/zinc SOD (Cu/ZnSOD) and manganese SOD (MnSOD) based on the metal co-factor in their active sites (Miller 2012). CAT is an oxidoreductase enzyme found in nearly all living organisms. CAT is known to catalyze the breakdown of H_2O_2 into oxygen and water, regulating the metabolism of H_2O_2. Hence it acts by protecting the cell against oxidative stress caused by H_2O_2 (Kaushal *et al.* 2018). Prx is a family of antioxidant enzymes that protect cells from oxidative damage by reducing H_2O_2, peroxynitrite, and lipid peroxidation, as well as by scavenging thiyl radicals. They catalyze the reduction of peroxides and alkyl peroxides with the help of thioredoxin, therefore receiving the name thioredoxin peroxidases (Valero *et al.* 2015).

The GPx enzyme contains selenium in its active site and removes inorganic and organic hydroperoxides, reducing them to water or alcohols coupled with reduced glutathione (GSH) oxidation. The GR enzyme recycles oxidized glutathione (GSSG) to GSH again. To reduce GSSG, GR uses the reducing power of NADPH, whose main source in animals is the pentose phosphate pathway, where NADPH is catalyzed by the enzyme glucose-6-phosphate dehydrogenase. The GST family of enzymes utilize GSH as a co-factor in the phase II metabolism of various xenobiotics, resulting in the formation of GSH–xenobiotic conjugates that are more water-soluble than the parent compound and subject to transporter-mediated efflux (Backos *et al.* 2012).

The observed changes in the activity or gene expression of enzymes related to the oxidative status of fish infected with bacteria of the genus *Aeromonas* are shown in Table 1. Although most studies point to an increase in SOD activity/expression after infection, a few indicate decreased or unchanged enzyme activity/expression. On the other hand, although most studies point to a decrease in GST activity/expression after infection, a few indicate increased or unchanged enzyme activity/expression. In addition, the CAT, GPx, GR, and Prx activities/expressions did not show a well-defined pattern of increase or decrease among the different studies analyzed. Therefore, the results of enzymes activities/expressions lack consistency.

Interestingly, there seems to be a species-dependent response in the activity/expression of SOD and CAT for some fish species infected with *A. hydrophila*. For example, striped snakehead (*Channa striata*) (Arockiaraj *et al.* 2014; Kumaresan *et al.* 2015; Samayanpaulraj *et al.* 2019) and rohu (*Labeo rohita*) (Pal *et al.* 2015; Fawole *et al.* 2016; Nandi *et al.* 2017; Nandi *et al.* 2018; Sharma *et al.* 2018; Pal *et al.* 2019) infected with *A. hydrophila* increased SOD activity/expression. However, Nile tilapia (*Oreochromis niloticus*) infected with *A. hydrophila* decreased SOD activity/expression (Zahran *et al.* 2018; Deepika *et al.* 2019; El-Habashi *et al.* 2019; Moustafa *et al.* 2020). In addition, rohu infected with *A. hydrophila* increased CAT activity/expression (Pal *et al.* 2015; Fawole *et al.* 2016; Nandi *et al.* 2017; Nandi *et al.* 2018; Sharma *et al.* 2018; Pal *et al.* 2019). On the other hand, gibel carp (*Carassius auratus gibelio*) (Liu *et al.* 2013; Yang *et al.* 2015b; Cao *et al.* 2018; Zhang P. *et al.* 2018; Zhang P. *et al.* 2019), grass carp (*Ctenopharyngodon idella*) (Harikrishnan *et al.* 2018a; Baldissera *et al.* 2019; Tang *et al.* 2019; Zhao *et al.* 2019), and blunt snout bream (*Megalobrama amblycephala*) (Liu *et al.* 2012; Xia *et al.* 2017; Abasubong *et al.* 2018; Liang *et al.* 2018; Zhang H. *et al.* 2018; Geng *et al.* 2019) infected with *A. hydrophila* did not demonstrate a well-defined pattern of activity/expression of SOD and CAT. Therefore, it is evident that the fish species has great influence on response of these two enzymes to infection, since for some species there was an increase, for another there was a decrease and for others there is no defined pattern of enzymatic activity/expression even considering the organ and the time of collect after infection. Thus, we suggest that future results comparisons of these enzymes be carried out taking into account only experiments with the same fish species.

Effect of fish aeromonosis on non-enzymatic markers of oxidative status

Among the non-enzymatic markers of oxidative status, we highlight ROS, malondialdehyde (MDA), protein carbonylation (PC), GSH, respiratory burst activity (RBA), and nitric oxide (NO). In a healthy aerobic organism, a balance between the ROS production and the system to protect cells from ROS exists. However, bacterial infections can lead to ROS accumulation in the tissues, which can compromise cell structure and function (Baldissera *et al.* 2018). Lipid peroxidation is involved in pathological disorders related to oxygen toxicity. The colorimetric reaction of thiobarbituric acid is used to measure the lipid peroxidation of a specific tissue through the mensuration of a secondary product called MDA (Ohkawa *et al.* 1979). Among a wide range of ROS-derived modifications, biomolecule carbonylation is known to be a major hallmark of oxidative stress. PC is an irreversible post-translational modification that yields a reactive carbonyl moiety in a protein, such as an aldehyde, ketone, or lactam. Specific carbonylation in certain tissues and organs has been attributed to oxidative stress and connected with various infections (Fedorova *et al.* 2014).

The observed changes in non-enzymatic markers of the oxidative status of fish infected with bacteria of the genus *Aeromonas* are shown in Table 2. Most of the analyzed studies pointed to an increase in ROS, MDA, and PC levels, indicating possible oxidative damage caused by the infection. For example, Baldissera *et al.* (2018) reported that the infection of silver catfish (*Rhamdia quelen*) by *A. caviae* increased the hepatic and renal ROS and MDA levels 4 days post-infection (dpi). In addition, Morselli *et al.* (2020) reported that *A. hydrophila* infection in grass carp increased the branchial ROS levels 7 dpi. The *A. hydrophila* infection in silver catfish increased the hepatic MDA and PC levels 2 dpi (Baldissera *et al.* 2017). Another example is the aeromonosis in sea trout (*Salmo trutta*), which led to an increase in hepatic PC levels (Tkachenko *et al.* 2014).

Reduced glutathione (GSH) plays a critical role in suppressing oxidative stress and maintaining cellular redox homeostasis. GSH is a tripeptide composed of glutamate, cysteine, and glycine, and its antioxidant and conjugation properties are derivative of the sulfhydryl moiety of the cysteine residue. GSH has the ability to directly scavenge cellular ROS in a non-enzymatic manner as well as serve as a co-factor for GPx in the reduction reaction that generates the oxidized form of glutathione (GSSG) (Backos *et al.* 2012).

The GSH levels did not show a well-defined pattern of increase or decrease among the different studies analyzed. In grass carp, Tang *et al.* (2019) reported that *A. hydrophila* infection decreased the intestinal GSH levels in 3 and 12 hours post-infection (hpi), but increased the intestinal GSH levels in 6 and 48 hpi. In common carp (*Cyprinus carpio*),

Chen *et al.* (2020) reported that the infection increased serum and intestinal GSH levels in 1 dpi, but decreased serum and intestinal GSH levels in 5 and 7 dpi. In this same study, a similar pattern was also observed in the liver and gills of common carp. In rohu, Pal *et al.* (2019) reported that the infection increased the hepatic and splenic GSH levels in 7 dpi and Roy *et al.* (2019) stated that the infection increased the serum GSH levels in 3 dpi. In Nile tilapia, Zahran *et al.* (2018) reported that the infection decreased the muscle GSH levels in 14 dpi and Vinosha *et al.* (2020) reported a decrease in hepatic GSH levels after infection. Therefore, the fish species, the sample collection time and the organ collected interfere with GSH results.

Respiratory burst activity (RBA) is the rapid release of ROS inside phagocytes in order to kill microorganisms (Grayfer *et al.* 2018). NO is derived from the amino acid L-arginine by the enzymatic activity of constitutive nitric oxide synthase (cNOS) and inducible nitric oxide synthase (iNOS). The immune-system cells are capable of generating reactive nitrogen species (RNS), such as NO, in response to bacterial infections to destroy pathogens (Bogdan 2001). Therefore, RBA and NO are part of the innate immune system of animals.

Several studies have indicated an increase in RBA activity, but a few indicate unchanged activity after infection. NO levels also increased after infection in most studies. For example, Patel *et al.* (2016) reported that the infection of catla (*Catla catla*) by *A. hydrophila* increased the RBA of thymus macrophages 2 and 3 dpi and the NO levels of thymus macrophages 3 dpi. Qin *et al.* (2018) reported that the aeromonosis in blunt snout bream increased the RBA and NO levels of macrophages 24 hpi. Another example is the infection of obscure pufferfish (*Takifugu obscurus*) with *A. hydrophila*, leading to an increase in RBA and NO levels of blood cells 3, 6, 12, 24, 48, and 72 hpi (Cheng *et al.* 2017). Therefore, it is suggested that the fish's immune system tries to fight a bacterial infection by releasing ROS and RNS.

Natural substances for prevention and treatment of redox imbalance in fish with aeromonosis

Studies regarding the use of different natural substances for prevention and treatment of redox imbalance in different fish species infected with *Aeromonas* has increased in the last years. The two main routes of drug administration for fish are by baths (Sutili *et al.* 2016; Bandeira Junior *et al.* 2019) and incorporation into the feed, administering this medicated feed daily before and/or after infection (Asakura *et al.* 2019;

Vinosha *et al.* 2020). Below are some examples of studies about the effect of natural substances on the redox balance of fish with aeromonosis. The use of plant essential oils by bath and dietary supplementation against fish bacterial pathogens was further discussed in da Cunha *et al.* (2018).

Many studies with natural compounds have focused on disease prevention. For example, prophylactic baths (1 h per day for one week) of tea tree (*Melaleuca alternifolia*) essential oil (50 µL/L) prevents oxidative damage in liver of silver catfish experimentally infected with *A. hydrophila* by preventing the increase in MDA and PC levels (Baldissera *et al.* 2017). Vinosha *et al.* (2020) reported that sulfated galactan from red seaweed (*Halymenia dilatata*) in the feed (20 g/kg of feed) for one week before infection prevents redox imbalance in tilapia with aeromonosis. Baldissera *et al.* (2019) reported that dietary supplementation with 8% of caffeine for grass carp for one month prevents lipid and protein damage caused by aeromonosis.

Other studies have focused on the use of natural substances for treating diseased fish. For example, exposure to neem (*Azadirachta indica*) oil nanoemulsion increases the activity of antioxidant enzymes (CAT and GST) in common carp infected with *A. culicicola* (Swathy *et al.* 2018). Another example is the influence of kuma bamboo grass (*Sasa veitchi*) extracts supplementation on SOD activity in the liver of goldfish (*Carassius auratus*) infected with *A. salmonicida* (Asakura *et al.* 2019). In addition to their use as antioxidants, natural substances are also widely researched in aquaculture as growth promoters, appetite stimulators, immunomodulators, antiparasitics, antibacterials, sedatives, anesthetics and antistress (Sutili *et al.* 2018; Souza *et al.* 2019).

A strategy widely used by researchers in recent years is the use of probiotics to prevent/combat the damage caused by aeromonosis in fish. For example, dietary supplementation of 10^5, 10^7, and 10^9 CFU/g of *Bacillus amyloliquefaciens* increased the activity of antioxidant enzymes (CAT and SOD) in the liver of rohu in pre-challenge period (70 days) and after *A. hydrophila* challenge (28 days) (Nandi *et al.* 2018). Tang *et al.* (2019) reported that 42 days of dietary supplementation of 10^7 CFU/g of *Bacillus subtilis* in grass carp can provide effective protection against oxidative stress damage induced by aeromonosis through increasing the activity/expression of antioxidant enzymes (SOD, CAT, GPx and total antioxidant capacity - TAC). In addition, Mohammadian *et al.* (2018) reported the immunostimulating effect of supplementation for two months of autochthonous probiotics (5×10^7 CFU/g of *Lactobacillus plantarum* and *Lactobacillus delbrueckii* ssp. *bulguricus*) in the diet of shabot fish (*Tor grypus*)

infected with *A. hydrophila*. Probiotics as antioxidants are also widely researched to be positive promoters of aquatic animal growth, survival and health (Hai 2015).

Aeromonas resistance against oxidative stress

Like the cells of eukaryotic organisms, prokaryotes are also affected by high levels of ROS. For example, bacteria exposed to high levels of H_2O_2 show cell damage that can lead to death (Landre *et al.* 2000). During infection, the host's phagocytes perform the phagocytosis of the microorganisms and release ROS in order to kill the invaders (Leung *et al.* 1995). However, some species of bacteria from the genus *Aeromonas* have antioxidant systems capable of overcoming the harmful effects of ROS in their cells. We can highlight the role of genes related to superoxide dismutase (*sodA* and *sodB*) in *A. hydrophila* resisting oxidative damage to survive in fish macrophages and escape for further infection (Zhang *et al.* 2019). In addition, the gene related to periplasmic binding protein (*preA*) in *A. veronii* has a key role in the regulation of virulence and resistance to oxidative stress (Yang *et al.* 2020). The gene related to the catalase enzyme (*katA*) was also characterized in this species (Rio *et al.* 2007). Therefore, bacteria of the genus *Aeromonas* may have different antioxidant mechanisms that make them more resistant to the hosts' immune systems.

Concluding remarks

The most studied enzymes were SOD and CAT. Moreover, the most studied non-enzymatic biomarkers were MDA and RBA. Many factors can influence the results, such as differences in methodologies used, species of fish and bacteria used in the experiments, organs collected, and sample collection times between studies. However, these findings suggest that the results of these antioxidants after infection should be interpreted with caution.

Increased levels of ROS, MDA, and PC indicate that, generally, *Aeromonas* infection causes oxidative damage. Thus, these three biomarkers are excellent indicators of oxidative stress during infection. In addition, the increase in RBA and NO levels indicates that the fish’s innate immune system is generally more active during infection.

Acknowledgments

G. Bandeira Junior received a PhD scholarship from the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil - finance code 001) and

B. Baldisserotto a research fellowship from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil).

Conflict of interest

The authors declare that they have no conflicts of interests to disclose.

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Table 1 Observed changes in the activity or gene expression (italic) of enzymes related to the oxidative status of fish infected with *Aeromonas*

***Aeromonas* species**	**Fish species**	**Enzymes**	**Changes**	**References**
A. caviae	*Rhamdia quelen*	SOD, GPx, GST, GR	≈ hepatic and renal SOD, GPx, and GR activities in 4 dpi. ↓ hepatic GST activity in 4 dpi. ≈ renal GST activity in 4 dpi.	Baldissera *et al.* (2018)
A. caviae	*Rhamdia quelen*	XO	↑ splenic XO activity in 4 dpi.	Souza *et al.* (2017)
A. culicicola	*Cyprinus carpio*	CAT, GR, GST	↓ CAT, GR, and GST activities in the gill, tissue, and liver.	Swathy *et al.* (2018)
A. hydrophila	*Acipenser ruthenus*	*MnSOD*	↑ expression of *MnSOD* in the head kidney in 6, 12, 24, and 48 hpi. ↑ expression of *MnSOD* in the spleen in 1, 2, 3, and 4 dpi.	Sun *et al.* (2019)
A. hydrophila	*Anguilla marmorata*	SOD, Cu/ZnSOD, *Cu/ZnSOD*, *MnSOD*	↑ branchial SOD activity in 1, 3, 6, 24, and 48 hpi. ↑ renal SOD activity in 1, 3, 12, 24, and 48 hpi. ↑ hepatic SOD activity in 1, 6, 24, and 48 hpi. ↑ branchial Cu/ZnSOD activity in 1, 3, 12, and 48 hpi. ↑ renal Cu/ZnSOD activity in 1, 3, 6, 12, 24, 48, and 72 hpi. ↑ hepatic Cu/ZnSOD activity in 1, 6, 12, 48, and 72 hpi. ↑ expression of *MnSOD* in the gill in 3 and 24 hpi. ↓ expression of *MnSOD* in the gill in 6 hpi. ↑ expression of *MnSOD* in the kidney in 1, 3, 24, and 48 hpi. ↓ expression of *MnSOD* in the kidney in 72 hpi. ↑ expression of *MnSOD* in the liver in 1 and 24 hpi. ↓ expression of *MnSOD* in the liver in 6 hpi.	Wang *et al.* (2016)

			↑ expression of *Cu/ZnSOD* in the gill in 1, 3, 24, and 48 hpi. ↓ expression of *Cu/ZnSOD* in the gill in 6 hpi. ↑ expression of *Cu/ZnSOD* in the kidney 1, 3, 12, and 24 hpi. ↓ expression of *Cu/ZnSOD* in the kidney in 48 and 72 hpi. ↑ expression of *Cu/ZnSOD* in the liver in 1 and 6 hpi. ↓ expression of *Cu/ZnSOD* in the liver in 12, 24, 48, and 72 hpi.	
A. hydrophila	*Carassius auratus*	Cu/ZnSOD, MnSOD, *Cu/ZnSOD, MnSOD*	↑ hepatic Cu/ZnSOD activity in 6, 12, and 24 hpi. ↑ branchial Cu/ZnSOD activity in 6 and 12 hpi. ↑ renal Cu/ZnSOD activity in 6, 12, 24, and 48 hpi. ↑ Cu/ZnSOD activities in the spleen and head kidney in 6 hpi. ↑ hepatic MnSOD activity in 6, 12, and 24 hpi. ↑ branchial MnSOD activity in 3, 6, 12, and 24 hpi. ↑ renal MnSOD activity in 6 hpi. ↑ splenic MnSOD activity in 6 and 12 hpi. ↑ MnSOD activity in the head kidney in 3, 6, and 12 hpi. ↑ expression of *Cu/ZnSOD* in the liver in 3, 12, 24, and 48 hpi. ↑ expression of *Cu/ZnSOD* in the gill in 3, 6, and 12 hpi. ↑ expression of *Cu/ZnSOD* in the kidney in 3, 6, 12, 24, and 48 hpi. ↑ expression of *Cu/ZnSOD* in the spleen in 3 and 48 hpi.	Kong *et al.* (2017)

			↓ expression of *Cu/ZnSOD* in the head kidney in 12 and 24 hpi. ↑ expression of *MnSOD* in the liver and gill in 3, 6, 12, 24, and 48 hpi. ↑ expression of *MnSOD* in the kidney in 12, 24, and 48 hpi. ↑ expression of *MnSOD* in the spleen in 3, 6, and 12 hpi. ↓ expression of *MnSOD* in the head kidney in 12 hpi. ↑ expression of *MnSOD* in the head kidney in 24 and 48 hpi.	
A. hydrophila	*Carassius auratus gibelio*	SOD	↓ plasmatic SOD activity in 7 hpi.	Zhang P. *et al.* (2019)
A. hydrophila	*Carassius auratus gibelio*	SOD	≈ plasmatic SOD activity in 12 hpi.	Cao *et al.* (2018), Zhang P. *et al.* (2018)
A. hydrophila	*Carassius auratus gibelio*	SOD, CAT, TAC	↑ plasmatic SOD activity in 6 hpi. ↓ plasmatic SOD activity in 1 and 7 dpi. ↑ plasmatic CAT activity in 3 dpi. ≈ plasmatic TAC activity in 7 dpi.	Yang *et al.* (2015b)
A. hydrophila	*Carassius auratus gibelio*	SOD, TAC	↓ hepatic SOD activity in 1 and 2 dpi. ≈ hepatic TAC activity in 7 dpi.	Liu *et al.* (2013)
A. hydrophila	*Carassius gibelio*	SOD, TAC	≈ plasmatic SOD and TAC activities in 10 hpi.	Xu *et al.* (2020)
A. hydrophila	*Catla catla*	*iNOS*	↑ expression of *iNOS* in the thymus macrophages in 2 and 3 dpi.	Patel *et al.* (2016)
A. hydrophila	*Catla catla*	SOD, CAT, GPx	↑ SOD, CAT, and GPx activities in the gut in 3 and 6 dpi.	Pal *et al.* (2016)
A. hydrophila	*Channa punctata*	SOD	↑ SOD activity in the intestine, liver, muscle, and kidney in 28 dpi.	Verma *et al.* (2015)
A. hydrophila	*Channa striata*	Cu/ZnSOD, *Cu/ZnSOD*	↑ hepatic Cu/ZnSOD activity in 3, 6, 12, 24, 48, and 72 hpi.	Kumaresan *et al.* (2015)

			↑ expression of *Cu/ZnSOD* in the liver in 6, 24, and 48 hpi.	
A. hydrophila	*Channa striata*	*MnSOD*, *CAT*, *GPx*	↑ expression of *MnSOD*, *CAT*, and *GPx* in the liver in 4 dpi.	Samayanpaulraj *et al.* (2019)
A. hydrophila	*Channa striata*	SOD, *MnSOD*	Apparently ↑ hepatic SOD activity in 3 and 6 hpi. ↑ expression of *MnSOD* in the liver in 12, 24, and 48 hpi.	Arockiaraj *et al.* (2014)
A. hydrophila	*Cirrhinus mrigala*	SOD, CAT	Apparently ↓ branchial and hepatic SOD and CAT activities in 15 dpi.	Kumar *et al.* (2018)
A. hydrophila	*Cirrhinus mrigala*	SOD, CAT	↑ hepatic SOD and CAT activities in 7 dpi.	Ray and Homechaudhuri (2014)
A. hydrophila	*Clarias batrachus*	SOD	≈ serum SOD activity in 28 dpi.	Harikrishnan *et al.* (2018b)
A. hydrophila	*Clarias gariepinus*	SOD	↑ intestinal and muscle SOD activities in 14-28 dpi. ≈ hepatic and renal SOD activities in 14-28 dpi.	Verma *et al.* (2013)
A. hydrophila	*Clarias gariepinus*	SOD, CAT	↑ SOD and CAT activities in the liver, kidney, gill, and intestine in 7 dpi.	Sellegounder *et al.* (2018)
A. hydrophila	*Ctenopharyngodon idella*	SOD	≈ serum SOD activity in 56 dpi.	Harikrishnan *et al.* (2018a)
A. hydrophila	*Ctenopharyngodon idella*	SOD, CAT, TAC	↓ hepatic SOD activity in 28 dpi. ↓ hepatic TAC activity in 56 dpi. ≈ hepatic CAT activity in 28 and 56 dpi.	Zhao *et al.* (2019)
A. hydrophila	*Ctenopharyngodon idella*	SOD, CAT, TAC, *MnSOD*, *CAT*, *GPx*	≈ serum and hepatic SOD activity in 72 hpi. ↑ intestinal SOD activity in 72 hpi. ≈ serum and hepatic CAT activity in 72 hpi. ↓ intestinal CAT activity in 24 hpi. ≈ serum and hepatic TAC activity in 72 hpi. ↓ intestinal TAC activity in 6, 12, and 72 hpi. ↑ intestinal TAC activity in 48 hpi. ↓ expression of *MnSOD* in the head kidney in 6 hpi.	Tang *et al.* (2019)

			≈ expression of *CAT* in the head kidney in 24 hpi. ↓ expression of *GPx* in the head kidney in 12 and 24 hpi. ≈ expression of *MnSOD* and *CAT* in the intestine in 24 hpi. ↓ expression of *GPx* in the intestine in 24 hpi.	
A. hydrophila	*Ctenopharyngodon idella*	SOD, GPx, GST, GR	↓ hepatic SOD, GPx, GST, and GR activities in 7 dpi.	Baldissera *et al.* (2019)
A. hydrophila	*Cyprinus carpio*	*Prx3*, *Prx4*	↑ expression of *Prx3* in the head kidney in 12 hpi. ↓ expression of *Prx3* in the head kidney in 36 hpi. ↑ expression of *Prx3* in the liver in 6, 12, 24, 36, 48, and 72 hpi. ↑ expression of *Prx3* in the spleen in 6, 12, 24, 48, and 72 hpi. ↓ expression of *Prx4* in the head kidney in 6, 12, 24, 36, 48, and 72 hpi. ↑ expression of *Prx4* in the liver in 24, 36, 48, and 72 hpi. ↑ expression of *Prx4* in the spleen in 6 and 12 hpi.	Yang *et al.* (2018)
A. hydrophila	*Cyprinus carpio*	SOD	↑ serum and branchial SOD activities in 3 dpi. ↓ serum and branchial SOD activities in 7 dpi. ↑ hepatic SOD activity in 3 dpi. ↓ hepatic SOD activity in 5 and 7 dpi. ↑ intestinal SOD activity in 1 dpi. ↓ intestinal SOD activity in 5 and 7 dpi.	Chen *et al.* (2020)
A. hydrophila	*Danio rerio*	*DUOX*	↑ expression of *DUOX* in the intestine in 1 and 3 hpi.	Yang *et al.* (2017)
A. hydrophila	*Ictalurus furcatus*	*Sel*, *GST theta 1b*, *Prx1*, *Prx2*	↓ expression of *Sel* precursor genes in the skin in 2 and 12 hpi. ↑ expression of *GST theta 1b* in the skin in 24 hpi.	Li *et al.* (2013a)

			↓ expression of *Prx1* and *Prx2* in the skin in 24 hpi.	
A. hydrophila	*Ictalurus punctatus*	*Sel, SOD*	↑ expression of *Sel* precursor genes and *SOD* in the skin in 12 hpi.	Li *et al.* (2013b)
A. hydrophila	*Ictalurus punctatus*	SOD, CAT, TAC	≈ plasmatic SOD, CAT, and TAC activities in 7 dpi.	Yang *et al.* (2015a)
A. hydrophila	*Labeo rohita*	*MnSOD, CAT, GPx3, iNOS*	↑ expression of *iNOS* in the skin in 1 and 2 dpi. ↑ expression of *iNOS* in the muscle in 6 hpi. ↑ expression of *MnSOD* in the muscle in 5 dpi. ↑ expression of *CAT* in the muscle in 1, 2, and 5 dpi. ↑ expression of *GPx3* in the skin and muscle in 6 hpi.	Sharma *et al.* (2018)
A. hydrophila	*Labeo rohita*	SOD, CAT	Apparently ↑ hepatic and branchial SOD and CAT activities in 7 dpi.	Fawole *et al.* (2016)
A. hydrophila	*Labeo rohita*	SOD, CAT	↑ hepatic SOD activity in 7, 14, 21, and 28 dpi. ↑ hepatic CAT activity in 7 and 14 dpi.	Nandi *et al.* (2017)
A. hydrophila	*Labeo rohita*	SOD, CAT	↑ hepatic SOD activity in 7, 14, 21, and 28 dpi. ↑ hepatic CAT activity in 7 and 14 dpi.	Nandi *et al.* (2018)
A. hydrophila	*Labeo rohita*	SOD, CAT	↑ hepatic SOD and CAT activities in 7 dpi.	Pal *et al.* (2015)
A. hydrophila	*Labeo rohita*	SOD, CAT, GPx, GR	↑ hepatic and splenic SOD, CAT, GPx, and GR activities in 7 dpi.	Pal *et al.* (2019)
A. hydrophila	*Labeo rohita*	SOD, CAT, GPx, *iNOS*	≈ serum SOD, CAT, and GPx activities in 56 dpi. ≈ expression of *iNOS* in the head kidney leukocytes in 56 dpi.	Devi *et al.* (2019)
A. hydrophila, A. salmonicida	*Labeo rohita*	GPx, GR	↑ serum GPx and GR activities in 3 dpi.	Roy *et al.* (2019)
A. hydrophila	*Lutjanus peru*	SOD, CAT	Apparently ↓ SOD activity of blood leukocytes in 24 hpi. Apparently ↑ CAT activity of blood leukocytes in 24 hpi.	Reyes-Becerril *et al.* (2018a)

A. hydrophila	*Megalobrama amblycephala*	*CAT, GSTm*	↓ protein expression of *CAT* in the hepatopancreas in 1 dpi. ↓ mRNA expression of *CAT* in the hepatopancreas in 1 and 7 dpi. ↑ mRNA expression of *CAT* in the hepatopancreas in 14 dpi. ↑ protein expression of *GSTm* in the hepatopancreas in 1 dpi. ↓ mRNA expression of *GSTm* in the hepatopancreas in 1, 7, and 14 dpi. ↑ mRNA expression of *GSTm* in the hepatopancreas in 4 dpi.	Zhang H. *et al.* (2018)
A. hydrophila	*Megalobrama amblycephala*	*iNOS*	↑ expression of *iNOS* in the head kidney after infection.	Qin *et al.* (2018)
A. hydrophila	*Megalobrama amblycephala*	NOS, iNOS, SOD, GPx, *NOS, iNOS, Cu/ZnSOD, MnSOD, GPx, AMPK*	↑ plasmatic NOS, iNOS, SOD, and GPx activities in 7 dpi. ↑ expression of *NOS, iNOS, Cu/ZnSOD, MnSOD, GPx,* and *AMPK* in the head kidney in 7 dpi.	Liang *et al.* (2018)
A. hydrophila	*Megalobrama amblycephala*	SOD, CAT	↑ hepatic SOD activity in 6 and 12 hpi. ≈ hepatic CAT activity in 48 hpi.	Liu *et al.* (2012)
A. hydrophila	*Megalobrama amblycephala*	SOD, CAT	↓ serum SOD and CAT activities in 1, 3, 5, 14, 21, and 28 dpi.	Xia *et al.* (2017)
A. hydrophila	*Megalobrama amblycephala*	SOD, CAT, *Cu/ZnSOD, CAT, GPx, GR, GCLC, NRF2*	↑ SOD activities in the liver and spleen (6 hpi) and the blood (12 hpi). ↓ SOD activities in the gut and blood (6 hpi), and in the kidney and gut (12 hpi). ↑ CAT activities in the liver and spleen (6 hpi).	Geng *et al.* (2019)

			↑ expression of *Cu/ZnSOD* in the kidney and gut (6 hpi), and the spleen and blood (12 hpi). ↑ expression of *GPx* in liver and spleen (12 hpi). ↑ expression of *GR* in kidney and gut (6 hpi). ↑ expression of *GCLC* in the liver (6 hpi) and spleen (6 and 12 hpi). ↑ expression of *NRF2* in the spleen (12 hpi). ≈ expression of *CAT* in 6 and 12 hpi.	
A. hydrophila	*Megalobrama amblycephala*	SOD, CAT, GPx	↑ hepatic SOD activity in 2 dpi. ↓ hepatic SOD activity in 4 dpi. ↑ hepatic CAT and GPx activities in 2 dpi.	Abasubong *et al.* (2018)
A. hydrophila	*Micropterus salmoides*	SOD, CAT	↑ plasmatic SOD activity in 12 hpi. ≈ plasmatic CAT activity in 12 hpi.	Gong *et al.* (2019)
A. hydrophila	*Mystus vittatus*	SOD	≈ serum SOD activity in 30 dpi.	Harikrishnan *et al.* (2019)
A. hydrophila	*Oncorhynchus mykiss*	SOD, CAT, GPx	↑ serum SOD activity in 10 dpi. ≈ serum CAT and GPx activities in 10 dpi.	Kiadaliri *et al.* (2020)
A. hydrophila	*Oreochromis niloticus*	SOD, CAT	↓ serum SOD and CAT activities in 7 dpi.	El-Habashi *et al.* (2019)
A. hydrophila	*Oreochromis niloticus*	SOD, CAT	↓ SOD and CAT activities in the gill, intestine, liver, and muscle in 30 dpi.	Deepika *et al.* (2019)
A. hydrophila	*Oreochromis niloticus*	SOD, CAT, GPx	≈ plasmatic SOD and GPx activities in 10 dpi. ↑ plasmatic CAT activity in 10 dpi.	Abdel-Razek *et al.* (2019)
A. hydrophila	*Oreochromis niloticus*	SOD, CAT, GPx	≈ serum SOD, CAT, and GPx activities in 10 dpi.	Dawood *et al.* (2020)
A. hydrophila	*Oreochromis niloticus*	SOD, CAT, GPx, GR, GST, G6PDH	≈ SOD, CAT, GPx, GR, GST, and G6PDH activities in the spleen in 7 dpi.	Dotta *et al.* (2018)
A. hydrophila	*Oreochromis niloticus*	SOD, CAT, GPx, GST, TAC	Apparently ↓ muscle SOD activity in 14 dpi. Apparently ↓ hepatic CAT activity in 14 dpi. Apparently ↑ muscle CAT activity in 14 dpi. Apparently ↓ muscle GPx activity in 14 dpi. Apparently ↓ hepatic GST activity in 14 dpi.	Zahran *et al.* (2018)

			Apparently ↓ serum TAC activity in 14 dpi.	
A. hydrophila	*Oreochromis niloticus*	SOD, GPx	↓ serum SOD and GPx activities in 6 and 24 hpi, and 7 dpi.	Moustafa *et al.* (2020)
A. hydrophila	*Oreochromis niloticus* × *O. aureus*	SOD, GPx	≈ serum SOD and GPx activities in 5 and 10 hpi.	Han *et al.* (2020)
A. hydrophila	*Oreochromis* spp.	SOD, CAT, GPx	↓ hepatic SOD, CAT, and GPx activities in the infected group.	Vinosha *et al.* (2020)
A. hydrophila	*Pelteobagrus fulvidraco, P. vachelli, P. fulvidraco* × *P. vachelli*	SOD, *Cu/ZnSOD*, *MnSOD*	↑ SOD activity and expression of *Cu/ZnSOD* and *MnSOD* in the liver in a few hours after infection.	Zhang J. *et al.* (2019)
A. hydrophila	*Puntius sarana*	*MnSOD*	↑ expression of *MnSOD* in the head kidney in 1, 3, and 12 hpi, and 14 dpi. ↓ expression of *MnSOD* in the liver in 4 and 7 dpi.	Das *et al.* (2011)
A. hydrophila	*Rhamdia quelen*	SOD, CAT	↓ hepatic CAT activity in 2 dpi. ≈ hepatic SOD activity in 2 dpi.	Baldissera *et al.* (2017)
A. hydrophila	*Rhamdia quelen*	SOD, GR, TAC	↓ muscle SOD, GR, and TAC activities in 7 dpi.	da Rosa *et al.* (2019)
A. hydrophila	*Rhodeus uyekii*	*Prx1*	↑ expression of *Prx1* in the liver in 3 hpi. ↓ expression of *Prx1* in the liver in 24 and 48 hpi. ↓ expression of *Prx1* in the intestine in 24 and 48 hpi.	Cho *et al.* (2014)
A. hydrophila	*Salmo trutta*	SOD, CAT, GR, GPx, TAC	↑ hepatic SOD activity in males. ↓ hepatic CAT, GPx, and TAC activities in males and females. ↓ hepatic GR activity in males.	Tkachenko *et al.* (2014)
A. hydrophila	*Takifugu obscurus*	*CAT*, *GR*	↑ expression of *CAT* in the head kidney in 6 hpi. ↓ expression of *CAT* in the head kidney in 12 and 24 hpi. ↓ expression of *GR* in the head kidney in 6 hpi. ↑ expression of *GR* in the head kidney in 3, 12, and 24 hpi.	Liu *et al.* (2020)

A. salmonicida	*Carassius auratus*	SOD	Apparently ↑ hepatic SOD activity in 2 hpi.	Asakura *et al.* (2019)
A. salmonicida	*Gadus morhua*	*Cu/ZnSOD, MnSOD, CAT, NRF2, NOX1*	↓ expression of *Cu/ZnSOD* in the macrophages in 6 hpi. ≈ expression of *MnSOD* in the macrophages in 1, 2, and 6 hpi. ↓ expression of *CAT* in the macrophages in 1, 2, and 6 hpi. ↓ expression of *NRF2* in the macrophages in 2 and 6 hpi. ↓ expression of *NOX1* in the macrophages in 1 hpi.	Soto-Dávila *et al.* (2019)
A. salmonicida	*Oncorhynchus mykiss*	SOD, CAT, *Cu/ZnSOD*, *CAT*	↓ serum SOD activity in 2 and 6 dpi. ↓ serum CAT activity in 2 and 4 dpi. ↑ serum CAT activity in 6 dpi. ↑ expression of *Cu/ZnSOD* in the head kidney in 2, 4, and 6 dpi. ↑ expression of *CAT* in the head kidney in 4 and 6 dpi.	Ji *et al.* (2017)
A. salmonicida	*Oreochromis mossambicus*	SOD	↑ SOD activity in gill, liver, and muscle in 15 dpi.	Thanigaivel *et al.* (2019)
A. salmonicida	*Salmo salar*	SOD, CAT	Apparently ↓ serum SOD activity in 1, 2, and 3 dpi. Apparently ↓ serum CAT activity in 1 dpi.	Wang *et al.* (2019)
A. salmonicida	*Salmo salar*	SOD, CAT, Prx, *Cu/ZnSOD*, *CAT*, *Prx1*	↑ serum SOD activity in 6 dpi. ↑ mucus SOD activity in 2 dpi. ↓ mucus SOD activity in 4 and 6 dpi. ↓ skin SOD activity in 4 dpi. ↓ expression of *Cu/ZnSOD* in the skin in 2, 4, and 6 dpi. ↓ serum CAT activity in 2 and 4 dpi. ↓ mucus CAT activity in 4 and 6 dpi. ↓ skin CAT activity in 4 dpi.	Du *et al.* (2015)

			↓ expression of *CAT* in the skin in 2, 4, and 6 dpi. ≈ Prx activity in serum and skin in 6 dpi. ↑ mucus Prx activity in 2 dpi. ↓ expression of *Prx1* in the skin in 4 dpi.	
A. salmonicida	*Scophthalmus maximus*	G6PDH, 6PGDH, GR	≈ hepatic G6PDH, 6PGDH, and GR activities in 8 dpi.	Rodríguez-Quiroga *et al.* (2017)
A. veronii	*Lutjanus peru*	SOD, CAT	↑ serum SOD and CAT activities in 1 and 2 dpi.	Reyes-Becerril *et al.* (2015)
A. veronii	*Totoaba macdonaldi*	*CAT*, *GPx1*, *GPx4*	↓ expression of *CAT* and *GPx4* in the head kidney leukocytes in 6 and 24 hpi. ↓ expression of *GPx1* in the head kidney leukocytes in 24 hpi. ↓ expression of *CAT* in the spleen leukocytes in 6 hpi. ↑ expression of *CAT* in the spleen leukocytes in 24 hpi. ↓ expression of *GPx1* in the spleen leukocytes in 6 and 24 hpi. ↑ expression of *GPx4* in the spleen leukocytes in 6 hpi.	Reyes-Becerril *et al.* (2018b)

↑, increased; ↓, decreased; ≈, non-significant variation.

AMPK, adenosine monophosphate-activated protein kinase; 6PGDH, 6-phosphogluconate dehydrogenase; CAT, catalase; dpi, days post-infection; DUOX, dual oxidase; G6PDH, glucose 6-phosphate dehydrogenase; GCLC, glutamate-cysteine ligase; GPx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione S-transferase; hpi, hours post-infection; iNOS, inducible nitric oxide synthase; NOS, nitric oxide synthase; NOX1, NADPH oxidase 1; NRF2, nuclear factor erythroid 2-related factor 2; Prx, peroxiredoxin; Sel, selenoprotein; SOD, superoxide dismutase; TAC, total antioxidant capacity; XO, xanthine oxidase.

Table 2 Observed changes in non-enzymatic markers of oxidative status of fish infected with *Aeromonas*

***Aeromonas* species**	**Fish species**	**Biomarkers**	**Changes**	**References**
A. caviae	*Rhamdia quelen*	ROS, MDA, NPSH, GSH	↑ hepatic and renal ROS and MDA levels in 4 dpi. ↓ hepatic and renal NPSH levels in 4 dpi. ≈ hepatic and renal GSH levels in 4 dpi.	Baldissera *et al.* (2018)
A. caviae	*Rhamdia quelen*	ROS, NO	↑ splenic ROS and NO levels in 4 dpi.	Souza *et al.* (2017)
A. culicicola	*Cyprinus carpio*	MDA	↑ MDA levels in the gill, tissue, and liver.	Swathy *et al.* (2018)
A. hydrophila	*Carassius auratus*	RBA, NO	↓ RBA and NO levels of blood leukocytes in 3, 6, and 12 hpi.	Harikrishnan *et al.* (2010)
A. hydrophila	*Carassius auratus gibelio*	MDA	↑ hepatic MDA levels in 1, 2, and 7 dpi.	Liu *et al.* (2013)
A. hydrophila	*Carassius auratus gibelio*	MDA	≈ plasmatic MDA levels in 12 hpi.	Cao *et al.* (2018)
A. hydrophila	*Carassius auratus gibelio*	MDA	≈ plasmatic MDA levels in 7 dpi.	Yang *et al.* (2015b)
A. hydrophila	*Carassius gibelio*	MDA	≈ plasmatic MDA levels in 10 hpi.	Xu *et al.* (2020)
A. hydrophila	*Catla catla*	GSH	↓ gut GSH levels in 3 and 6 dpi.	Pal *et al.* (2016)
A. hydrophila	*Catla catla*	RBA, NO	↑ RBA of thymus macrophages in 2 and 3 dpi. ↑ NO levels of thymus macrophages in 3 dpi.	Patel *et al.* (2016)
A. hydrophila	*Channa punctata*	NO	↑ NO levels of head kidney and spleen phagocytes in 14 and 28 dpi.	Verma *et al.* (2015)
A. hydrophila	*Channa striata*	$O_2^{\bullet-}$	Apparently ↓ blood $O_2^{\bullet-}$ levels in 3, 6, 12, 24, 48, and 72 hpi.	Arockiaraj *et al.* (2014)
A. hydrophila	*Channa striata*	ROS, NO	↑ ROS levels of blood leukocytes in 5 and 10 dpi. ↑ NO levels of blood leukocytes in 5 dpi.	Priyadarshini *et al.* (2017)
A. hydrophila	*Cirrhinus mrigala*	ROS	↑ intracellular levels of ROS in heart cells in 7 dpi.	Ray and Homechaudhuri (2019)
A. hydrophila	*Cirrhinus mrigala*	ROS	↑ intracellular levels of ROS in liver cells in 7 dpi.	Ray and Homechaudhuri (2014)
A. hydrophila	*Clarias batrachus*	RBA	≈ RBA of head kidney leukocytes in 28 dpi.	Harikrishnan *et al.* (2018b)

A. hydrophila	*Clarias gariepinus*	MDA, NO	↑ MDA levels in the intestine, liver, muscle, and head kidney in 14-28 dpi. ↓ NO levels of spleen phagocytes in 14 and 28 dpi.	Verma *et al.* (2013)
A. hydrophila	*Clarias gariepinus*	RBA	≈ RBA of blood leukocytes in 24 hpi.	Mbokane and Moyo (2020)
A. hydrophila	*Clarias gariepinus*	RBA	↑ RBA of blood leukocytes in 24 hpi.	Nafiqoh *et al.* (2020)
A. hydrophila	*Clarias gariepinus*	ROS	↑ ROS levels in the liver, kidney, gill and intestine in 7 dpi.	Sellegounder *et al.* (2018)
A. hydrophila	*Ctenopharyngodon idella*	H_2O_2, MDA, GSH	↑ hepatic H_2O_2 levels in 28 dpi. ↓ hepatic H_2O_2 levels in 56 dpi. ↑ hepatic MDA levels in 28 and 56 dpi. ≈ hepatic GSH levels in 28 and 56 dpi.	Zhao *et al.* (2019)
A. hydrophila	*Ctenopharyngodon idella*	MDA, GSH	↑ serum MDA levels in 24, 48, and 72 hpi. ↑ intestinal MDA levels in 3, 6, and 24 hpi. ↑ hepatic MDA levels in 3 and 12 hpi. ↓ hepatic MDA levels in 24 hpi. ↓ serum GSH levels in 48 hpi. ↓ intestinal GSH levels in 3 and 12 hpi. ↑ intestinal GSH levels in 6 and 48 hpi. ↑ hepatic GSH levels in 3 hpi.	Tang *et al.* (2019)
A. hydrophila	*Ctenopharyngodon idella*	RBA	≈ RBA of head kidney leukocytes in 56 dpi.	Harikrishnan *et al.* (2018a)
A. hydrophila	*Ctenopharyngodon idella*	ROS	↑ branchial ROS levels in 7 dpi.	Morselli *et al.* (2020)
A. hydrophila	*Ctenopharyngodon idella*	ROS, LOOH, PC, ACAP	↑ hepatic ROS, LOOH, and PC levels in 7 dpi. ↓ hepatic ACAP levels in 7 dpi.	Baldissera *et al.* (2019)
A. hydrophila	*Cyprinus carpio*	GSH, MDA	↑ serum and intestinal GSH levels in 1 dpi. ↓ serum and intestinal GSH levels in 5 and 7 dpi. ↑ hepatic GSH levels in 3 dpi. ↓ hepatic GSH levels in 5 and 7 dpi. ↑ branchial GSH levels in 3 dpi. ↓ branchial GSH levels in 7 dpi.	Chen *et al.* (2020)

			↑ serum MDA levels in 3, 5, and 7 dpi. ↑ hepatic MDA levels in 1, 3, 5, and 7 dpi. ↑ intestinal and branchial MDA levels in 5 and 7 dpi.	
A. hydrophila	*Cyprinus carpio*	RBA, NO	≈ RBA of head kidney macrophages in 28 dpi. ≈ NO levels of blood leukocytes in 28 dpi.	Anbazahan *et al.* (2014)
A. hydrophila	*Danio rerio*	ROS	↑ intestinal ROS levels in 3 hpi.	Yang *et al.* (2017)
A. hydrophila	*Ictalurus punctatus*	MDA	≈ plasmatic MDA levels in 7 dpi.	Yang *et al.* (2015a)
A. hydrophila	*Labeo rohita*	MDA	↑ hepatic MDA levels in 7, 14, 21, and 28 dpi.	Nandi *et al.* (2017)
A. hydrophila	*Labeo rohita*	MDA	↑ hepatic MDA levels in 7, 14, 21, and 28 dpi.	Nandi *et al.* (2018)
A. hydrophila	*Labeo rohita*	MDA, RBA	↑ serum MDA levels in 42 and 56 dpi. ≈ RBA of head kidney leukocytes in 56 dpi.	Devi *et al.* (2019)
A. hydrophila	*Labeo rohita*	RBA	Apparently ↑ RBA of blood leukocytes in 7 dpi.	Fawole *et al.* (2016)
A. hydrophila	*Labeo rohita*	RBA	≈ RBA of blood leukocytes in 10 dpi.	Gora *et al.* (2018)
A. hydrophila	*Labeo rohita*	ROS	↑ hepatic intracellular ROS levels in 7 dpi.	Pal *et al.* (2015)
A. hydrophila	*Labeo rohita*	ROS, GSH	↑ hepatic and splenic ROS and GSH levels in 7 dpi.	Pal *et al.* (2019)
A. hydrophila, *A. salmonicida*	*Labeo rohita*	GSH	↑ serum GSH levels in 3 dpi.	Roy *et al.* (2019)
A. hydrophila	*Lates calcarifer*	RBA	↑ RBA of blood leukocytes in 3, 6, 12, and 24 hpi.	Kathirkaman *et al.* (2018)
A. hydrophila	*Lutjanus peru*	NO	Apparently ↑ NO levels of blood leukocytes in 24 hpi.	Reyes-Becerril *et al.* (2018a)
A. hydrophila	*Megalobrama amblycephala*	MDA	≈ hepatic MDA levels in 4 dpi.	Abasubong *et al.* (2018)
A. hydrophila	*Megalobrama amblycephala*	MDA	↑ hepatic MDA levels in 48 hpi.	Liu *et al.* (2012)
A. hydrophila	*Megalobrama amblycephala*	MDA, GSH, GSSG	↑ MDA levels in the spleen (6 hpi). ↑ GSH levels in the gut (6 hpi) and kidney (12 hpi). ↓ GSH levels in the liver and spleen (6 hpi), and the liver, gut, and blood (12 hpi).	Geng *et al.* (2019)

			↑ GSSG levels in the kidney (12 hpi). ↓ GSSG levels in the liver, spleen, gut, and blood (6 hpi), and the spleen, gut, and blood (12 hpi).	
A. hydrophila	*Megalobrama amblycephala*	MDA, NO	↑ plasmatic MDA and NO levels in 7 dpi.	Liang *et al.* (2018)
A. hydrophila	*Megalobrama amblycephala*	RBA, NO	↑ RBA and NO levels of macrophages in 24 hpi.	Qin *et al.* (2018)
A. hydrophila	*Micropterus salmoides*	MDA	↑ plasmatic MDA levels in 12 hpi.	Gong *et al.* (2019)
A. hydrophila	*Mystus vittatus*	RBA, NO	≈ RBA of head kidney leukocytes in 30 dpi. ≈ serum NO levels in 30 dpi.	Harikrishnan *et al.* (2019)
A. hydrophila	*Oreochromis mossambicus*	RBA	≈ RBA of blood leukocytes in 24 hpi.	Mbokane and Moyo (2018)
A. hydrophila	*Oreochromis niloticus*	MDA	↑ MDA levels in the gill, intestine, liver, and muscle in 30 dpi.	Deepika *et al.* (2019)
A. hydrophila	*Oreochromis niloticus*	MDA	↑ serum MDA levels in 10 dpi.	Dawood *et al.* (2020)
A. hydrophila	*Oreochromis niloticus*	MDA	↑ serum MDA levels in 7 dpi.	El-Habashi *et al.* (2019)
A. hydrophila	*Oreochromis niloticus*	MDA, NO, GSH	Apparently ↑ hepatic and muscle MDA levels in 14 dpi. Apparently ↑ hepatic NO levels in 14 dpi. Apparently ↓ muscle GSH levels in 14 dpi.	Zahran *et al.* (2018)
A. hydrophila	*Oreochromis niloticus*	MDA, RBA	↑ serum MDA levels in 6 and 24 hpi, and 7 dpi. ↓ RBA of blood leukocytes in 6 and 24 hpi, and 7 dpi.	Moustafa *et al.* (2020)
A. hydrophila	*Oreochromis niloticus*	MDA, RBA, NO	≈ RBA of blood leukocytes in 10 dpi. ≈ plasmatic MDA levels in 10 dpi. ↑ plasmatic NO levels in 10 dpi.	Abdel-Razek *et al.* (2019)
A. hydrophila	*Oreochromis niloticus*	RBA	↑ RBA of blood leukocytes in 6 and 24 hpi.	Charlie-Silva *et al.* (2019)
A. hydrophila	*Oreochromis niloticus* × *O. aureus*	ROS	≈ serum ROS levels in 5 and 10 hpi.	Han *et al.* (2020)
A. hydrophila	*Oreochromis* spp.	AA, GSH	↓ hepatic AA and GSH levels in the infected group.	Vinosha *et al.* (2020)
A. hydrophila	*Piaractus mesopotamicus*	RBA	↑ RBA of blood leukocytes in 3 hpi.	Claudiano *et al.* (2019)

			↓ RBA of blood leukocytes in 6 hpi.	
A. hydrophila	*Piaractus mesopotamicus*	RBA	↑ RBA of blood leukocytes in 7 dpi.	Biller-Takahashi *et al.* (2013)
A. hydrophila	*Puntius sarana*	RBA	↑ RBA of blood leukocytes in 4 and 7 dpi.	Das *et al.* (2011)
A. hydrophila	*Rhamdia quelen*	MDA, PC	↑ hepatic MDA and PC levels in 2 dpi.	Baldissera *et al.* (2017)
A. hydrophila	*Rhamdia quelen*	NO, $O_2^{\bullet-}$, GSH, GSSG, PSSX, PSH, LOOH, MDA, AA	↑ muscle NO, $O_2^{\bullet-}$, GSSG, PSSX, LOOH, and MDA levels in 7 dpi. ↓ muscle GSH, PSH, and AA levels in 7 dpi.	da Rosa *et al.* (2019)
A. hydrophila	*Salmo trutta*	PC, MDA	↑ hepatic PC levels. ≈ hepatic MDA levels.	Tkachenko *et al.* (2014)
A. hydrophila	*Takifugu obscurus*	RBA, NO	↑ RBA and NO levels of blood cells in 3, 6, 12, 24, 48, and 72 hpi.	Cheng *et al.* (2017)
A. hydrophila	*Takifugu obscurus*	ROS	↓ blood ROS levels in 12 hpi.	Liu *et al.* (2020)
A. hydrophila	*Tor grypus*	RBA	≈ RBA of blood leukocytes in 7 dpi.	Mohammadian *et al.* (2018)
A. salmonicida	*Gadus morhua*	RBA	≈ RBA of macrophages in 1 and 6 hpi.	Soto-Dávila *et al.* (2019)
A. salmonicida	*Oreochromis mossambicus*	MDA	↑ MDA levels in the gill, liver, and muscle in 15 dpi.	Thanigaivel *et al.* (2019)
A. salmonicida	*Perca fluviatilis*	RBA	↓ RBA of the spleen in 3 dpi. ≈ RBA of the head kidney in 3 dpi.	Geay *et al.* (2015)
A. salmonicida	*Scophthalmus maximus*	NADP, NADPH	≈ hepatic NADP and NADPH levels in 8 dpi.	Rodríguez-Quiroga *et al.* (2017)

↑, increased; ↓, decreased; ≈, non-significant variation.

AA, ascorbic acid; ACAP, antioxidant capacity against peroxyl radicals; dpi, days post-infection; GSH, reduced glutathione; GSSG, oxidized glutathione; H_2O_2, hydrogen peroxide; hpi, hours post-infection; LOOH, lipid hydroperoxides; MDA, malondialdehyde; NADP, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; NPSH, non-protein sulfhydryl; $O_2^{\bullet-}$, superoxide anion; PC, protein carbonylation; PSH, cysteine residues of proteins; PSSX, thiol-protein mixed disulfides; RBA, respiratory burst activity; ROS, reactive oxygen species.

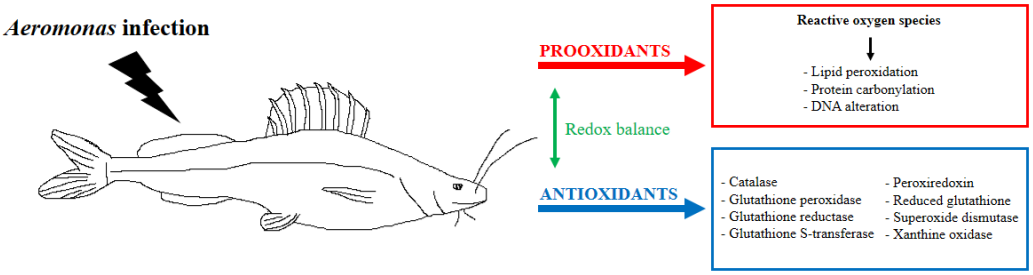

Figure 1 Prooxidants and antioxidants factors associated with fish *Aeromonas* infection

3.2 ARTIGO ORIGINAL 1

O primeiro artigo original intitulado “*Aeromonas hydrophila* infection in silver catfish causes hyperlocomotion related to stress” foi publicado na revista científica “Microbial Pathogenesis” (fator de impacto 3.738). Endereço de acesso: https://doi.org/10.1016/j.micpath.2019.05.017 (BANDEIRA JUNIOR et al., 2019).

Highlights

Aeromonas hydrophila infection in silver catfish causes increased locomotor activity.
This infection also causes increased brain expression of *hspa12a* and *crh*.
The disease pathophysiology involves changes in the HPI axis.

Aeromonas hydrophila infection in silver catfish causes hyperlocomotion related to stress

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Abstract

Aeromonosis is a fish disease that leads to haemorrhagic septicaemia and high mortality. The detection of early behavioural changes associated to this disease could be helpful in anticipating the initiation of treatment, increasing the probability of success. The influence of this disease on the hypothalamic-pituitary-interrenal (HPI) axis and on the brain expression of heat shock proteins (HSP) is little known. Therefore, the aim of this study was to evaluate the effect of *Aeromonas hydrophila* infection on individual behaviour and brain expression of genes related to stress (*slc6a2*, *hsp90*, *hspa12a*, *hsd20b*, *hsd11b2*, *crh*) in silver catfish (*Rhamdia quelen*). Thirty fish were divided into healthy and infected groups. The fish of the infected group were inoculated intramuscularly with 50 μL of bacterial suspension (6.4 x 10^8 CFU/mL), while control animals received 50 μL of saline. On day five post-infection, animals were submitted to the novel tank test, euthanized, and the brain was collected for molecular analysis. Infected fish swam more in the unknown aquarium and presented an increase in brain expression of genes related to HSP (*hspa12a*) and the route of cortisol synthesis (*crh*) when compared to uninfected fish. Therefore, this disease causes hyperlocomotion related to stress.

Keywords: *Rhamdia quelen*, *Aeromonas hydrophila*, novel tank test, gene expression, heat shock protein, HPI axis.

1. Introduction

Bacteria of the genus *Aeromonas* are oxidase-positive, facultatively anaerobic, gram-negative rods that grow readily on basic laboratory media, such as heart infusion agar. Moreover, many strains are β-haemolytic and biofilm producers [1]. These bacteria are known to cause septicaemia in aquatic organisms, and cause gastroenteritis and extraintestinal diseases, such as septicaemia, skin, eye, wound and respiratory tract infections, in humans [2]. Among the pathogenic species for fish, *A. hydrophila* has been widely studied for causing cutaneous ulcerations and haemorrhagic septicaemia in freshwater fish, such as silver catfish (*Rhamdia quelen*) [3].

The treatment of fish aeromonosis is complicated due to the emergence of multiresistant strains [4]. Also, the use of unlicensed drugs is excessive, since only oxytetracycline, florfenicol and sulfadimethoxine/ormetoprim are antimicrobials approved by the Food and Drug Administration for use in aquaculture [5]. In addition, fish producers often detect the disease when it is in an advanced state, initiating the treatment late, which reduces the possibility of therapeutic success. Treatment usually starts when fish exhibit marked clinical signs, such as skin ulcers, fin necrosis, and erratic swimming. If it were possible to detect behavioural changes early, it would be possible to anticipate the beginning of treatment, increasing the chances of healing. The novel tank test can be used to evaluate changes in the locomotor activity of fish infected with bacteria. On this subject, *Pseudomonas aeruginosa* infection in silver catfish, *Rhamdia quelen*, is known to cause cerebral changes and hyperlocomotion [6]. However, there is a lack of data on behavioural changes in fish infected with *A. hydrophila*.

Behavioural changes may indicate that the fish is experiencing some stressful condition. The interrenal gland contains chromaffin cells, which produce catecholamines (adrenaline and noradrenaline) that are involved in the primary and faster response to acute stress. Stressful stimuli may promote the release of serotonin in the central nervous system (CNS) or acetylcholine in the preganglionic fibres of the sympathetic nervous system, stimulating the release of catecholamines by chromaffin cells [7]. The *slc6a2* (sodium-dependent norepinephrine transporter) gene encodes a multi-pass transmembrane protein present in the fish brain, which is responsible for the reuptake of noradrenaline at the presynaptic nerve terminals and is a regulator of noradrenaline homeostasis [8].

In response to stress, the corticotropin-releasing hormone (CRH) is secreted by the paraventricular nucleus of the hypothalamus, binds to specific receptors and prompts the pituitary gland to release adrenocorticotropic hormone (ACTH) or corticotrophin, which stimulates the cortisol synthesis and secretion by the interrenal cells of the interrenal gland. The CRH is an important regulator of homeostasis, mediating autonomic, behavioural and neuroendocrine responses to stress [7,9]. The *crh* gene encodes a family member of ACTH release factors. The genes *hsd11b2* (11β-hydroxysteroid dehydrogenase type 2) and *hsd20b* (20β-hydroxysteroid dehydrogenase) encode two enzymes involved in the transformation of cortisol into inactive cortisone [8]. Therefore, these three genes influence the amount of cortisol in the fish body. However, there is a lack of studies on the effect of fish bacterial infections on the hypothalamic-pituitary-interrenal (HPI) axis.

Heat shock proteins (HSP) are a family of proteins expressed in response to a wide range of biotic and abiotic stressors and may also be referred to as stress proteins. Among the biotic stressors are bacterial challenge [10]. In the fish brain, the genes *hspa12a* (heat shock protein 70, member 12) and *hsp90* (heat shock protein 90) are two examples of genes encoding HSP members [8].

Taking into account the aforementioned mechanisms, the aim of this study was to evaluate the effect of *A. hydrophila* infection on the individual behaviour and brain expression of genes related to stress (*slc6a2*, *hsp90*, *hspa12a*, *hsd20b*, *hsd11b2*, *crh*) in silver catfish.

2. Materials and methods

2.1. Clinical isolate

The *A. hydrophila* MF 372510 was isolated from a naturally infected juvenile silver catfish and identified by biochemical and molecular tests, as previously described by Bandeira Junior et al. [11].

2.2. Animals and water quality parameters

Apparently healthy (no external signs of lesions) silver catfish (males and females, n = 30, 12 ± 1 cm, 8 ± 2 g) were obtained from a local fish farm and acclimated for one week in 250 L tanks. The fish were fed twice per day with an extruded commercial feed, 1.7 mm in size (Supra^{TM} Juvenil, 46% protein). Food and faeces that had accumulated at

the bottom of tanks were removed daily, and 25% of the water was renewed each day. Water quality parameters (temperature, pH, dissolved oxygen and total ammonia concentration) were evaluated daily.

2.3. Experimental design

The fish were divided into two groups: healthy (n = 15) and infected (n = 15). For the inoculum, *A. hydrophila* MF 372510 was grown on Tryptone Soya Agar (TSA) (Himedia Laboratories, Mumbai, India). The inoculum was prepared for the infection model in sterile saline (NaCl 0.9%) with the turbidity adjusted to an optical density (OD_{600}) of 0.8, equivalent to 6.4×10^8 colony forming units (CFU)/mL. The fish of the infected group were inoculated intramuscularly in the right dorsolateral region with 50 μL of bacterial suspension, according to Cunha et al. [12]. Similarly, 50 μL of saline was used to inoculate fish of the healthy group. Immediately after inoculation, the fish were transferred to individual 1.5 L aquariums and mortality was evaluated throughout the experiment. On day five post-infection, the surviving animals were submitted to the behavioural test (novel tank). Subsequently, the fish were euthanized by spinal cord transection, and the brain was collected, stored in Eppendorf tubes containing TRIzol™ reagent and maintained at -80°C for molecular analysis.

2.4. Novel tank test

The experimental apparatus consisted of a glass aquarium (24 × 8 × 20 cm, length × width × height). The fish (n = 12 per group) were individually transported to the unknown aquarium and filmed for 6 min with a video camera (Logitech™ HD Webcam C525) positioned in front of the apparatus. The analysis of the video recordings was performed using ANY-maze™ video monitoring software (Stoelting Co., USA). Two zones (upper and bottom) were evaluated. The parameters analysed were distance travelled, mean speed, number of crossings, freezing time (immobile for more than 2 seconds), number of entries in the upper zone and dwelling time in the upper zone [13].

2.5. Gene expression

From brain tissue, expression of genes directly related to stress (*slc6a2*, *hsp90*, *hspa12a*, *hsd20b*, *hsd11b2* and *crh*) was evaluated (n = 6 per group). The primer design and the qPCR analysis were performed according to the protocol already established by Souza et al. [8].

2.6. Ethical statement

The experimental protocols involving animals was approved by the Comissão de Ética no Uso de Animais (CEUA) at the Universidade Federal de Santa Maria, UFSM, Santa Maria, RS, Brazil (Protocol 6723271118/2019) and met the guidelines of Conselho Nacional de Controle de Experimentação Animal (CONCEA).

2.7. Statistical analysis

Data were reported as mean ± standard error (SEM). The homogeneity of variances between groups was determined with the Levene test. When the data were parametric, comparisons between different groups were made through Student's t-test. In the case of nonparametric data, the two-tailed Mann-Whitney U test was used to verify differences between groups (Statistica 7.0^{TM}; StatSoft Inc., Tulsa, OK, USA). The data were considered statistically significant when $p < 0.05$.

3. Results

3.1. Water quality parameters and mortality

The water quality parameters were maintained as follows: temperature 19 ± 1°C, pH 7.90 ± 0.05, dissolved oxygen 9.00 ± 0.70 mg/L, and total ammonia concentration ≤ 0.50 mg/L (corresponding to non-ionized ammonia ≤ 0.018 mg/L). All fish in the healthy group survived throughout the experimental period. As for the fifteen fish of the infected group, one fish died on days 2, 4 and 5, and consequently, twelve fish survived throughout the experiment.

3.2. Novel tank test

Silver catfish infected with *A. hydrophila* showed a greater distance travelled, higher average speed, greater number of crosses between zones, greater number of entries in the upper zone, longer dwell time in the upper zone and shorter freezing time when compared to uninfected fish (Fig. 1). Therefore, the infected fish presented hyperlocomotion.

3.3. Gene expression

Silver catfish infected with *A. hydrophila* presented an increase in *hspa12a* and *crh* expression in the brain, compared to uninfected fish. However, infected fish had no significant difference in the expression of the genes *slc6a2*, *hsp90*, *hsd20b* or *hsd11b2* in the brain, compared to uninfected fish (Fig. 2).

4. Discussion

Fish infectious disorders are very prevalent in intensive aquaculture, and the bacterial and parasitic diseases generate direct and indirect economic losses that were estimated at US $84 million per year in Brazilian fish farms [14]. Among the fish pathogenic bacteria, we can highlight the *Aeromonas* genus due to its high prevalence and pathogenicity [1]. This bacteria genus produce a diverse and heterogeneous range of virulence factors. Expression of membrane components, toxins, enzymes and several molecules contribute to bacterial pathogenicity and act in different ways, such as tissue adhesion, immune response evasion, and involvement of host cells [15]. However, the effect of aeromonosis on behaviour, CNS and stress in fish remains poorly studied.

Behavioural tests may be useful to detect infectious diseases in fish and their stage, especially those that lead to the stress of these animals [16]. In the current study, fish infected with *A. hydrophila* swam more in an unknown aquarium on day five post-infection than uninfected fish. A similar pattern was observed in silver catfish infected with *P. aeruginosa*, because infected fish exhibited hyperlocomotion and brain alterations on days 6 and 7 post-infection [6]. This bacterium also causes a disruption of the blood-brain barrier in silver catfish, which may contribute to disease pathogenesis in the CNS [17]. However, a bacterial infection in advanced stage lead to a lethargic state, as observed in silver catfish infected with *Citrobacter freundii* on day 18 post-infection [18]. Therefore, we can conclude that, in general, recently infected fish present hyperlocomotion, and the aggravation of the disease leads to lethargy due to the debilitated state of the animal.

The *crh* overexpression in the CNS means increased activation of the cortisol synthesis and secretion pathway [7]. In this study, we observed *crh* upregulation in the brain of silver catfish infected with *A. hydrophila* compared to uninfected fish, indicating higher activation of the aforementioned pathway. Another study reported that infection by the protozoan *Trypanoplasma borreli* in common carp (*Cyprinus carpio*) leads to a downregulation of the CRH receptor genes in the peripheral tissues (gill and skin), which

may have happened due to the negative feedback exerted by high concentrations of plasma cortisol [19]. The *hsd20b* and *hsd11b2* genes encode enzymes involved in the cortisol inactivation into cortisone [8]. *Citrobacter freundii* infection led to *hsd11b2* upregulation in zebrafish (*Danio rerio*) skin, indicating an increase in plasma cortisol in infected animals, requiring more enzyme to inactivate it [20]. In our study, despite the *crh* upregulation, the genes *hsd20b* and *hsd11b2* presented the same expression level between the healthy and infected groups. Our hypothesis is that we detected the beginning of the increase in *crh* expression, which was not sufficient to significantly increase cortisol to the level necessary to elevate the expression of the inactivating enzymes. However, Ellis et al. [21] reported that acute infections by Viral Haemorrhagic Septicaemia virus and *Aeromonas salmonicida* elevate water cortisol concentrations in rainbow trout (*Oncorhynchus mykiss*) tanks, which reflected in elevated plasma cortisol concentrations. Therefore, plasma cortisol levels should be evaluated in future experiments.

The HSP are protein molecules typically involved in protein homeostasis. Among their functions are the protection of proteins against denaturing stressors. They can be activated by the stress and immune response caused by a bacterial infection [22]. In aquatic animals, the HSP have been shown to play an important role in health, in relation to development of inflammation and the specific and non-specific immune responses to bacterial and viral infections in both finfish and shrimps [23]. In the present study, although there was no difference between groups in the brain expression of *hsp90*, infected fish presented higher brain expression of *hspa12a* when compared to uninfected fish, indicating the stressing capacity of aeromonosis in silver catfish. Similar results were described by Song et al. [24], which reported a significant increase in expression of the HSP70 family of genes in channel catfish (*Ictalurus punctatus*) gills after infection with *Flavobacterium columnare*. In another study, induced expression of HSP family was observed within a few hours after *F. columnare* and *Edwardsiella ictaluri* infection in channel catfish, but suppressed HSP genes expression was observed at later stages after bacterial infection (three days post-infection) [25].

This is the first study that investigated the influence of a bacterial infection on the expression of the *slc6a2* gene in fish. This gene is a regulator of noradrenaline homeostasis [8]. In our work, there was no difference in brain expression of the *slc6a2* gene between infected and uninfected animals. One possible explanation is the fact that catecholamines are involved in the primary and faster response to acute stress [7].

Therefore, this acute stress may have ceased after five days of infection. Further study is needed to investigate the expression of this gene within a few hours after infection to confirm this hypothesis.

In summary, the *A. hydrophila* infection in silver catfish causes hyperlocomotion related to stress and changes the HPI axis, increasing the brain expression of *hspa12a* and *crh*, which are related to heat shock proteins and the route of cortisol synthesis, respectively.

Competing financial interests

The authors declare no competing financial interests.

Acknowledgements

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES - finance code 001) of Brazil.

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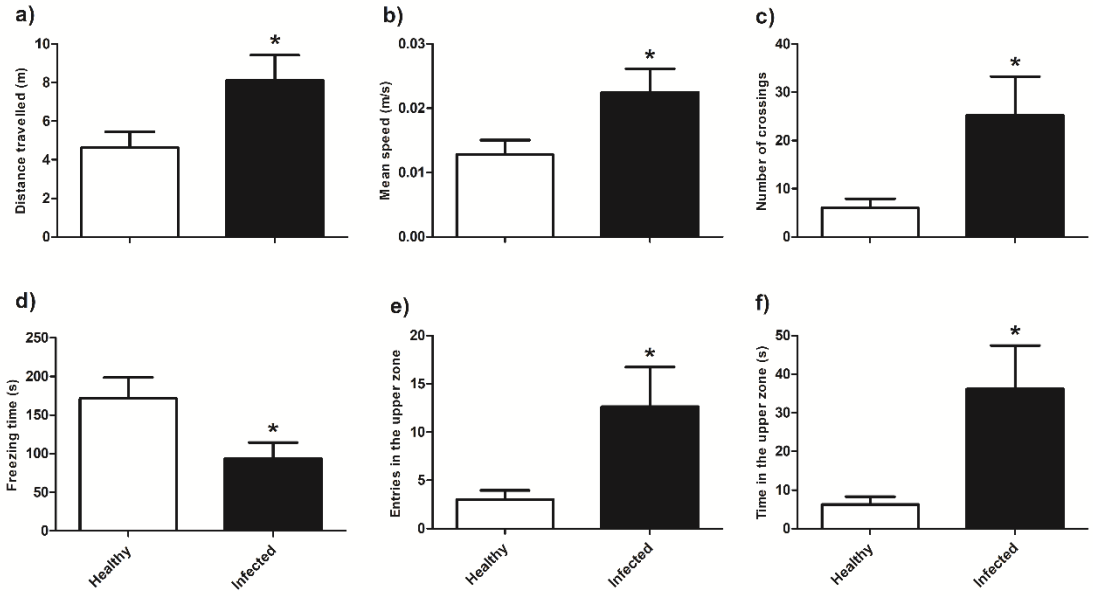

Fig. 1. Novel tank test in silver catfish (*Rhamdia quelen*, n = 12) experimentally infected with *Aeromonas hydrophila* according to locomotor activity: (a) total distance travelled, (b) mean speed, (c) number of crossings between zones, (d) total time in freezing, (e) number of entries in the upper zone and (f) total time in the upper zone. Values are expressed as means ± SEM. An asterisk indicates a significant difference compared to the healthy group ($p < 0.05$).

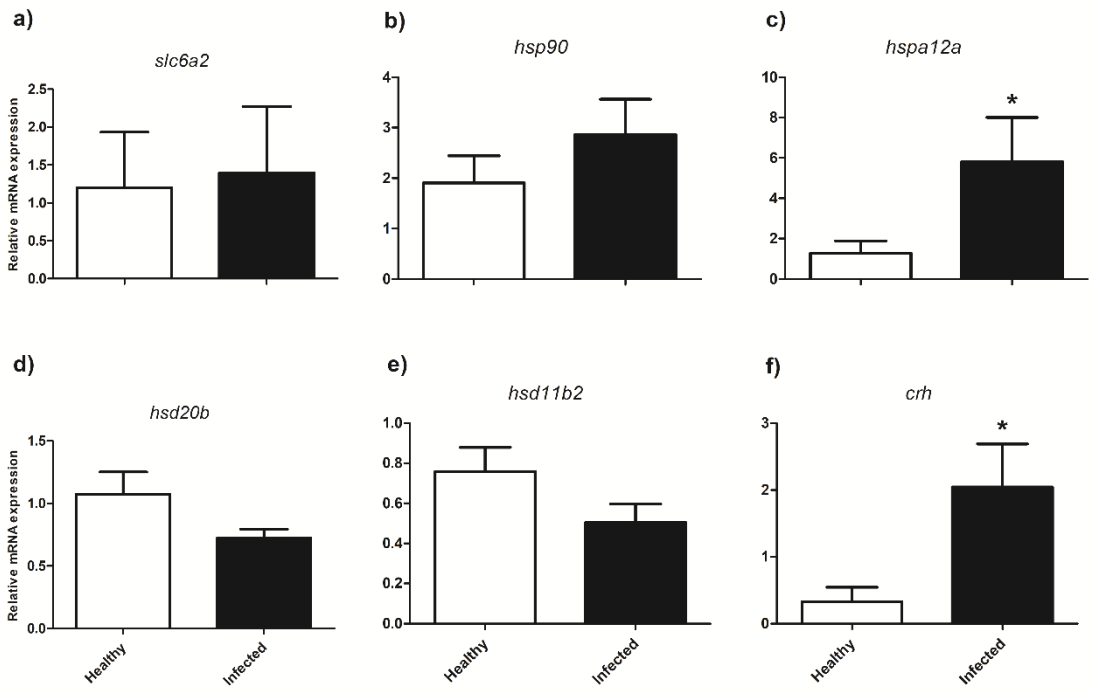

Fig. 2. Expression of (a) *slc6a2* (sodium-dependent norepinephrine transporter), (b) *hsp90* (heat shock protein 90), (c) *hspa12a* (heat shock protein 70, member 12), (d) *hsd20b* (20β-hydroxysteroid dehydrogenase), (e) *hsd11b2* (11β-hydroxysteroid dehydrogenase type 2) and (f) *crh* (corticotropin-releasing hormone) in brain tissues of silver catfish (*Rhamdia quelen*, n = 6) that were healthy or infected with *Aeromonas hydrophila*. Values are expressed as means ± SEM. An asterisk indicates a significant difference compared to the healthy group ($p < 0.05$).

3.3 ARTIGO ORIGINAL 2

O segundo artigo original intitulado “Combined effect of florfenicol with linalool via bath in combating *Aeromonas hydrophila* infection in silver catfish (*Rhamdia quelen*)” foi publicado na revista científica “Aquaculture” (fator de impacto 4.242). Endereço de acesso: https://doi.org/10.1016/j.aquaculture.2021.737247 (BANDEIRA JUNIOR et al., 2021).

Highlights

- Florfenicol administered via bath is effective against fish aeromonosis.
- Bath with florfenicol and linalool decreases TBARS levels in infected fish.
- Bath with florfenicol and linalool prevents histological changes in gills and liver.
- There was still a satisfactory concentration of linalool 1 d after the bath.
- The use of florfenicol with linalool is indicated to combat fish aeromonosis.

Combined effect of florfenicol with linalool via bath in combating *Aeromonas hydrophila* infection in silver catfish (*Rhamdia quelen*)

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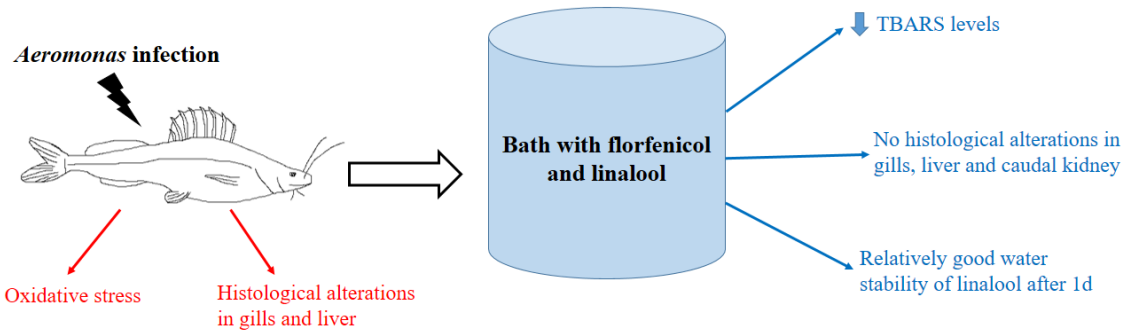
Abstract

The phytochemical linalool has synergistic activity with florfenicol against *Aeromonas hydrophila* in an *in vitro* study. However, there is a lack of *in vivo* studies to confirm this finding. Therefore, the aim of this study was to evaluate the *in vivo* effect of the combination of florfenicol with linalool via bath against aeromonosis in silver catfish (*Rhamdia quelen*) and their responses in redox balance and histology of gills, liver, and caudal kidney. In a previous test, a survival curve of silver catfish infected with *A. hydrophila* and treated with different concentrations of florfenicol via bath was performed. Florfenicol via bath at low concentration (12 mg/L) was able to increase the survival of infected fish. In the main test, the combined effect of florfenicol with linalool was evaluated. Bath with both substances decreased thiobarbituric acid reactive substances - TBARS levels and prevented histological alterations in gills and liver of infected fish. However, the use of these substances via bath was not able to modify the changes observed in other parameters related to the redox balance of infected fish. There was still a satisfactory concentration of linalool 1 d after the bath. In general, the use of florfenicol with linalool would be an appropriate combination for treatment of fish aeromonosis.

Keywords: Aeromonosis; Evaporative loss; Histopathology; Oxidative stress; Survival curve



Graphical abstract

1. Introduction

The increase in the prevalence of bacterial infections in fish farming leads producers to use antimicrobial therapy or prophylaxis indiscriminately (Cabello, 2006). Studies have shown that the development of bacterial resistance to conventional antimicrobials has been particularly motivated by the use of these drugs as growth promoters or as unspecific means of preventing infections in veterinary practices (Economou and Gousia, 2015; Dodds, 2017). Thus, these substances remain in the aquatic environment, exerting selective pressure for long periods of time, resulting in the emergence of multiresistant bacteria (Belém-Costa and Cyrino, 2006; Yang et al., 2018). The indiscriminate use of antimicrobials also creates public health concerns, as it increases the amount of their residues in fish meat products, and many pathogens isolated from the meat of these animals have bacterial resistance to various antimicrobials listed by the World Health Organization (Cabello, 2006; Done et al., 2015). In Brazil, only the antimicrobials florfenicol and oxytetracycline are approved for use in fish for human consumption (SINDAN, 2021). In the United States, the situation is similar, as only the antimicrobials florfenicol, oxytetracycline, sulfamerazine, and sulfadimethoxine/ormetoprim are legalized by the Food and Drug Administration (FDA) for use in the production of fish for human consumption (FDA, 2021).

In view of this scenario, studies have emerged focusing on natural substances with antimicrobial properties, as they are an alternative source in bacteriosis treatment and prevention (Baldissera et al., 2017; Bandeira Junior et al., 2018; da Cunha et al., 2019). Plants have been used in traditional medicine since ancient times, with pharmacological properties being attributed to their secondary metabolites, mainly phenols. There are several benefits of using these compounds, mainly involving antimicrobial, antioxidant, nutritional, and pharmaceutical activities (Lai and Roy, 2004). Citarasu (2010) highlighted herbal medicines as important alternatives to be used in aquaculture as growth promoters, immunostimulants, appetite stimulators, stress preventers, in addition to being very effective alternatives to antibiotics and other synthetic compounds. The same author highlights the antimicrobial activity of compounds isolated from plants against bacteria, fungi, and viruses. Furthermore, this author emphasizes the anti-inflammatory activity of some plant-derived substances in fish.

Several plant essential oils (EOs), such as basil (*Ocimum basilicum*), rosewood (*Aniba rosaeodora*) and "cidreira" herb (*Lippia alba*), have linalool as one of the major

compounds (Sampaio et al., 2012; Avetisyan et al., 2017; Souza et al., 2017). Linalool is a monoterpenoid, so its biosynthetic origin derives from the condensation of two isoprene units (C5), with ten carbons in its chemical structure (Bakkali et al., 2008). This phytochemical has antibacterial properties against *Aeromonas hydrophila*, in addition to sedative and anesthetic properties in silver catfish (*Rhamdia quelen*) (Dorman and Deans, 2000; Heldwein et al., 2014; Silva et al., 2017). The anti-inflammatory action of linalool has already been described in rats (Peana et al., 2002). However, so far there are no reports on this subject in fish.

Silver catfish is one of the most produced native fish in southern Brazil (PEIXE BR, 2020). Bacteria are the main disease-causing agents, and the genus *Aeromonas* is one of the most prevalent in this fish species (Shama et al., 2000).

Aeromonas hydrophila is widely known to cause skin ulcerations and hemorrhagic septicemia in fish, especially silver catfish (Barcellos et al., 2008). Fish aeromonosis generally leads to oxidative damage, increasing the levels of reactive oxygen species (ROS), malondialdehyde (MDA), and protein carbonylation (PC) (Bandeira Junior and Baldisserotto, 2020). Baldissera et al. (2017) reported oxidative damage in the liver of silver catfish after *A. hydrophila* infection, increasing the MDA and PC levels, and decreasing the activity of the enzyme catalase (CAT). In addition, this infection leads to histological alterations, mainly involving the gills, liver, and caudal kidney (Abdelhamed et al., 2017).

Sometimes, antimicrobial therapy alone has not been effective in combating this disease. Therefore, studies evaluating the synergistic activity of conventional antimicrobials with substances isolated from plants are emerging with the purpose of increasing therapeutic success, overcoming resistance mechanisms, and reducing the use of conventional antimicrobials and, consequently, their accumulation in the aquatic environment and in fish meat (de Souza et al., 2017; Deepika et al., 2019; Assane et al., 2021). In this line, it has been reported that the phytochemical linalool has synergistic activity with florfenicol against *A. hydrophila* in an *in vitro* study (Bandeira Junior et al., 2018). However, there is a lack of *in vivo* studies to confirm this finding. Therefore, the aim of this study was to evaluate the *in vivo* effect of the combination of florfenicol with linalool via bath against aeromonosis in experimentally infected silver catfish and their responses in redox balance and histology of gills, liver, and caudal kidney.

2. Materials and methods

2.1. Bacterial strains

Strain *A. hydrophila* ATCC 7966 was used as a standard. The clinical isolate *A. hydrophila* MF 372510 was obtained from a juvenile silver catfish naturally infected and identified by biochemical and molecular tests, as described by Bandeira Junior et al. (2018).

2.2. Chemical substances

Commercial antimicrobial (Maxflor™ 40%) was purchased from a local market. Its formulation is 40 g of florfenicol and vehicle q.s.p. 100 mL. Florfenicol (analytical standard) and linalool (racemic mixture of the enantiomers S-(+)- and R-(-)-linalool, ≥ 97% purity) were purchased from Sigma-Aldrich™.

2.3. In vitro antibacterial activity

The minimum inhibitory concentrations (MICs) of pure florfenicol (analytical standard) and the commercial antimicrobial Maxflor™ 40% were verified against the two strains to determine if they had similar antibacterial activity. The microdilution method was performed in accordance with the guidelines of the Clinical and Laboratory Standards Institute, document VET04-A2 (CLSI, 2014). Florfenicol was diluted in 95% ethanol (according to the manufacturer's recommendation) and incorporated into Mueller-Hinton broth (MHB) (HiMedia Laboratories™) at concentrations of 0.25-520 μg/mL (in triplicate). The inoculum was prepared from cultures grown in Mueller-Hinton agar (MHA) (HiMedia Laboratories™) at 28 °C for 24 h and dissolved in 0.9% sterile saline with turbidity equivalent to the McFarland 0.5 scale (≈1 × 10^8 colony-forming units [CFU] per mL) and an optical density at 600 nm (OD_{600}) of 0.13 ± 0.01. From the inoculum solution, 1 mL was diluted in 9 mL of MHB (≈1 × 10^7 CFU/mL). From this suspension, 10 μL was inoculated into each well for a total volume of 100 μL/well (≈1 × 10^6 CFU/mL). The microplates were incubated at 28 °C for 24 h under aerobic conditions. The same procedure was performed on an ethanol control. Ten microliters of 0.1% resazurin dye (Sigma-Aldrich™) was added to each well to assist in the MIC reading,

which was defined as the lowest concentration of the antimicrobial that inhibited visible bacterial growth.

2.4. Pilot experiment 1

The studies carried out until then highlighted the sedative and anesthetic activities of linalool dissolved in water for bath in silver catfish; however, they did not highlight what would be the minimum concentration necessary to cause sedation, considering that all tested concentrations caused sedation or anesthesia. Concentrations equal to or less than 20 mg/L have not been tested (Heldwein et al., 2014; Silva et al., 2017). In this study, it was not desirable for the fish to enter a state of deep sedation or anesthesia. Therefore, a previous test was performed to determine the concentration of linalool to be used, selecting the highest concentration that did not cause deep sedation or anesthesia in the animals.

2.4.1. Animals

Apparently healthy (no external signs of lesions) juvenile silver catfish (males and females, n = 12, 13 ± 1.5 cm, 10 ± 3 g) were bought from a local fish farm and acclimated for 1 week in 250 L tanks. The fish were fed once a day with an extruded commercial feed that was 4 mm in size (Puro Trato^{TM}, 36% protein). Residues of food and feces from the bottom of tanks were removed daily, and 20% of the water was renewed daily.

2.4.2. Water quality parameters

Water quality parameters were evaluated daily and maintained as follows: temperature, 18.5 ± 0.5 °C; pH, 7.35 ± 0.25; dissolved oxygen, 8.55 ± 0.80 mg/L; and total ammonia concentration, ≤ 0.50 mg/L (corresponding to a nonionized ammonia concentration of ≤ 0.018 mg/L). These parameters remained within the range stipulated as ideal for the species (Gomes et al., 2000).

2.4.3. Experimental design

The animals were divided into four groups of three fish each, as follows: linalool at 5 mg/L, linalool at 10 mg/L, linalool at 15 mg/L, and linalool at 20 mg/L. For the calculation, the density of linalool (0.86 g/mL) was taken into account. Linalool (lipophilic) was solubilized in ethanol (1:10) before being mixed in water. The fish were

exposed to the substance individually in 2 L aquariums. The animals were observed continuously in the first 30 minutes and then observed every 6 h for 24 h. The sedation stages were defined according to Small (2003).

2.5. Pilot experiment 2

Taking into account that the use of linalool combined with florfenicol in the *in vitro* test led to a 75% decrease in the MIC of the conventional antimicrobial compared to its isolated use, we tested 25% of the effective florfenicol concentration to combat aeromonosis in silver catfish in the *in vivo* study (Bandeira Junior et al., 2018). However, to date, there have been no studies testing different concentrations of florfenicol via bath to combat bacteriosis. Therefore, a previous study was carried out by testing different concentrations of this antimicrobial in a bath to establish the ideal florfenicol concentration for the treatment of bacteriosis in silver catfish.

2.5.1. Animals

Apparently healthy (no external signs of lesions) juvenile silver catfish (males and females, n = 162, 12.85 ± 1.35 cm, 10 ± 3 g) were obtained and fed as described in item 2.4.1.

2.5.2. Water quality parameters

The water quality parameters were similar to those reported in item 2.4.2 and remained within the range stipulated as ideal for the species (Gomes et al., 2000).

2.5.3. Experimental design

The fish were divided into boxes of 25 L of water in 6 groups of 9 fish each, in triplicate, as follows: negative control (fish inoculated with 0.9% sterile saline); positive control (untreated fish inoculated with a bacterial suspension of *A. hydrophila* MF 372510); fish inoculated with a bacterial suspension and treated with 12 mg/L florfenicol (low concentration, corresponding to 30 µL/L Maxflor^{TM} 40%); fish inoculated with a bacterial suspension and treated with 36 mg/L florfenicol (medium concentration, corresponding to 90 µL/L Maxflor^{TM} 40%); fish inoculated with a bacterial suspension and treated with 72 mg/L florfenicol (high concentration, corresponding to 180 µL/L Maxflor^{TM} 40%); and fish inoculated with a bacterial suspension and treated with diluent

(ethanol). These florfenicol concentrations were classified as low, medium, and high based on the average concentrations used in the control groups from previous studies (da Cunha et al., 2018, 2019). Groups of fish inoculated with 0.9% sterile saline and treated with different florfenicol concentrations were not tested since it is already known that florfenicol, when used via bath, does not cause mortality in silver catfish (da Cunha et al., 2019). The infected fish received 100 µL of the bacterial suspension (prepared at an OD_{600} of 1.2) intramuscularly in the right dorsolateral region, which corresponds to approximately 9.6×10^8 CFU/mL. Healthy fish received 100 µL of 0.9% sterile saline through the same route, similar to Bandeira Junior et al. (2019). Florfenicol (Maxflor™ 40%) was diluted in ethanol (1:3) since it is lipophilic. The compounds were dissolved in water one day after inoculation. Twenty percent of the water was renewed each day, and fish mortality was evaluated for two weeks.

2.6. Main experiment

After the two pilot tests, we carried out the main test, which is the *in vivo* test of florfenicol with linalool via bath against aeromonosis in experimentally infected silver catfish.

2.6.1. Animals

Apparently healthy (no external signs of lesions) juvenile silver catfish (males and females, n = 192, 7.5 ± 1 cm, 4 ± 1 g) were obtained and fed as described in item 2.4.1.

2.6.2. Water quality parameters

The water quality parameters were similar to those reported in item 2.4.2 and remained within the range stipulated as ideal for the species (Gomes et al., 2000).

2.6.3. Experimental design

The fish were divided into 25 L boxes in groups of 8 fish, in triplicate, as follows: negative control (fish inoculated with 0.9% sterile saline); positive control (untreated fish inoculated with a bacterial suspension); fish inoculated with sterile saline and treated with florfenicol (3 mg/L, 25% of therapeutic concentration, corresponding to 7.5 µL/L of Maxflor™ 40%); fish inoculated with sterile saline and treated with linalool (20 mg/L, highest concentration that did not cause deep sedation); fish inoculated with sterile saline

and treated with the same concentrations of florfenicol and linalool; fish inoculated with bacteria and treated with florfenicol; fish inoculated with bacteria and treated with linalool; and fish inoculated with bacteria and treated with florfenicol and linalool. According to Bandeira Junior et al. (2018), the use of linalool combined with florfenicol in the *in vitro* test led to a 75% decrease in the MIC of florfenicol compared to the required MIC of the isolated antimicrobial. Therefore, we tested 25% of the effective florfenicol concentration to combat aeromonosis in silver catfish in the *in vivo* study (12 mg/L, see results). Thus, we used 3 mg/L of florfenicol in this experiment.

The inoculum was prepared from the strain *A. hydrophila* ATCC 7966 at an OD_{600} of 1.2, corresponding to 9.6 x 10^8 CFU/mL. Fifty microliters of the inoculum or 0.9% sterile saline was intramuscularly inoculated in the right dorsolateral region, similar to Bandeira Junior et al. (2019). This strain was selected because it was used in a previous *in vitro* experiment, where synergistic activity of the combination of the antimicrobial florfenicol with the phytochemical linalool was verified through the "checkerboard" test (Bandeira Junior et al., 2018). We now test whether this effect remains in an *in vivo* trial.

Florfenicol (Maxflor^{TM} 40%) and linalool were diluted in ethanol (1:3 and 1:10, respectively) since they are lipophilic. The compounds were dissolved in water one day after inoculation. Twenty percent of the water was renewed each day, but compounds were not added again. Mortality was assessed daily, and when the first fish of the infected control group died (5 days after infection), euthanasia was performed by spinal cord transection, and the gills, liver, and caudal kidney were collected for histological and oxidative stress analysis. The samples for oxidative stress analysis were kept in a freezer at -80°C until processing. Water samples were collected at 0 h, 12 h and 24 h after linalool administration to verify the loss of phytochemical to the environment, considering that linalool is a volatile compound.

2.6.4. Oxidative stress analysis

The gills, liver, and caudal kidney of all animals were collected and ten samples per group were randomly selected for oxidative stress analysis. Homogenate aliquots for oxidative stress analysis were prepared by homogenizing the tissues in 0.3 M sodium phosphate buffer + 140 mM KCl pH 7.4 at a 1:10 ratio.

As an index of lipid peroxidation, the formation of thiobarbituric acid reactive substances (TBARS) during an acid heating reaction was carried out as described by Ohkawa et al. (1979) with some modifications, as described in detail by Baldissera et al.

(2016). TBARS levels were determined by absorbance at 532 nm and expressed as MDA equivalents (nmol MDA/g of tissue).

The ROS levels were determined by the dichlorodihydrofluorescein (DCF) oxidation method described by LeBel et al. (1992), and the results were expressed as μmol DCF/mg of protein.

The enzymatic activity of superoxide dismutase (SOD) was evaluated spectrophotometrically, according to Misra and Fridovich (1972), described in detail by Baldissera et al. (2016). The enzymatic activity was expressed as U SOD/mg of protein.

The enzymatic activity of glutathione S-transferase (GST) was determined according to Habig et al. (1974). The extinction coefficient used for 2,4-dinitrochlorobenzene (DNCB) was 9.6 mM/cm^{-1}, and the activity was expressed as U GST/mg of protein.

2.6.5. Histological analysis

Three samples per group were randomly selected for histological analysis. Samples of gills, liver, and caudal kidney were fixed in 10% formaldehyde solution and submitted to histological routine. Histological sections (5 μm) were stained with hematoxylin-eosin (H&E). Two histopathologists examined all slides to detect microarchitecture and histopathological alterations using a light microscope.

2.6.6. Gas chromatography with flame ionization detection (GC-FID)

The water samples from the groups that received linalool were quantified by GC-FID at 0 h, 12 h, and 24 h. After 24 h, no quantification was performed, as the water was renewed due to the removal of food and feces that had accumulated at the bottom of tanks. Water samples were also collected from a tank continuously aerated and without fish, in which linalool was added to verify the influence of compound accumulation or metabolism by the fish on the linalool concentration in the water.

Linalool was extracted from the water by adding 1 mL of hexane to 2 mL of water. Subsequently, the sample was stirred and left to stand for spontaneous separation of the supernatant (hexane), which was filtered through a PTFE membrane (0.45 μm). Hexane (500 μL) together with the internal standard (thymol 50 μg/mL) were subjected to chromatographic analysis.

For the standard curve, known linalool concentrations (50-0.1 μg/mL) were added to 2 mL samples of water in triplicate and subjected to the extraction process described

above. The linear regression analysis of the curve provided the following equation: Y= 0.84283*X - 0.012280, with R^2= 0.998, where y is the division between the linalool concentration and the internal standard and x is the division between the peak area of linalool and the internal standard. The accuracy (recovery), coefficient of variation and limits of detection (LD) and quantification (LQ) were 93.54%, 6.65%, 0.019 μg/mL and 0.057 μg/mL, respectively.

After the hexane phase extraction, quantitative analysis of linalool in water was performed by GC-FID, according to Bianchini et al. (2020).

2.7. Statistical analysis

Regarding pilot experiment 2, fish survival was compared using Kaplan-Meier survival analysis with the log-rank test (GraphPad Prism 5.0 Software). Regarding the oxidative stress analysis, the homogeneity of variances between groups was determined using the Levene test. When the variances were homogeneous, two-way ANOVA and Tukey's post hoc test were used, and the results were expressed as the mean ± standard error (Statistica 7.0 Software). When the variances were not homogeneous, the Scheirer-Ray-Hare extension of the Kruskal-Wallis test was used, followed by the Nemenyi test, and the results were expressed as the median ± interquartile range. The minimum significance level was set at $p < 0.05$.

2.8. Ethical note

The experimental protocol involving animals was approved by the Comissão de Ética no Uso de Animais (CEUA) at the Universidade Federal de Santa Maria, Santa Maria, RS, Brazil (Protocol 6723271118/2019) and met the guidelines of Conselho Nacional de Controle de Experimentação Animal (CONCEA, Brazil).

3. Results

3.1. In vitro antibacterial activity

The MIC of both Maxflor^{TM} 40% (considering its florfenicol concentration) and pure florfenicol (analytical standard) was 1.02 µg/mL for the clinical isolate and the standard strain.

3.2. Pilot experiment 1

All tested concentrations of linalool did not cause deep sedation in these animals but only superficial sedation. Therefore, we selected the highest tested concentration (20 mg/L) for the main experiment.

3.3. Pilot experiment 2

The survival curve for fish infected and treated with 40% Maxflor^{TM} is shown in Fig. 1. All fish in the negative control survived, while only two fish in the positive control and three fish in the infected ethanol group remained alive. The survival rates of fish infected and treated with florfenicol were as follows: 40.74% for 12 mg/L, 48.15% for 36 mg/L, and 55.56% for 72 mg/L florfenicol. Through the log-rank test, we found that the three concentrations of florfenicol tested were able to significantly increase fish survival compared to the positive control group and the group infected and treated with only the diluent, and there was no significant difference between them.

Therefore, a florfenicol concentration of 12 mg/L was selected as a parameter for the main experiment.

3.4. Oxidative stress analysis

The infection increased TBARS levels in the gills. The combination of florfenicol with linalool increased TBARS levels in the gills of healthy fish but decreased TBARS levels in the gills of infected fish compared to their respective controls (Fig. 2a). The infection increased TBARS levels in the liver. The use of linalool increased TBARS levels in the livers of healthy and infected fish compared to their respective controls. However, the combined use of florfenicol with linalool led to a decrease in TBARS levels in the liver of infected fish compared to the control group (Fig. 2b). Regarding the caudal kidney, there was no significant difference between the treatments and control group in healthy fish. However, all treatments (drugs alone or combined) led to a decrease in

TBARS levels in the caudal kidney of infected fish compared to the control group (Fig. 2c).

Florfenicol increased ROS levels in the gills of healthy and infected fish compared to their respective control groups. The infection also led to increased ROS levels in the gills of fish from all treatments. Linalool increased ROS levels in the gills of infected fish compared to the control group, but the combined use of florfenicol and linalool showed no difference from control group (Fig. 2d). The isolated use of linalool and florfenicol decreased ROS levels in the liver of healthy fish compared to the control group. In addition, the combined use of the two drugs potentiated this decrease in ROS levels in the liver of healthy fish. However, the liver of the infected fish also had lower ROS levels (Fig. 2e). The combined use of the two drugs decreased ROS levels in the caudal kidney of healthy fish compared to the control group. However, infection increased ROS levels in the caudal kidney of the treated animals (Fig. 2f).

There was no significant difference in SOD activity in the gills between the different treatments for healthy fish and between the different treatments for infected fish. However, the isolated and combined use of the two drugs increased SOD activity in the gills of infected fish compared to the same treatments of healthy animals (Fig. 3a). There was no significant difference in SOD activity in the liver between the different treatments for healthy fish and between the different treatments for infected fish. However, the isolated use of linalool and the combined use of the two drugs decreased SOD activity in the liver of infected fish compared to the same treatments of healthy animals (Fig. 3b). Regarding the caudal kidney, there was no significant difference between the groups in SOD activity (Fig. 3c).

There was no significant difference in GST activity in the gills between the different treatments for healthy fish and between the different treatments for infected fish. However, the isolated use of the two drugs increased GST activity in the gills of infected fish compared to the same treatments of healthy animals (Fig. 3d). The GST activity in the liver of healthy animals treated with the two isolated drugs was lower than that in the healthy control group. There was no significant difference in GST activity in the liver between the different treatments for infected fish. However, the infected control group and the group infected and treated with both substances had lower GST activity in the liver compared to the same treatments of healthy animals (Fig. 3e). Regarding the caudal kidney, there was no significant difference between the groups in GST activity (Fig. 3f).

3.5. Histological analysis

The healthy animals of the control group and of all treatments had normal gills (Fig. 4aceg). The fish in the infected control group had gills with a high degree of epithelial detachment (Fig. 4b). The fish of the group infected and treated with florfenicol had gills with hyperplasia of the filamentous epithelium, migration of inflammatory cells, and lamella fusion (Fig. 4d). The fish of the group infected and treated with linalool had gills with slight hyperplasia of the filamentary epithelium and epithelial detachment (Fig. 4f). Finally, animals infected and treated with both substances (florfenicol and linalool) at the same time had normal gills (Fig. 4h).

The healthy animals of the control group and of all treatments had normal liver and hepatocytes with good energy reserves (Fig. 5aceg). The fish of the infected control group had hepatocytes with little energy reserve, obstruction of vessels due to inflammatory infiltrate and consequent sinusoidal dilation, in addition to loss of tissue architecture (Fig. 5b). The fish of the groups infected and treated with florfenicol or linalool showed liver with a slight loss of tissue architecture (Fig. 5df). Finally, animals infected and treated with both substances (florfenicol and linalool) at the same time had normal tissue architecture and good energy reserve (Fig. 5h).

No relevant histopathological alterations were observed in the caudal kidney of the analyzed fish (Fig. 6).

3.6. Gas chromatography with flame ionization detection (GC-FID)

After 12 h of linalool addition, there was a loss of 11.15% linalool in water without fish and 16.43% in water with fish. After 24 h of linalool addition, there was a loss of 27.07% linalool in water without fish and 35.12% in water with fish. Therefore, the loss of linalool by metabolism or accumulation in the fish body was approximately 5.28% at 12 h and 8.05% at 24 h.

4. Discussion

The MIC result is similar to those previously published for pure florfenicol with the same strains (Bandeira Junior et al., 2018). Therefore, the *in vitro* test validated the use of Maxflor^{TM} 40% (commercial injectable formulation) via bath since it proved that

it has the same effectiveness as the analytical standard florfenicol. Obviously, the concentration of the diluted active ingredient in the commercial solution must be taken into account to make this comparison.

The use of florfenicol via feed has already been shown to be effective against bacteriosis in Atlantic salmon (*Salmo salar*) (Inglis et al., 1991), cod (*Gadus morhua*) (Samuelsen and Bergh, 2004), and channel catfish (*Ictalurus punctatus*) (Gaunt et al., 2004). However, the effectiveness of using this antimicrobial via bath in fish with bacteriosis remains poorly studied, despite its use as a control group at a single concentration in some studies (da Cunha et al., 2018, 2019). Nevertheless, these studies did not cite a reference for the concentration used. This was the first study that tested different concentrations of florfenicol via bath in fish to determine the most appropriate concentration. We can conclude that florfenicol is a good pharmacological option to be used via bath for the treatment of aeromonosis in silver catfish and is recommended even at low concentrations (12 mg/L). Thus, this study may serve as a reference for the recommended concentration of this drug in future studies.

According to the FDA, the use of florfenicol is approved only via premix feeding (FDA, 2021). Administration via feed has the advantage of being more practical. However, debilitated fish may not eat or may eat smaller amounts of feed, which means that they do not come into contact with the active ingredient at therapeutic concentrations. Bath administration has the advantage of ensuring that all animals come into contact with the same drug concentration, including animals with anorexia. Therefore, this study opens the possibility of a new route of administration of this substance.

Fish bacterial diseases can induce oxidative stress, provoked by excessive generation or impaired removal of free radicals, leading to tissue damage, loss of biological function and homeostatic imbalance (Baldissera et al., 2018). To neutralize excess free radicals, antioxidant defense mechanisms can be modulated by fish by increasing the activity of specific enzymes (Martínez-Álvarez et al., 2005).

Lipid peroxidation is related to pathological disorders linked to oxygen toxicity. Thiobarbituric acid levels are used to measure the lipid peroxidation of a specific tissue through the measurement of a secondary product called MDA (Ohkawa et al., 1979). According to Bandeira Junior and Baldisserotto (2020), most of the studies on fish aeromonosis pointed to an increase in MDA levels in organs of the infected animals, indicating possible oxidative damage. In general, in our study, the infection increased TBARS levels in the organs tested, and the combined use of florfenicol with linalool via

bath reduced these levels, showing good therapeutic efficacy in silver catfish. Similar to our study, Duarte et al. (2016) reported that linalool has an exceptional ability to inhibit lipid peroxidation. In addition, Souza et al. (2018) reported that anesthesia of silver catfish with linalool chemotype of *Lippia alba* EO show antioxidant capacity and does not cause damage to lipids or proteins.

A balance between ROS production and the system to protect cells from ROS exists in healthy aerobic organisms. However, ROS accumulation in tissues can be provoked by bacterial infections and can compromise cell structure and function (Baldissera et al., 2018). According to Bandeira Junior and Baldisserotto (2020), most of the studies on fish aeromonosis pointed to an increase in ROS levels in organs of the infected animals, indicating possible oxidative damage. Similarly, in our study, infection increased ROS levels in the gills and caudal kidney of silver catfish, and our treatment was not able to prevent this increase.

Antioxidant enzymes SODs can prevent oxidative stress by catalyzing the dismutation reaction of superoxide anion ($O2^{\bullet-}$) to oxygen and hydrogen peroxide (H_2O_2) in living organisms. SODs are classified into manganese SOD (MnSOD) and copper/zinc SOD (Cu/ZnSOD) based on the metal cofactor in their active sites (Miller, 2012). Bandeira Junior and Baldisserotto (2020) indicated that most of the studies on fish aeromonosis pointed to an increase in SOD activity/expression in organs of the infected animals, and a few indicated decreased or unchanged enzyme activity/expression. Interestingly, in our study, infection increased SOD activity in the gills but decreased SOD activity in the liver of silver catfish treated with the drugs, indicating that the enzymatic response is different in each organ.

The GST family of enzymes utilizes reduced glutathione (GSH) as a cofactor in the phase II metabolism of various xenobiotics, resulting in the formation of GSH–xenobiotic conjugates (Backos et al., 2012). Bandeira Junior and Baldisserotto (2020) indicated that most of the studies on fish aeromonosis pointed to a decrease in GST activity/expression in organs of the infected animals, and a few indicated increased or unchanged enzyme activity/expression. Similarly, in our study, infection decreased GST activity in the liver of silver catfish, and our treatment was not able to avoid this decrease.

We observed a high degree of epithelial detachment in the gills of silver catfish with aeromonosis. However, fish infected and treated with the two substances via bath showed normal gills. Marinho-Neto et al. (2019) also reported detachment of epithelial cells from the lamella base, in addition to congestion of large vases and secondary

lamellae in the gills of pacu (*Piaractus mesopotamicus*) infected with *A. hydrophila*. Saharia et al. (2018) reported clubbing and fusion of filaments and dilatation of the central venous sinus, excessive mucous secretion, fusion of secondary lamella, hypertrophy of epithelial cells, and unilateral lamella hyperplasia in the gills of rohu (*Labeo rohita*) with aeromonosis. Furthermore, Abdelhamed et al. (2017) reported gills with thickening and clubbing of the primary and secondary lamellae with moderate numbers of lymphocytic infiltration in channel catfish infected with *A. hydrophila*.

In our study, the group infected and treated with florfenicol showed great inflammatory cell migration in the branchial lamellae, and the group infected and treated with linalool did not show this alteration. One hypothesis for this finding is that linalool has bactericidal action, while florfenicol has bacteriostatic action. A bacteriostatic drug does not kill the bacteria but simply prevents it from multiplying so that the host's immune system needs to kill it. Thus, the immune system of the fish treated with florfenicol was trying to combat the infection, which generated the migration of large numbers of inflammatory cells to the site. This hypothesis is corroborated by our previous study that tested the MIC and minimum bactericidal concentration (MBC) of linalool and florfenicol against the same bacterial strain used in the present study. In this previous study, we observed that the MIC and MBC values for linalool were the same, suggesting bactericidal action, while the MBC value was much higher than the MIC value for florfenicol, suggesting bacteriostatic action (Bandeira Junior et al., 2018).

On the other hand, there is an alternative hypothesis that does not involve the bactericidal effect of linalool. The anti-inflammatory activity of this phytochemical in rats and also its ability to inhibit the virulence factors of *A. hydrophila* have been reported in previous studies (Peana et al., 2002; Bandeira Junior et al., 2018). Therefore, it is likely that exposure of fish to linalool has reduced the inflammatory process in the gills caused by the infection.

The silver catfish with aeromonosis showed loss of energy reserve of hepatocytes, sinusoid obstruction due to inflammatory infiltrate and consequent sinusoidal dilation, in addition to loss of tissue architecture of the liver. However, fish infected and treated with the two substances via bath showed normal liver. According to Barcellos et al. (2010), the liver represents a primary energy source in periods of food restriction. Fish with aeromonosis have anorexia. Therefore, we suggest that the loss of energy reserve in the liver of infected animals observed in this study is due to the fact that infected fish eat lower amounts of feed, needing to use their primary source of energy reserve. Similar to

our study, Marinho-Neto et al. (2019) reported congestion of hepatic sinusoids, leukocyte infiltrates, hemorrhagic areas, necrotic areas, and disorganization of hepatic tissue architecture in the perivascular region of the liver of pacu with aeromonosis. Islam et al. (2008) reported hepatic abscess, focal necrosis, hemorrhagic areas, vacuolation, and massive atrophy in the liver of shing (*Heteropneustes fossilis*) infected with *A. hydrophila*. Gupta et al. (2008) reported moderately degenerate hepatocytes, edema and leucocytic infiltration in the liver of rohu infected with *A. hydrophila*. In addition, Abdelhamed et al. (2017) reported a liver with a focal area of lymphocytes migrating from dilated blood vessels to necrotic hepatic cells in channel catfish with aeromonosis.

Marinho-Neto et al. (2019) reported abundant melanomacrophage accumulation between renal tubules, necrosis with karyolysis of tubular cell nuclei, interstitial hemorrhage in renal tissue, and loss of cytoplasmic delimitation between renal tubule epithelial cells in the caudal kidney of Pacu infected with *A. hydrophila*. Abdelhamed et al. (2017) reported caudal kidney with hyaline droplet accumulation in tubular epithelium, diffuse necrosis of renal tubule, separation of renal tubular epithelium from its basement membrane with karyolysis of its nucleus, and severe interstitial nephritis in channel catfish with aeromonosis. Saharia et al. (2018) reported vacuolation and degeneration of the tubular epithelial cells and glomerular atrophy in the caudal kidney of rohu infected with *A. hydrophila*. Furthermore, Barcellos et al. (2008) reported interstitial mononuclear infiltrate and basophilic foci compatible with bacterial colonies in the interstitium of the caudal kidney of silver catfish infected with *A. hydrophila*. Interestingly, in this study, we did not find any relevant histopathological alterations in the renal tubules or hematopoietic tissue of the caudal kidney in infected fish.

Regarding the GC-FID analysis, the loss by evaporation to the environment was greater than the loss by metabolism or accumulation in the animals' bodies. This was expected, as the animals used in the experiment were small and the density was low (1.28 kg/m^3). However, we believe that this loss by evaporation was not significant because it is a monoterpenoid (volatile compound) and that there was still a satisfactory concentration of this phytochemical after 24 h of linalool addition in the water. Soltanbeigi (2020) has already reported that the presence of water and atmospheric gases leads to the processes of evaporation and conversion of this phytochemical. The positive point in comparison with conventional antimicrobials is that linalool does not pose a risk of environmental bioaccumulation, as it undergoes rapid biodegradation in aquatic environments, soil and the atmosphere (OECD, 2021).

In this study, the combined use of florfenicol with linalool had a beneficial effect on TBARS levels and histopathological analyses. However, the use of these substances via bath was not able to modify the changes observed in other parameters related to the redox balance of infected fish. An *in vivo* beneficial effect of the combination of linalool with florfenicol is desirable, as it would be possible to develop protocols for bacteriosis treatments using lower amounts of the conventional antimicrobial, decreasing its levels in the aquatic environment and, consequently, in fish meat and slowing down the process of bacterial resistance.

5. Conclusion

In general, the use of florfenicol with linalool would be an appropriate combination for treatment of fish aeromonosis, as it showed promising results in both *in vitro* and *in vivo* tests. Further studies on the treatment of fish bacteriosis via bath are necessary to establish the most suitable protocol. Finally, combinations of conventional drugs with natural substances are extremely promising, as they have good efficacy and are eco-friendly.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, finance code 001) of Brazil.

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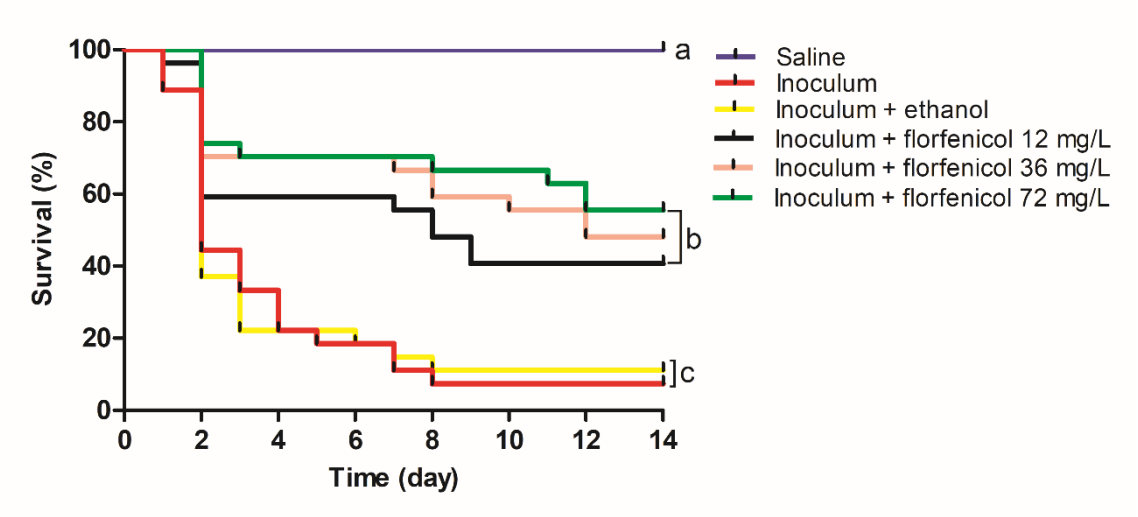

Fig. 1. Survival of silver catfish infected with *A. hydrophila* and treated with different concentrations of florfenicol (Maxflor^{TM} 40%) via bath. Kaplan-Meier survival analysis with the log-rank test. Different letters indicate a significant difference between groups ($p < 0.05$).

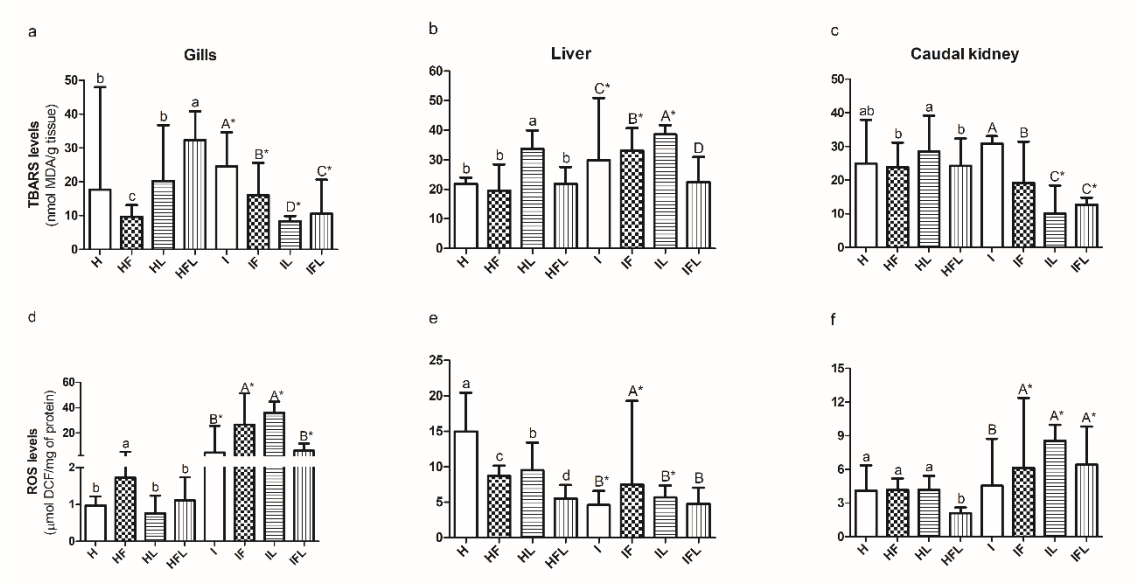

Fig. 2. Levels of thiobarbituric acid reactive substances (TBARS) in gills (a), liver (b), and caudal kidney (c) and reactive oxygen species (ROS) in gills (d), liver (e), and caudal kidney (f) of silver catfish experimentally infected with *A. hydrophila* and treated with different substances by bath. (H) Healthy fish. (HF) Healthy fish treated with florfenicol. (HL) Healthy fish treated with linalool. (HFL) Healthy fish treated with florfenicol and linalool. (I) Infected fish. (IF) Infected fish treated with florfenicol. (IL) Infected fish treated with linalool. (IFL) Infected fish treated with florfenicol and linalool. Different lowercase letters indicate significant differences between treatments in healthy fish. Different capital letters indicate significant differences between treatments in infected fish. Asterisks indicate significant differences between healthy and infected fish within the same treatment. Data expressed as median ± interquartile range ($p < 0.05$).

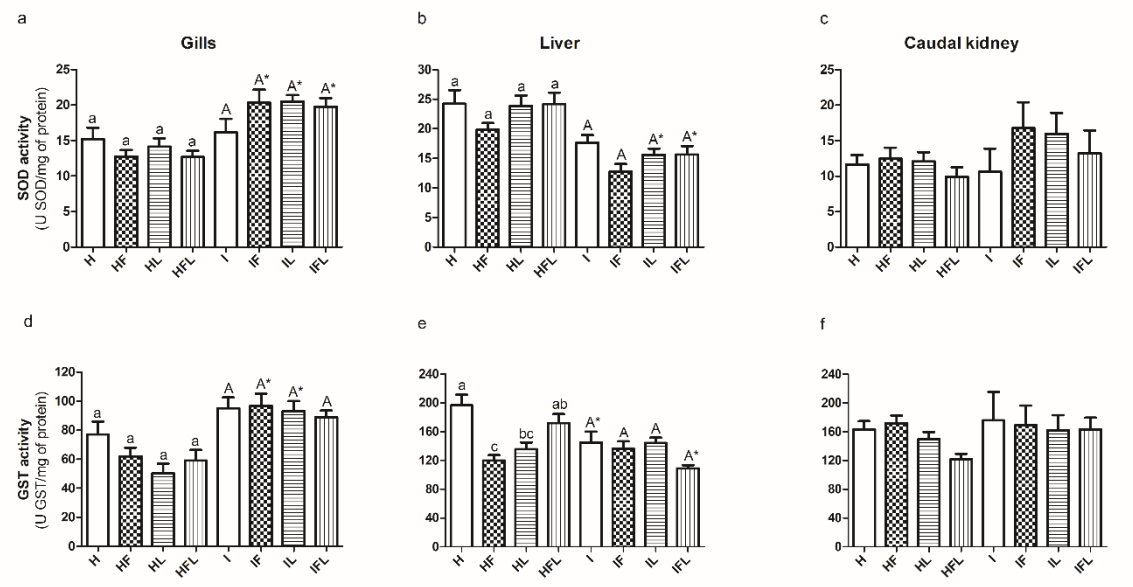

Fig. 3. Activities of superoxide dismutase (SOD) in gills (a), liver (b), and caudal kidney (c), and glutathione S-transferase (GST) in gills (d), liver (e), and caudal kidney (f) of silver catfish experimentally infected with *A. hydrophila* and treated with different substances by bath. (H) Healthy fish. (HF) Healthy fish treated with florfenicol. (HL) Healthy fish treated with linalool. (HFL) Healthy fish treated with florfenicol and linalool. (I) Infected fish. (IF) Infected fish treated with florfenicol. (IL) Infected fish treated with linalool. (IFL) Infected fish treated with florfenicol and linalool. Different lowercase letters indicate significant differences between treatments in healthy fish. Different capital letters indicate significant differences between treatments in infected fish. Asterisks indicate significant differences between healthy and infected fish within the same treatment. Data expressed as mean ± standard error ($p < 0.05$).

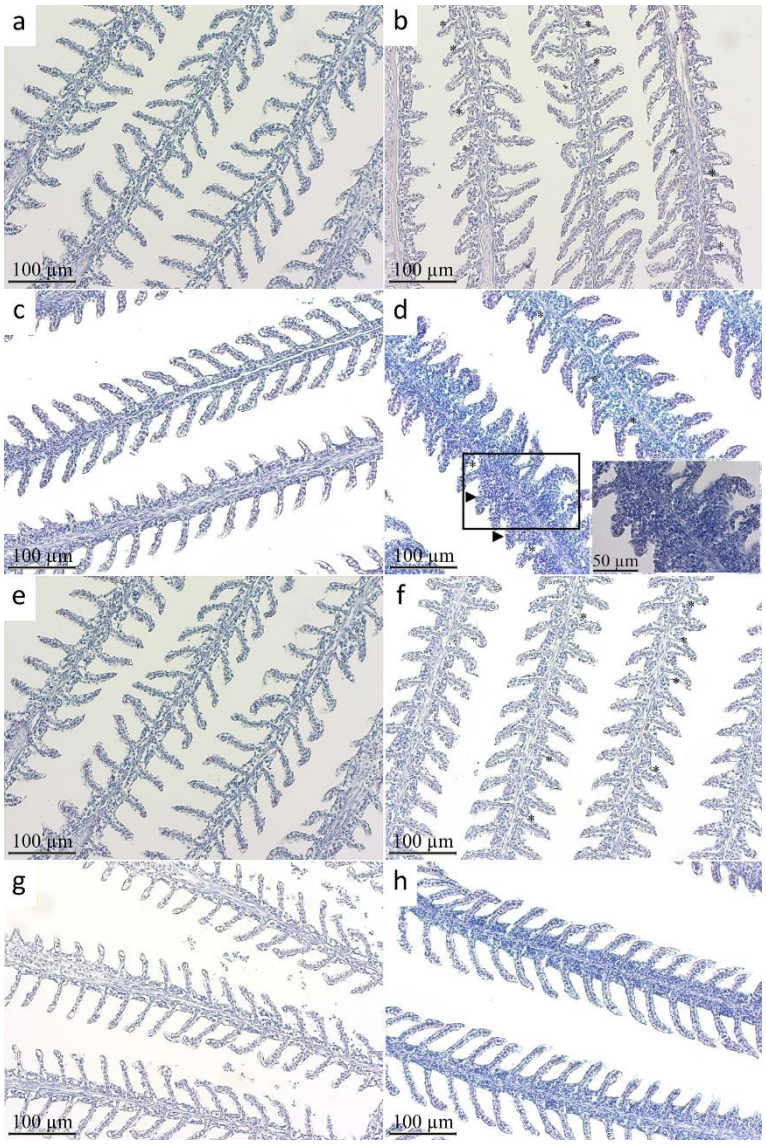

Fig. 4. Gill histopathology of silver catfish experimentally infected with *A. hydrophila* and treated with different substances by bath. (a) Healthy fish. (b) Infected fish. (c) Healthy fish treated with florfenicol. (d) Infected fish treated with florfenicol. Higher magnification shows cell proliferation and inflammatory cell migration. (e) Healthy fish treated with linalool. (f) Infected fish treated with linalool. (g) Healthy fish treated with florfenicol and linalool. (h) Infected fish treated with florfenicol and linalool. Arrowhead - lamellar fusion; asterisk - epithelium detachment. Section of 5 µm, stained with H&E.

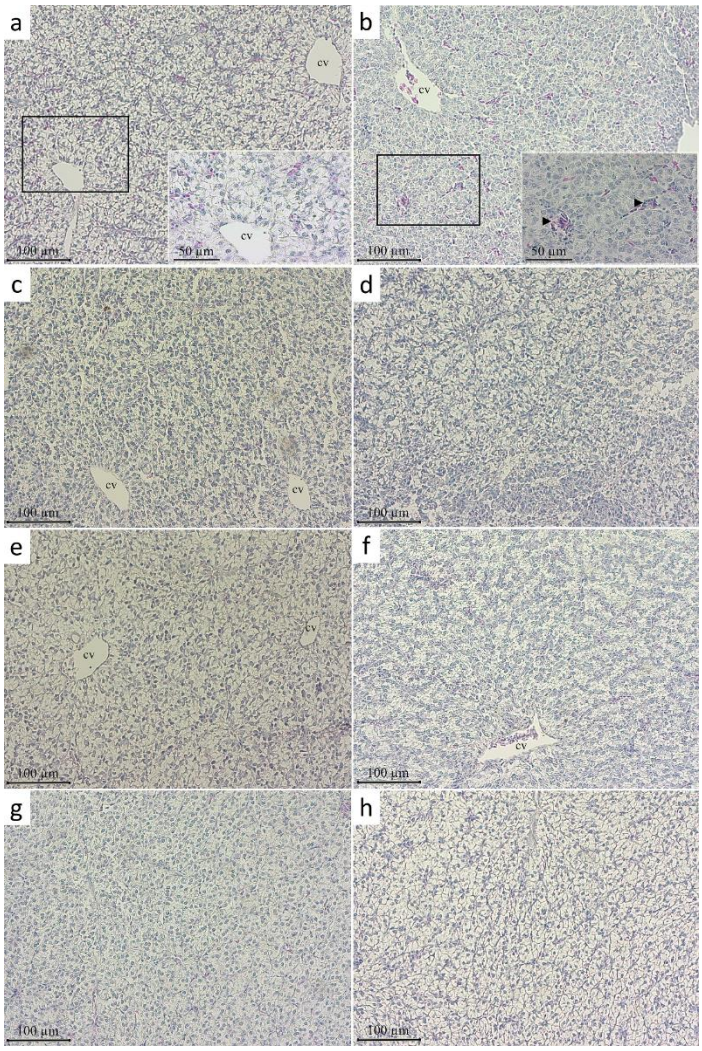

Fig. 5. Liver histopathology of silver catfish experimentally infected with *A. hydrophila* and treated with different substances by bath. (a) Healthy fish. Higher magnification showing normal hepatic sinusoids and hepatocytes with good energy reserve. (b) Infected fish. Higher magnification showing sinusoid obstruction and hepatocytes with little energy reserve. (c) Healthy fish treated with florfenicol. (d) Infected fish treated with florfenicol. (e) Healthy fish treated with linalool. (f) Infected fish treated with linalool. (g) Healthy fish treated with florfenicol and linalool. (h) Infected fish treated with florfenicol and linalool. Arrowhead - sinusoid obstruction; cv - centrilobular vein. Section of 5 µm, stained with H&E.

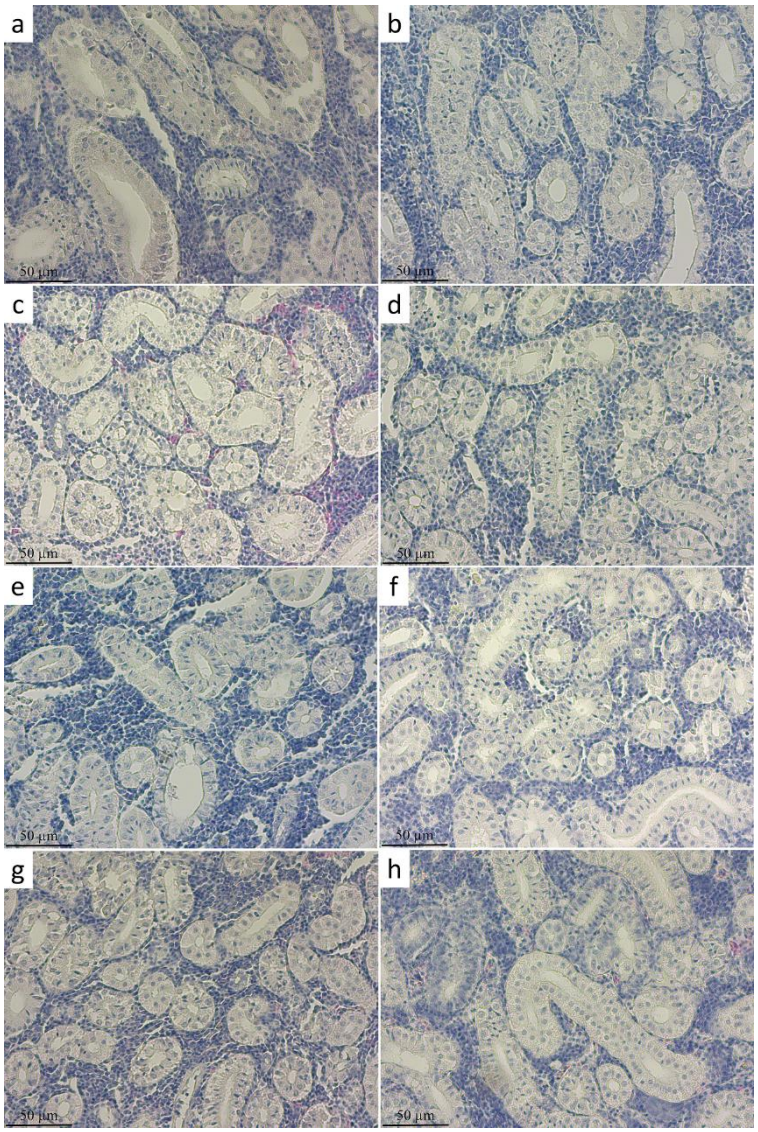

Fig. 6. Caudal kidney histopathology of silver catfish experimentally infected with *A. hydrophila* and treated with different substances by bath. (a) Healthy fish. (b) Infected fish. (c) Healthy fish treated with florfenicol. (d) Infected fish treated with florfenicol. (e) Healthy fish treated with linalool. (f) Infected fish treated with linalool. (g) Healthy fish treated with florfenicol and linalool. (h) Infected fish treated with florfenicol and linalool. Section of 5 µm, stained with H&E.

4 DISCUSSÃO GERAL

Essa tese foi dividida em três partes: um artigo de revisão e dois artigos originais. O artigo de revisão teve como foco condensar o conhecimento atual à respeito do efeito da aeromonose no equilíbrio redox do organismo dos peixes, dando embasamento teórico para os trabalhos posteriores. O primeiro artigo original focou em avaliar os efeitos estressores, tanto a nível comportamental quanto a nível molecular, da infecção por *A. hydrophila* em jundiás, explicando melhor a patogenia dessa doença. Por fim, o segundo artigo original teve como foco estabelecer um protocolo de tratamento via banho dessa bacteriose utilizando a combinação do fitoquímico linalol com o antimicrobiano florfenicol, apresentando uma forma de mitigar os eventos estressores causados pela infecção.

Em relação ao artigo de revisão, os resultados encontrados sobre as atividades/expressões das enzimas antioxidantes após a infecção são inconsistentes, pois enquanto muitos trabalhos apontaram para um aumento, outros muitos apontaram para uma diminuição ou inalteração desses biomarcadores. Por outro lado, a maioria dos trabalhos analisados apontaram para um aumento nos níveis de EROs, MDA e PC, indicando um possível dano oxidativo causado pela infecção. Portanto, parece que as análises não enzimáticas de equilíbrio redox fornecem resultados mais consistentes quando se trata de infecções bacterianas em peixes se comparadas às análises enzimáticas.

À respeito do primeiro artigo original, percebemos que peixes infectados com *A. hydrophila* apresentaram um aumento na expressão cerebral de genes relacionados à proteínas de choque térmico (*hspa12a*) e à rota de síntese de cortisol (*crh*) no quinto dia após a infecção, quando comparados com animais não infectados, indicando um possível efeito estressor dessa doença. Além disso, os peixes infectados nadaram mais em um aquário desconhecido quando comparados aos animais sadios. Isso é interessante, pois demonstra que a infecção atenuou o medo dos animais pelo desconhecido, deixando-os alheios ao ambiente. Um padrão similar foi observado em jundiás infectados com *P. aeruginosa*, os quais apresentaram hiperlocomoção e alterações cerebrais nos sexto e sétimo dia após a infecção (BALDISSERA et al., 2017b). Esse microrganismo também já foi descrito como capaz de causar disrupção da barreira hematoencefálica em jundiás, o que pode contribuir para a patogenia da doença no SNC (BALDISSERA et al., 2018).

Em relação ao segundo artigo original, descrevemos que o banho com florfenicol e linalol impediu o aumento dos níveis de TBARS e as alterações histológicas nas

brânquias e no fígado dos peixes infectados. No entanto, o tratamento não foi capaz de impedir as alterações que a infecção causou nos marcadores enzimáticos de estresse oxidativo. De maneira semelhante ao nosso estudo, outros trabalhos também relataram aumento nos níveis de TBARS (BALDISSERA et al., 2017a; NANDI et al., 2018), além de alterações histológicas nas brânquias (ABDELHAMED et al., 2017; SAHARIA et al., 2018) e no fígado (GUPTA et al., 2008; MARINHO-NETO et al., 2019) de peixes infectados com *A. hydrophila*. Ainda em nosso estudo, podemos concluir que permanecia uma concentração satisfatória de linalol na água mesmo um dia após o banho, apesar da considerável perda por evaporação devido à volatilidade do composto. O uso de nanoemulsão ou de nanoencapsulação são alternativas para proteger OEs e fitoquímicos da volatilização (FLORES et al., 2011). Desta forma, é interessante sugerir estudos futuros visando testar a perda evaporativa de nanoestruturas de linalol.

Ao compararmos os resultados do artigo de revisão com os resultados do segundo artigo original, percebemos que o aumento encontrado nos níveis de TBARS após a infecção no nosso experimento já foi relatado por vários estudos sobre aeromonose em outras espécies de peixes. Nesta mesma comparação, também corroboramos a inconsistência dos resultados relacionados às atividades de enzimas antioxidantes após a infecção, visto que elas não seguem um padrão bem definido. Porém, o que nos chama a atenção nesta comparação é a discrepância em relação aos níveis de EROs, pois o artigo de revisão apontou para uma tendência de aumento dos níveis após a infecção, o que não ocorreu em nosso experimento. Obviamente, os resultados são influenciados pelas diferentes metodologias, espécies de peixes e de bactérias, órgãos analisados e tempos de coleta entre os variados estudos.

Por fim, acredito que esta tese poderá contribuir para o desenvolvimento de uma aquicultura mais ambientalmente sustentável, reforçando a possibilidade do uso de combinações de fármacos antimicrobianos convencionais com substâncias naturais, o que proporciona uma diminuição nas concentrações utilizadas dos primeiros.

5 CONCLUSÕES

Níveis aumentados de EROs, MDA e PC indicam que a infecção por *Aeromonas* geralmente causa estresse oxidativo. Portanto, esses três biomarcadores são excelentes indicadores de estresse oxidativo durante a infecção. Em adição, o aumento na atividade de *burst* respiratório e nos níveis de óxido nítrico indicam que o sistema imune inato dos peixes é geralmente mais ativo durante a infecção.

A infecção por *A. hydrophila* em jundiá causa hiperlocomoção relacionada ao estresse e alterações no eixo HPI, aumentando a expressão cerebral de *hspa12a* e *crh*, os quais são relacionados às proteínas de choque térmico e à rota de síntese de cortisol, respectivamente.

O uso combinado de florfenicol com linalol via banho apresentou um efeito benéfico sobre os níveis de TBARS e análises histopatológicas em brânquias e fígado de jundiás experimentalmente infectados com *A. hydrophila*. No entanto, o uso dessas substâncias não foi capaz de modificar as alterações observadas em outros parâmetros relacionados ao equilíbrio redox de peixes infectados. Ainda permaneceu uma concentração satisfatória de linalol um dia após o banho. Em geral, o uso de florfenicol com linalol é indicado para combater a aeromonose em peixes. Um efeito benéfico *in vivo* da combinação de florfenicol com linalol é desejável, pois seria possível desenvolver protocolos de tratamento de bacterioses utilizando menores quantidades de antimicrobiano convencional, diminuindo seus níveis no ambiente aquático e, consequentemente, na carne dos peixes e retardando o processo de resistência bacteriana.

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