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Elisia Gomes da Silva

HISTOLOGIA BRANQUIAL DE *Rhamdia quelen* SAUDÁVEIS E INFECTADOS COM *Aeromonas hydrophila* EXPOSTOS A DIFERENTES ISOFORMAS DE LIMONENO

Santa Maria, RS
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Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Farmacologia, Área de concentração Farmacologia Aplicada à Produção Animal, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de **Mestre em Farmacologia.**

Orientador: Prof. Dr. Bernardo Baldisserotto

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2019

DEDICATÓRIA

À minha família, mineira,
por acreditar que a educação
é a herança mais valiosa que se pode herdar.

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Fica sempre um pouco de perfume nas mãos que oferecem rosas,
nas mãos que sabem ser generosas...

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Profª Juliana Felipetto Cargnelutti

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Rejane Roratto Foletto

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Muito obrigada!

RESUMO

HISTOLOGIA BRANQUIAL DE *Rhamdia quelen* SAUDÁVEIS E INFECTADOS COM *Aeromonas hydrophila* EXPOSTOS A DIFERENTES ISOFORMAS DE LIMONENO

AUTORA: Elisia Gomes da Silva
ORIENTADOR: Prof. Dr. Bernardo Baldisserotto

O crescimento da piscicultura brasileira e o consequente aumento de doenças causadas por bactérias, como a *Aeromonas hydrophila*, tem provocado o uso abusivo de fármacos sintéticos e produtos químicos. Além do uso indiscriminado dos antimicrobianos desencadear resistência bacteriana, seu emprego favorece a deposição de resíduos nos tecidos do animal, provoca imunossupressão e contamina o ambiente aquático. Com base nisso, a aquicultura tem demonstrado um maior interesse no uso de óleos essenciais de plantas como fonte de medicamentos profiláticos e terapêuticos. A maioria dos óleos essenciais contêm compostos que possuem propriedades antimicrobianas, antiparasitárias, antifúngicas e anti-inflamatórias. O limoneno é um monoterpeno presente em óleos essenciais de plantas cítricas, disponível na natureza nas formas enantioméricas R-(+)-limoneno e S-(-)-limoneno. Diante da importância que o combate a patógenos tem na aquicultura, o objetivo deste trabalho foi avaliar os efeitos das isoformas R-(+)-limoneno e S-(-)-limoneno sobre os parâmetros histológicos de brânquias de *Rhamdia quelen* saudáveis e infectados por *Aeromonas hydrophila*. No primeiro experimento, peixes saudáveis foram divididos em sete grupos: controle, 10 mg/L de gentamicina, 90 μL/L de etanol, 10 μL/L R-(+)-limoneno, 20 μL/L R-(+)-limoneno, 10 μL/L S-(-)-limoneno, 20 μL/L S-(-)-limoneno, e expostos uma vez aos tratamentos. No segundo experimento os peixes foram divididos em dez grupos: controle, 10 mg/L de gentamicina, 90 μL/L de etanol, 10 μL/L R-(+)-limoneno, 20 μL/L R-(+)-limoneno, expostos uma vez aos tratamentos e, inoculados ou não, com *A. hydrophila.* A exposição ao S-(-)-limoneno provocou a morte de alguns peixes e induziu sinais de inflamação no tecido branquial, como edema lamelar, fusão lamelar, infiltração de células inflamatórias no tecido conjuntivo, proliferação de células mucosas e ionócitos. Por outro lado, nas brânquias expostas à isoforma R-(+)-limoneno não foram observadas alterações nesses parâmetros. As brânquias dos peixes infectados com *A. hydrophila* também apresentaram sinais de inflamação, aumento na espessura do epitélio filamentoso, lamelar e tecido conjuntivo. As lamelas sofreram redução no comprimento e espaço interlamelar. A formação de aneurisma, aumento do número de células mucosas e de ionócitos e do tamanho das células mucosas foram observados. No entanto, nos peixes infectados e tratados com R-(+)-limoneno as alterações foram menos intensas. Em conclusão, o S-(-)-limoneno demonstra ser irritante para o jundiá, provocando mortes e induzindo inflamações e alterações histopatológicas. Por outro lado, o R-(+)-limoneno protege as brânquias da inflamação posicionando-se como o mais adequado para peixes saudáveis e infectados com *A. hydrophila*, preferencialmente na concentração de 10 μL/L R-(+)-limoneno.

Palavras-chave: Peixes. Bactérias. Plantas cítricas. Monoterpeno. Histopatologia.

ABSTRACT

GILL HISTOLOGY OF *Rhamdia quelen* HEALTHY AND INFECTED WITH *Aeromonas hydrophila* AND EXPOSED DIFFERENT LIMONENE ISOFORMS

AUTHOR: Elisia Gomes da Silva
ADVISOR: Prof. Dr. Bernardo Baldisserotto

The growth of Brazilian fish culture and the consequent increase of diseases caused by bacteria, such as *Aeromonas hydrophila*, has caused the abuse of synthetic drugs and chemicals. In addition to the indiscriminate use of antimicrobials to trigger bacterial resistance, their use favors the deposition of residues in animal tissues, causes immunosuppression and contaminates the aquatic environment. Based on this, aquaculture has shown a greater interest in the use of plant essential oils as a source of prophylactic and therapeutic medicine. Most essential oils contain compounds that have antimicrobial, antiparasitic, antifungal and anti-inflammatory properties. Limonene is a monoterpene present in essential oils of citrus plants, available in nature in the enantiomeric forms R-(+)-limonene and S-(-)-limonene. Given the importance of pathogen control in aquaculture, the objective of this study was to evaluate the effects of the R-(+)-limonene and S-(-)-limonene isoforms on the histological parameters of the gills of healthy and *Aeromonas hydrophila* infected *Rhamdia quelen*. In the first experiment, healthy fish were divided into seven groups: control, 10 mg/L gentamicin, 90 μL/L ethanol, 10 μL/L R-(+)-limonene, 20 μL/L R-(+)-limonene, 10 μL/L S-(-)-limonene, 20 μL/L S-(-)-limonene and exposed once to treatments. In the second experiment the fish were divided into ten groups: control, 10 mg/L gentamicin, 90 μL/L ethanol, 10 μL/L R-(+)-limonene, 20 μL/L R-(+)-limonene, exposed once to treatments and inoculated or not with *A. hydrophila*. Exposure to S-(-)-limonene caused the death of some fish and also induced signs of gill tissue inflammation, such as lamellar edema, lamellar fusion, infiltration of inflammatory cells in connective tissue, proliferation of mucous cells and ionocytes. On the other hand, in the gills exposed to the R-(+)-limonene isoform no changes were observed in these parameters. The gills of fish infected with *A. hydrophila* also showed signs of inflammation, in addition to increased thickness of filamentous epithelium, lamellar and connective tissue. The lamellae were reduced in length and interlamellar space. Aneurysm formation, increased number of mucous cells and ionocytes and in the size of mucous cells were observed. However, in fish infected and treated with R-(+)-limonene the changes were less intense. In conclusion, S-(-)-limonene was shown to be irritating to silver catfish, causing deaths and inducing inflammation and histopathological changes. On the other hand, R-(+)-limonene protected against gill inflammation, being the most suitable for fish infected with *A. hydrophila*, preferably at 10 μL/L R-(+)-limonene concentration.

keywords: Fish. Bacteria. Citrus plants. Terpene. Histopathology.

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1. INTRODUÇÃO

A evolução da piscicultura brasileira em 2018 resultou na produção de 722.560 toneladas de peixes, resultado 4,5% superior ao ano de 2017. De acordo com o Departamento de Economia Rural (Deral) da Secretaria de Agricultura e Abastecimento, o estado do Paraná, segundo maior produtor de tilápia do país, projeta crescimento de 20% na atividade neste ano, com a expectativa de chegar a 170.000 toneladas de carne de peixe (SNA, 2019). Estima-se que a produção de peixes na América Latina, incluindo o Brasil, e no Caribe alcance cerca de 3,7 milhões de toneladas em 2025 (PEIXES BR, 2018; FAO, 2016).

Na região Sul do Brasil, o jundiá (*Rhamdia quelen*), teleósteo de água doce, é umas das espécies de peixes nativas mais cultivadas (EMBRAPA, 2017; BALDISSEROTTO, 2009), posicionando-se como a mais promissora para a piscicultura na região, especialmente no estado de Santa Catarina (EPAGRI/CEDAP, 2018). É um peixe que tem apresentado boa produtividade em cativeiro e aceitação no mercado consumidor (CARNEIRO, 2004).

Aliada à expansão da produção de peixes, crescem os desafios frente às inúmeras doenças bacterianas em função de inadequadas práticas de manejo, baixa qualidade da água e falta de cuidado com o solo (EMBRAPA, 2003), que causam estresse e comprometem a produção (SANTOS; LUDKE; LIMA, 2009).

No ecossistema aquático, Aeromonas *hydrophila* é considerada o principal patógeno no desenvolvimento de doenças em peixes (GHATAK et al., 2016). É uma bactéria gram-negativa e anaeróbia facultativa da família *Aeromonodaceae*. Peixes infectados com *A. hydrophila* apresentam lesões que podem progredir para úlceras e necrose na pele e em órgãos internos, além de quadros de septicemia (LAITH; NAJIAH, 2013; JANDA; ABBOTT, 2010). As brânquias dos peixes quando expostos à *A. hydrophila* apresentam aumento na espessura do filamento e lamelas, que conduzem à fusão lamelar, bem como hipersecreção das células mucosas (AZADBAKHT et al., 2019). Essas alterações são prejudiciais e podem interferir substancialmente nas trocas gasosas e na osmorregulação branquial (MALLATT et al., 1985).

A utilização contínua de fármacos sintéticos e produtos químicos no combate a patógenos em peixes tem apresentado impactos negativos, como depósitos de resíduos nos tecidos, imunossupressão e multirresistência aos fármacos pelos microrganismos (GRENNI; ANCONA; CARACCIOLOA, 2018; RASUL; MAJUMDAR, 2017). Nesse contexto, o uso de plantas medicinais e seus compostos majoritários têm se apresentado como uma nova alternativa ao uso de antibióticos e produtos químicos na piscicultura (SUTILI et al., 2015; DEBBARMA et al., 2012).

O uso de plantas medicinais é uma estratégia antiga utilizada para o tratamento de diversas doenças, bem como para a pesquisa e desenvolvimento de novos fármacos (GUPTA; BLEAKLEY; GUPT, 2008). O metabolismo secundário das plantas é capaz de originar óleos essenciais (OEs), constituídos de misturas voláteis de compostos, principalmente monoterpenos e sesquiterpenos com diversas funções, como atividade antimicrobiana (CUNHA; HEINZMANN; BALDISSEROTTO, 2018).

O limoneno é um monoterpeno do metabolismo secundário das plantas do gênero *Citrus*, disponível nas isoformas R-(+)-limoneno e S-(-)-limoneno (DEGENHARD; KÖLLNER; GERSHENZON, 2009). Dos terpenos no gênero *Citrus*, o R-(+)-limoneno é o mais abundante, podendo corresponder até a 96% do óleo total volátil e é o de maior interesse para a indústria na fabricação de vários produtos, como cosméticos (WISSING; MÜLLER, 2003) e inseticidas (IBRAHIM et al., 2001).

A atividade antimicrobiana de monoterpenos tem sido descrita através de pesquisas em diversas espécies de plantas e microrganismos testados (NGUGI; OYOO-OKOTH; MUCHIRI, 2017; SOUZA et al., 2016; SUTILI et al., 2015). De acordo com Greay e Hammer (2015), alguns monoterpenos interferem na integridade e funcionamento da membrana celular bacteriana. Além disso podem apresentar outros mecanismos de ação incluindo vazamento do conteúdo intracelular por coagulação do citoplasma (GUSTAFSON et al., 1998), inibição de síntese de ATP intracelular (BURT, 2004) e desequilíbrio da homeostase do K^+ (XU et al., 2008). Duarte et al. (2015), ao analisarem as propriedades do linalool, verificaram que este monoterpeno inibia o quorum-sensing (QS), sistema de comunicação entre bactérias envolvido na formação de biofilme.

Com base nisso, este trabalho buscou avaliar os efeitos das isoformas R-(+)-limoneno e S-(-)-limoneno sobre os parâmetros histológicos branquiais de peixes saudáveis e infectados com *A. hydrophila*.

2. REVISÃO BIBLIOGRÁFICA

2.1 *Rhamdia quelen*

O jundiá, *Rhamdia quelen* (Figura 1), é um teleósteo de água doce, da ordem Siluriformes e família Heptapteridae, encontrado desde o centro da Argentina até o sul do México (FISH BASE, 2006; BALDISSEROTTO; RADÜNZ NETO, 2004; SILFVERGRIP, 1996). De acordo com a EMBRAPA (2017), o jundiá tem sido amplamente cultivado na região sul do Brasil. Detém considerável aceitação comercial, além de características desejáveis para produção e ótimo crescimento, dentre elas boa resistência a baixas temperaturas (FIGUEREDO et al., 2014; BALDISSEROTTO, 2009; BALDISSEROTTO; RADÜNZ NETO, 2004).

Figura 1: Espécime de jundiá (*Rhamdia quelen*)

Fonte: Elisia Gomes da Silva

Em função disso, tem recebido atenção de inúmeros pesquisadores sul-americanos na investigação de seus sistemas reprodutivo, imunológico e digestório, além de sua resposta rápida a estressores (BANDEIRA JUNIOR et al., 2019; BALDISSERA; DE FREITAS SOUZA; BALDISSEROTTO, 2018; SUTILI et al., 2014, 2016; SOUZA et al., 2017; RODRIGUES et al., 2012). No entanto, sua criação, manutenção e reprodução em grande escala vem enfrentando desafios, atribuídos às infecções bacterianas (BARCELLOS et al., 2008).

Os jundiás preferem ambientes de águas mais calmas, são bentônicos, vivem em substrato de lagos e poços fundos dos rios, entre pedras e troncos apodrecidos, de onde saem à noite, à procura de alimentos (GUEDES, 1980). Apesar do hábito alimentar onívoro, o jundiá alimenta-se também de peixes, crustáceos, restos vegetais e detritos orgânicos (MEURER;

ZANIBONI FILHO, 1997). Em ambientes claros, a coloração do corpo tende a ficar mais clara, inversamente quando em ambientes escuros (GOMES et al., 2000). Podem ser considerados euritérmicos, pois suportam temperaturas de 15°C a 34°C (BALDISSEROTTO; RADÜNZ NETO, 2004; ZANIBONI FILHO, 2004).

O crescimento varia de acordo com a elevação da temperatura, o qual é mais proeminente nos primeiros anos de vida. Machos possuem taxa de crescimento até o terceiro e quarto ano de vida, período no qual as fêmeas crescem aceleradamente, com comprimento e pesos superiores aos dos machos (BALDISSEROTTO, 2009). A maturidade sexual é atingida por volta de um ano de idade nos dois sexos. Os machos iniciam o processo de maturação gonadal com 13,4cm e as fêmeas com 16,5cm. A partir das medidas 16,5cm e 17,5cm, machos e fêmeas, respectivamente, estão fisiologicamente aptos para reprodução (NARAHARA et al., 1985).

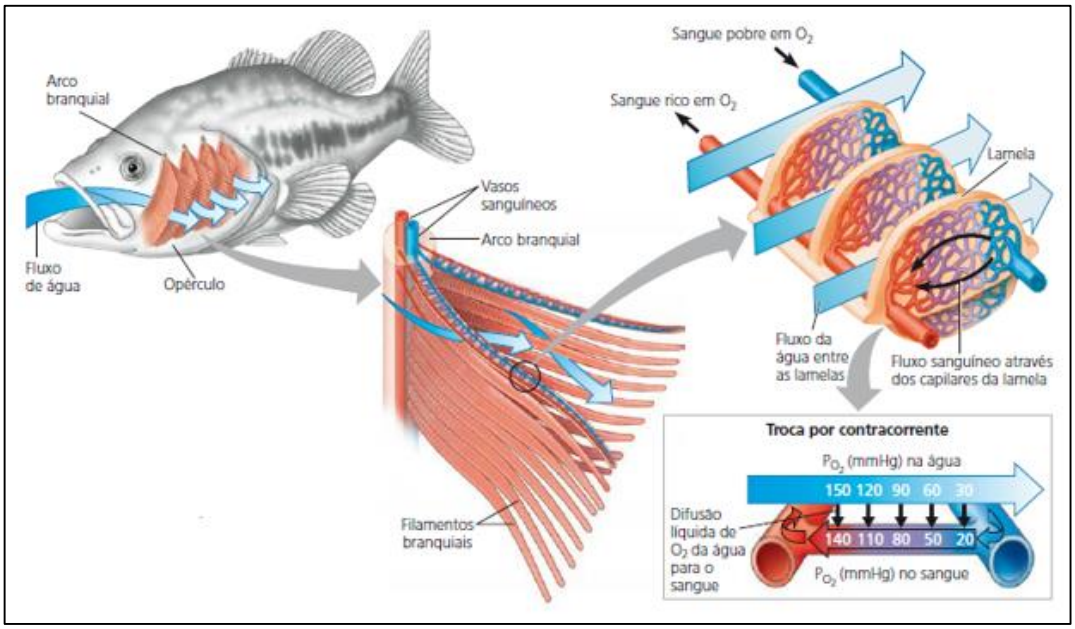
2.2 ESTRUTURA BRANQUIAL DOS PEIXES TELEÓSTEOS

As brânquias dos peixes são uma estrutura multifuncional responsável por realizar trocas gasosas, regulação iônica, equilíbrio ácido-base e excreção de resíduos nitrogenados (EVANS; PIERMARINI; CHOE, 2005; SAKURAGUI; SANCHES; FERNANDES, 2003; GOSS et al., 1992). A eficiência nas trocas gasosas ocorre dependendo primeiro de uma estrutura branquial saudável (HUGHES; BYCZKOWSKA-SMYK, 1974) e do seu sistema de circulação sanguínea e, segundo, pelo fluxo contínuo da água no tecido branquial (SAKURAGUI; SANCHES; FERNANDES, 2003; GREENWOOD, 1975). Pelas brânquias circula um fluxo contínuo de água que entra pela boca e sai através da abertura opercular, por meio de um fluxo contracorrente (Figura 2) (BALDISSEROTTO, 2013).

O sangue, rico em CO_2 e pobre O_2, é trazido dos tecidos, bombeado pelo coração até as brânquias onde passa entre as lamelas respiratórias em sentido contrário ao fluxo de água, possibilitando as trocas gasosas. Quando o sangue sai da lamela branquial consegue remover de 80% a 90% do oxigênio dissolvido na água (BALDISSEROTTO, 2013; PIIPER, 1998).

A água tem uma pressão parcial de oxigênio (PO_2) mais alta que o sangue que entra nas brânquias, permitindo a transferência de O_2. À medida que o sangue continua passando, sua PO_2 aumenta gradativamente. Portanto, ao longo do capilar, o gradiente de pressão parcial favorece a difusão de O_2 da água para o sangue (WITHERS, 1992). Os peixes são capazes de utilizar 80% de oxigênio dissolvido da água que circula através das brânquias, diferentemente dos humanos que utilizam somente 25% do oxigênio inalado (BALDISSEROTTO, 2013).

Figura 2: Trocas gasosas nas brânquias

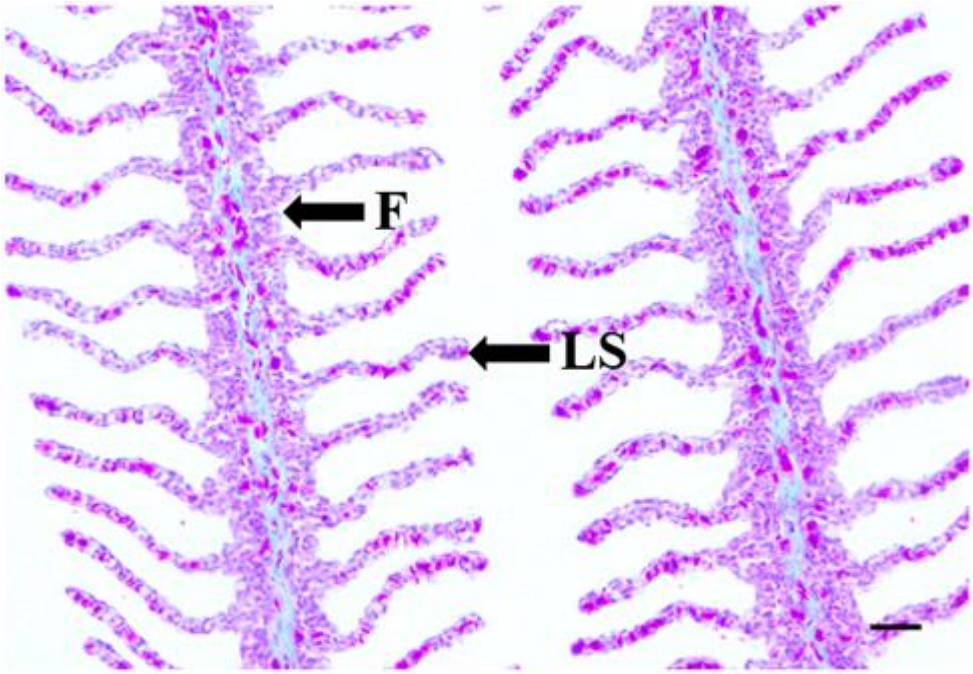


Fonte: Biologia de Campbell, 2015.

Por atuarem como interface entre o meio externo e interno, as brânquias são as principais vias de contaminação nos peixes (HEATH, 1997). São importantes na análise histológica dos efeitos à exposição de agentes tóxicos e patógenos, e alterações em sua estrutura podem afetar várias funções fisiológicas (SOUZA et al., 2016; PERRY; LAURENT, 1993; LAURENT; PERRY, 1991). A histologia branquial tem se revelado útil na detecção de lesões histopatológicas e na avaliação da saúde dos peixes (HINTON et al., 1992), sendo por isso um método de alta sensibilidade (DUTTA, 1996), refletindo alterações prévias às fisiológicas e até mesmo bioquímicas (NERO et al., 2006).

Como a maioria dos teleósteos, as brânquias dos jundiás possuem quatro arcos branquiais de cada lado da faringe. Em cada arco branquial projetam-se duas fileiras de filamentos ou lamelas primárias. Intercaladas nos lados dorsal e ventral do filamento branquial, encontram-se as lamelas secundárias (Figura 3) (COSTA et al., 2017).

Figura 3: Fotomicrografia do filamento (F) e lamela secundária (LS) de brânquias de *Rhamdia quelen*

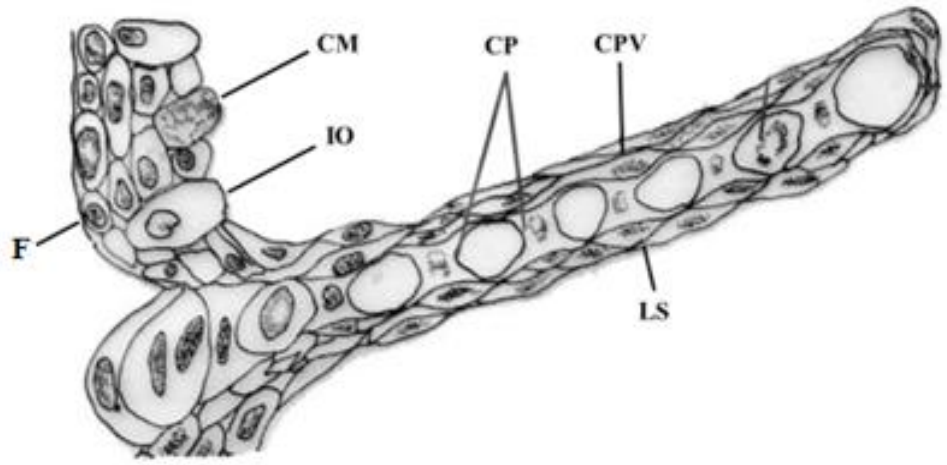


Fonte: Elisia Gomes da Silva

A lamela secundária, também denominada de epitélio respiratório, é composta por dobras transversais sobre a superfície do filamento, como finas lâminas triangulares, com a parte mais alta voltada para o lado da entrada da água, apoiada sobre redes vasculares, de modo a facilitar as trocas gasosas (HUGHES, 1982; HUGHES; BYCZKOWSKA-SMYK, 1974). O epitélio respiratório é formado pelas células pilares e células pavimentosas (Figura 4).

As células pilares quando em arranjos funcionam como canais para circulação do sangue, controlam o fluxo de outras células no interior da lamela e atuam como colunas evitando o abaulamento provocado pela alta pressão sanguínea. Filamentos contráteis semelhantes à actomiosina são encontrados no citoplasma das células pilares, e desempenham uma função contrátil para estas células (RANDALL; BURGGREN; FRENCH, 2000, HUGHES; BYCZKOWSKA-SMYK, 1974; BETTEX-GALLAND; HUGHES, 1973). Alterações na estrutura destas células causam interferência na dinâmica vascular (BETTEX-GALLAND; HUGHES, 1973).

Figura 4: Desenho esquemático das células do filamento (F) e lamela secundária (LS). CP – células pilares, CPV – células pavimentosas, CM – células mucosas, IO – ionócitos



Fonte: Mallat, 1985.

As células pavimentosas estão envolvidas nas trocas gasosas e mantém íntimo contato com a água (PERRY, 1998). São células poligonais, com um complexo sistema de Golgi e abundante retículo endoplasmático (BOYD et al., 1980). Estão dispostas em mosaico, recobrindo o epitélio externamente, formando dobras, denominadas de micropregas (HUGHES, 1979). As micropregas estão presentes em todos os peixes e acredita-se que sua função esteja relacionada em aumentar a superfície respiratória, mantendo muco sobre o epitélio para protegê-lo de agressões ambientais (MALLATT, 1985).

O filamento é formado, além das células pavimentosas, das células mucosas e ionócitos (Figura 4) (DIAZ et al., 2005; PERRY, 1997). As células mucosas encontram-se preferencialmente na região basal do filamento e região interlamelar. São células grandes, com núcleos geralmente localizados na região basal (KUMARI et al., 2009; LAURENT; HEBIBI, 1989). O muco produzido por estas células é composto por diferentes categorias de mucopolissacarídeos e está envolvido com o sistema de defesa dos peixes, podendo em larvas de teleósteos participar indiretamente na osmorregulação (SHEPHARD, 1994). No epitélio de revestimento corporal, o muco atua prevenindo injúrias e infecções, e nas brânquias representa um importante mecanismo de proteção às superfícies lamelares à exposição de agentes

patógenos e substâncias tóxicas, como uma barreira física, química e imunológica (DIAZ et al., 2005; SHEPHARD, 1994; POWELL; SPEARE; BURKA, 1992; MALLATT, 1985).

Os ionócitos, previamente denominados células de cloreto ou células ricas em mitocôndrias, são células que possuem ampla aérea de superfície e encontram-se distribuídas na base da lamela secundária e no epitélio do filamento (DYMOWSKA; HWANG; GOSS, 2012; SAKURAGUI; SANCHES; FERNANDES, 2003; PERRY, 1997). Têm função primordial na osmorregulação para manter o equilíbrio iônico nos peixes (HIROSE et al., 2003; PERRY, 1997). São responsáveis pela absorção de íons em água doce (FW) e secreção de íons em água salgada (SW). Por atuarem no transporte transepitelial, processo intenso em energia, são ricas em mitocôndrias para produção de ATP (HWANG; LEE; LIN, 2011).

Nas membranas basolateral e apical destas células estão presentes as enzimas Na^+/K^+-ATPase e Ca^+-ATPase, trocadores e canais iônicos, desempenhando papel ativo no transporte iônico (DYMOWSKA; HWANG; GOSS, 2012). Na membrana basolateral a bomba Na^+/K^+ (Na^+/K^+-ATPase) retira Na^+ do meio intracelular e cria um gradiente favorável à entrada desse íon na célula (WILSON; LAURENT, 2002). A entrada de Ca^{2+} ocorre na membrana apical por difusão, a favor de gradiente, e por meio de uma bomba de Ca^{2+} e transportador Na^+/ Ca^{2+} ocorre sua saída pela membrana basolateral (DYMOWSKA; HWANG; GOSS, 2012; DUNCAN; SILVA; FERNANDES, 2011; EVANS; PIERMARINI; CHOE, 2005; BALDISSEROTTO, 2013).

Exposição aos agentes patógenos ou tóxicos pode provocar alterações nas brânquias, comprometendo a saúde e sobrevivência do animal (ALAGAPPAN et al., 2009; FERNANDES et al., 2007). A preservação do filamento e da lamela secundária é fundamental para o desempenho respiratório dos peixes. Nas situações nas quais ocorre hiperplasia do filamento (AZADBAKHT et al., 2019; SOUZA et al., 2016), esse aumento do epitélio filamentar pode conduzir à fusão lamelar, comprometendo a integridade morfofuncional das brânquias, resultando na ineficiência das trocas gasosas (MALLATT, 1985). Apesar dessas alterações serem consideradas um mecanismo de defesa do animal, quando intensas e constantes, prejudicam as trocas gasosas na respiração (FERGUSON, 2006).

Diversos agentes estressores também podem influenciar o número e tamanho das células do filamento e lamela secundária (AZADBAKHT et al., 2019; TEH; ADAMS; HINTON, 1997; MALLATT, 1985). O aumento na proliferação de ionócitos pode funcionar como um mecanismo para aumentar a capacidade do epitélio branquial no transporte iônico (PERRY; LAURENT, 1989, 1993). No entanto, essa proliferação pode comprometer a distância água-

sangue entre as lamelas (PERRY; LAURENT, 1993), reduzindo a absorção de O_2 da água e, como resultado, sua transferência para o sangue (PERRY, 1997; SAKURAGUI; SANCHES; FERNANDES, 2003).

Da mesma maneira, alterações no tamanho e número de células mucosas são entendidas como uma resposta de defesa ao agente estressor. O muco secretado pelas células mucosas atua como barreira contra absorção de agentes patógenos e irritantes, porém, quando em excesso, interfere nas trocas gasosas, prejudicando a osmorregulação branquial (SHEPHARD; 1994; MALLATT, 1985).

2.3 *Aeromonas hydrophila*

No ecossistema aquático, *Aeromonas hydrophila* constitui o principal agente bacteriano com potencial patogênico e é capaz de provocar perdas econômicas consideráveis na aquicultura (SARKAR; RASHID, 2012; JANDA; ABBOTT, 2010). Normalmente são encontradas no intestino dos peixes, águas e sedimentos de lagos ricos em matéria orgânica (AOKI, 1999). Podem também colonizar produtos como carnes e peixes, afetando a saúde humana, com infecções gastrintestinais e até septicemia (TOMÁS, 2012).

Aeromonas hydrophila é uma bactéria gram negativa e anaeróbia facultativa da família *Aeromonadaceae*. Apresenta morfologia em forma de bacilos ou cocobacilos independentes, aos pares ou em cadeias curtas e não formam esporos. Cresce preferencialmente em temperaturas entre 20°C e 25°C, mas em meios de cultura também pode crescer a 37°C (JANDA; ABBOTT, 2010; JOSEPH; CARNAHAN, 2000).

A virulência de *A. hydrophila* é multifatorial, pois é capaz de produzir e secretar diversas substâncias extracelulares biologicamente ativas como hemolisinas, citotoxinas, proteases, fosfolipases, DNAses, colinesterases e endotoxinas, e estruturas celulares que possibilitam a formação de biofilme para facilitar a adesão e invasão nos tecidos dos hospedeiros (BEAZ-HIDALGO; FIGUERAS, 2013; OLIVEIRA; GOUVEIA; COSTA, 2012; JANDA; ABOTT, 2010).

Segundo Sutili et al. (2014), *A. hydrophila* é uma bactéria ubíqua em ambientes aquáticos, afetando patogenicamente peixes de água doce. A infecção bacteriana nos peixes é transmitida de modo horizontal por excretas ou lesões na pele (AOKI, 1999). Os principais sinais clínicos apresentados variam de lesões de pele (Figura 5), superficiais ou profundas, à hemorragia nos opérculos, anemia e quadros de septicemia (ABDELHAMED et al., 2017; BAUMGARTNER; FORD; HANSON, 2017; GHATAK et al., 2016; SUTILI et al., 2014,

AUSTIN; AUSTIN, 2010; BARCELLOS et al., 2008; CIPRIANO; BULLOCK; PYLES, 2001). Podem ainda ocorrer hiperlocomoção dos peixes relacionada ao estresse causado pela bactéria (BANDEIRA JUNIOR et al., 2019), perda do equilíbrio, perda do apetite e persistência dos animais no fundo das caixas. Os peixes infectados por *A. hydrophila* normalmente morrem entre 2 e 10 dias após o início dos sinais clínicos (BOIJINK; BRANDÃO, 2001).

Figura 5: Peixe infectado com *Aeromonas hydrophila*

Fonte: Elisia Gomes da Silva

Peixes infectados com *A. hydrophila* podem apresentar sinais clínicos no tecido branquial, como hemorragia, e alterações histológicas na arquitetura das brânquias (CARRASCHI et al., 2012). Entre os tipos de alterações histológicas nas brânquias comumente observadas em estudos com infecção por *A. hydrophila* estão a hiperplasia do filamento e lamelas secundárias (ABDELHAMED et al., 2017; YUN et al., 2017; SOUZA et al., 2016), fusão lamelar (SOUZA et al., 2016; KHALIL; MANSOUR, 1997), proliferação de células mucosas (AZADBAKHT et al., 2019; CARRASCHI et al., 2012,) e de ionócitos (AZADBAKHT et al., 2019).

2.4 ÓLEOS ESSENCIAIS E LIMONENO

Os óleos essenciais (OEs) são uma mistura complexa de diferentes compostos voláteis, originados do metabolismo secundário das plantas (CARSON; HAMMER, 2011; BAKKALI et al., 2008). São caracterizados por forte odor e estão presentes em vários órgãos vegetais como

partes aéreas, cascas, troncos, raízes, frutos, flores, sementes e resinas (SIMÕES; SPITZER, 2003).

Os OEs estão entre os mais importantes compostos naturais estudados em peixes devido sua vasta atividade antimicrobiana (CUNHA; HEINZMANN; BALDISSEROTTO, 2018; BANDEIRA JUNIOR et al., 2017; OZOGUL et al., 2015; SUTILI et al., 2014; LANG; BYCHBAUER, 2012), e também por serem biodegradáveis e menos propensos a causar o desenvolvimento de resistência bacteriana (YAP et al., 2014). Além da atividade antimicrobiana, vários estudos têm relatado outras propriedades desses compostos, dentre elas propriedades antiparasitárias (BALDISSERA; DE FREITAS SOUZA; BALDISSEROTTO, 2018), antifúngicas (PERIĆ et al., 2019; VIUDA-MATOS et al., 2008a), anti-inflamatória (HIROTA et al., 2010), antioxidantes (SACCOL et al., 2016), anestésica (SOUZA et al., 2017) e imunomoduladora (SUTILLI et al., 2016; BABA et al., 2016).

Os OEs de plantas possuem em sua composição um conjunto de compostos químicos, principalmente da classe dos terpenos (BAKKALI et al., 2008), que podem agir de maneira individual, aditiva, antagonista ou sinérgica melhorando a eficácia de fármacos antimicrobianos e aumentando o seu mecanismo de ação (BANDEIRA JUNIOR et al., 2018).

O limoneno (C_6H_{10}) é um monoterpeno cíclico e insaturado presente majoritariamente na composição do óleo essencial de plantas cítricas, sendo o principal constituinte das frações terpenoides dos óleos de limão e de laranja (DEGENHARD; KÖLLNER; GERSHENZON, 2009). O gênero *Citrus* apresenta destaque mundial e seus OEs estão entre os mais utilizados e comercializados. O Brasil, por exemplo, é o maior produtor mundial de frutas cítricas e ocupa lugar de destaque na produção de OEs (EMBRAPA, 2015).

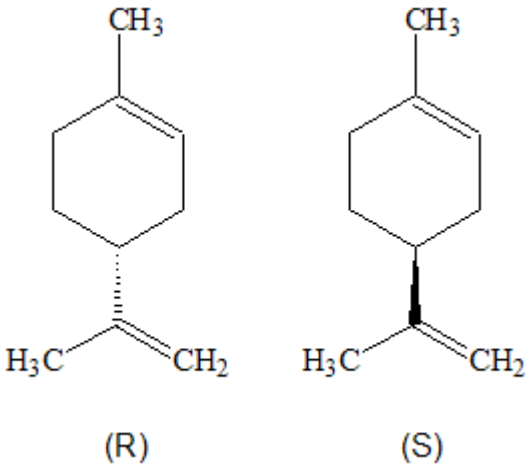
Além disso, vários pesquisadores têm relatado efeitos antimicrobianos do gênero *Citrus*. Viuda-Matos et al. (2008b), constataram que os óleos essenciais de limão (*Citrus lemon* L.), tangerina (*Citrus reticulata* L.), toranja (*Citrus paradisi* L.) e laranja (*Citrus sinensis* L.), inibiram o crescimento de diversas bactérias de origem alimentar. Acar et al. (2015), ao utilizarem óleo de *Citrus sinensis*, verificaram redução do crescimento bacteriano de *Streptococcus iniae* em tilápia nilótica. Inibição bacteriana também pode ser observada por Kirbaslar et al. (2009) utilizando óleos essenciais extraídos de cascas de limão.

Por possuir um centro quiral, ou seja, apresenta um carbono assimétrico, o limoneno (Figura 6) ocorre na natureza sob a forma de dois isômeros ópticos: R-(+)-limoneno e S-(-)-limoneno (DEGENHARD; KÖLLNER; GERSHENZON, 2009). Os dois isômeros são incolores e têm odores diferentes: o S-(-)-limoneno tem cheiro de terebintina e limão e o R-(+)-

limoneno tem um agradável aroma de laranja. A isoforma R-(+)-limoneno está presente principalmente na laranja, limão, tangerina e lima, enquanto o *S*-(-)-limoneno está presente em óleos de bergamota, eucalipto e ervas como *Mentha* spp. (MALKO; WRÓBLEWSKA, 2016, DEGENHARD; KÖLLNER; GERSHENZON, 2009).

Entre os terpenos presentes em *Citrus*, o R-(+)-limoneno é o fitoquímico mais abundante, com presença de até 96% do óleo total volátil (GAD; HAKKINEN, 2005).

Figura 6: Estruturas químicas dos isômeros do limoneno



Fonte: https://theflavourofchemistry.wordpress.com/2011/06/04/limonene/

Não existem dados na literatura sobre a absorção de limoneno em peixes através das brânquias. No entanto, sabe-se que o R-(+)-limoneno, dependendo da sua concentração, pode ser parcialmente solúvel em água e é armazenado nos tecidos de peixes e organismos aquáticos (FALK-FILIPSSON; BARD; KARLSSON, 1998), enquanto nos seres humanos é absorvido até 70% por inalação e se acumula no tecido adiposo (FALK-FILIPSSON et al., 1993).

A isoforma R-(+)-limoneno é produzida e utilizada em produtos cosméticos (WISSING; MÜLLER, 2003), alimentos (TONGNUANCHAN; BENJAKUL; PRODPRAN, 2012), fabricação de resinas, como agente umectante e dispersante, e no controle de insetos (IBRAHIM et al., 2001).

Na literatura existem diversos relatos da isoforma R-(+)-limoneno em relação às suas bioatividades. Zahi et al. (2015), em seus estudos com nanoemulsão de R-(+)-limoneno,

mostraram que este fitoquímico destruiu a integridade da membrana das células bacterianas de *Escherichia coli, Bacillus subtilis, Staphylococcus aureus* e *Saccharomyces cerevisiae.* Enquanto nos estudos de Giarratana et al. (2015), R-(+)-limoneno inibiu o endoparasito de peixes *Anisakis larvae*.

O fato de R-(+)-limoneno e S-(-)-limoneno serem isômeros indicam que eles possuem os mesmos constituintes atômicos, porém suas disposições na molécula são diferentes, conferindo consequentemente características químicas e atividades biológicas distintas. Lis-Balchin et al. (1996), ao estudarem a bioatividade dos enantiômeros do limoneno em relação à atividade antibacteriana, constataram que o R-(+)-limoneno foi mais ativo que o S-(-)-limoneno. Nesse mesmo estudo, frente à bactéria *Listeria monocytogenes*, o R-(+)-limoneno foi ativo contra treze cepas desta bactéria, ao passo que o S-(-)-limoneno apresentou atividade inibitória somente para sete cepas. Por outro lado, pesquisas com extratos de casca de laranja e limão contra os fungos *Candida albicans, Aspergillus niger, Aspergillus* sp. e *Penicillium* sp. demonstraram que o S-(-)-limoneno teve maior efeito inibitório nos fungos examinados que o R-(+)-limoneno (OMRAN et al., 2011).

3. OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar os efeitos das isoformas R-(+)-limoneno e S-(-)-limoneno sobre os parâmetros histológicos das brânquias de *Rhamdia quelen* saudáveis e infectados com *A. hydrophila*.

3.2 OBJETIVOS ESPECÍFICOS

- Avaliar a espessura do filamento e do tecido conjuntivo das brânquias de *Rhamdia quelen* quando expostos às isoformas de limoneno;

- Avaliar a espessura e comprimento da lamela e espaço interlamelar das brânquias de *Rhamdia quelen* quando expostos às isoformas de limoneno;

- Quantificar o número de células mucosas e de ionócitos das brânquias de *Rhamdia quelen* quando expostos às isoformas de limoneno;

-Analisar o tamanho das células mucosas das brânquias de *Rhamdia quelen* quando expostos às isoformas de limoneno;

.

4 MANUSCRITO

O manuscrito está disposto conforme as normas requisitadas pela revista Aquaculture, o qual foi submetido para publicação.

R-(+)-limonene protects the silver catfish gills (*Rhamdia quelen*) from *Aeromonas hydrophila*-induced histopathological changes

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Abstract

Limonene is a monoterpene available under two enantiomers, R-(+)-limonene and S-(-)-limonene. There are no studies in the literature reporting the effects of the chirality of the limonene in aquaculture. This research aimed to assess the effects of the R-(+)-limonene and S-(-)-limonene on the morphology of the gills of healthy silver catfish (*Rhamdia quelen*) and fish challenged with *Aeromonas hydrophila*. For this purpose, in the first experiment, healthy fish were split into seven groups: control, 10 mg/L gentamicin, 90 μL/L ethanol, 10 μL/L R-(+)-limonene (R10), 20 μL/L R-(+)-limonene (R20), 10 μL/L S-(-)-limonene (S10), 20 μL/L S-(-)-limonene (S20). They were exposed once to the different treatments. After five days, fish were anesthetized and then euthanized for removal of gill arches for histological analysis. For the second experiment, fish were split into ten groups and also exposed once to water, 10 mg/L gentamicin, 90 μL/L ethanol, R10 and R20 limonene and maintained during a week until their infection or not with *A. hydrophila*, which lasted an additional week. Then, animals from all groups were anesthetized and euthanized for removal of gill arches for histological analysis. Some of the non-infected fish exposed to S10 and S20 died, showed inflammation signs in their gills as edema and inflammatory cells infiltration into the connective tissue, exhibited an increase in the filamentous epithelium thickness (FT) and lamellar thickness (LT), lamellar fusion, as well as a reduction in the lamellar length (LL) and interlamellar space (IS), a rise in the number of mucous cells and ionocytes and the size of mucous cells. However, non-infected fish exposed to R10 and R20 showed lower alterations and no alterations, respectively, in these parameters. Regarding *A. hydrophila*-infected fish, they revealed inflammation signs and also an increase in the FT, connective tissue thickness, LT as well as a reduction in the LL and IS, lamellar fusion, aneurysm, a rise in the number of mucous cells and ionocytes and the size of

mucous cells. Conversely, R10- and R20-infected fish showed lower changes in these parameters, specially those from the R10 group. Therefore, S-(-)-limonene seemed to be irritant to silver catfish, triggering some deaths, inflammation and altering most morphometric and quantitative parameters evaluated through histology. On the other hand, R-(+)-limonene acted as anti-inflammatory and preserved the structure of the gills, being more proper for utilization in healthy and *A. hydrophila*-challenged silver catfish, specially at 10 μL/L.

Keywords: monoterpene, histology, anti-inflammatory, infection, fish.

1 Introduction

Fish are exposed to stressors in aquaculture conditions. Such stressors are components of modern intensive fish culture, as grading, transportation and vaccination (Iwama, 1998). Under such stressful situations, their defense mechanisms can be compromised, thus allowing pathogens to gain access to their tissues, which are rich in potential nutrients (Ellis, 2001). *Aeromonas hydrophila (A. hydrophila)*, a ubiquitous, free-living, Gram-negative bacterium, stands out among these opportunistic pathogens, resulting in high mortality levels in farmed and feral fishes (Harikrishnan and Balasundaram, 2005), including silver catfish (*Rhamdia quelen*), a fast-growing species native to South Brazil with great economic importance (Sutili et al., 2016, 2015, 2014, 2013).

It was shown, using a green fluorescent protein as a marker, that gills lesions are a main route for *A. hydrophila* entrance. Gills have a rich vascular structure for respiration that is separated from the external environment through a thin mucus layer. Such features make them good pathway for bacterial systemic invasion of fish (Chu and Lu, 2008). Thus, gills are also a frequent focus of histopathological changes during *A. hydrophila* infection (Abdelhamed et al., 2017; Azadbakht et al., 2019; Carraschi et al., 2012; Harikrishnan et al., 2008; Souza et al., 2016).

The application of antibiotics is essential for disease management. However, this practice has triggered the emergence of antibiotic resistant strains in pathogens, immunosuppression and environmental contamination (Harikrishnan and Balasundaram, 2005). Although it is clear that most *A. hydrophila* isolates from silver catfish are highly sensible to gentamicin (Andrade et al., 2006; Barcellos et al., 2008; Costa et al., 2008), it is known that such antibiotic causes histopathological changes in Nile tilapia kidney

(*Oreochromis nilotica*) (Augusto et al., 1996). Thus, an emerging trend is medicinal plant research (Harikrishnan and Balasundaram, 2005). It has been shown, for example, that essential oils containing limonene in their composition are highly effective against *A. hydrophila* (Souza et al., 2016; Ngugi et al., 2017; Parlatan et al., 2009; Pintore et al., 2009). Furthermore, recent studies from our group have shown that besides their antimicrobial properties (Souza et al., 2016; Sutili et al., 2015), essential oils containing limonene have also exhibited anti-inflammatory (Souza et al., 2016), antioxidant (Baldissera et al., 2017b) and immunostimulatory activities in *A. hydrophila*-infected silver catfish (Baldissera et al., 2017b, 2017a).

Limonene is a monoterpene available in nature under two enantiomeric forms, R-(+)-limonene and S-(-)-limonene. R-(+)-limonene is the principal component of the essential oils present in the peel of citrus fruits, including orange, lemon, mandarin, grapefruit and lime (Mann et al., 1994). The other optical isomer, S-(-)-limonene, has a turpentine smell and is also found in plants as the major component of volatiles emitted by oaks and pines (Schween et al., 1997). A recent research from our group showed that chirality can interfere in the biological properties of monoterpenes isolated from essential oils, in silver catfish (Silva et al., 2017). However, there are no studies in the literature reporting the effects of the chirality of limonene in aquaculture. Thus, the present investigation aimed to determine through histological analysis the effects of the R-(+)-limonene and S-(-)-limonene on the morphology of the gills of healthy and *A. hydrophila*-challenged silver catfish.

2 Materials and Methods

100

101 **2.1 Reagents**

102 R-(+)-limonene (#W263303) and S-(-)-limonene (#W504505) were purchased from
103 Sigma-Aldrich (St. Louis, USA). Unless indicated in the text, other reagent-grade chemicals
104 were also obtained from Sigma-Aldrich (St. Louis, USA).

105

106 **2.2 Fish and culture conditions**

107 Silver catfish (60 ± 1g) were purchased from a local fish farm and acclimatized for seven
108 days in 250 L tanks, 16 fish per tank, with continuous aeration. Through acclimation and
109 experiments fish were fed to satiation once daily with commercial feed (Supra Juvenil, São
110 Leopoldo, Brazil). The dissolved oxygen (7.88 ± 0.38 mg/L) and temperature (21.2 ± 0.5°C)
111 were measured daily with a Y 5512 oxygen meter (YSI, Yellow Springs, USA). Total ammonia
112 (0.035 ± 0.005 mg/L) and nitrite levels (0.01 ± 0.01 mg/L) were also checked daily using
113 commercial kits (Labcon Test, Camboriú, BR). The pH (7.26 ± 0.13) was evaluated weekly
114 with a K39-2014B pH-meter (Kasvi, São José dos Pinhais, BR). Feces and residues were
115 removed every day. The water in the boxes was 20% renewed every day.The Ethics on Animals
116 Use Commission from Federal University of Santa Maria approved all animal management
117 procedures (#4475070318).

118

119 **2.3 Experimental design**

120

121 **2.3.1 Experiment #1**

Silver catfish (*n*=112) were distributed in 14 plastic boxes (40 L) and divided into 7 groups as follows: control, gentamicin, ethanol, 10 μL/L R-(+)-limonene (R10), 20 μL/L R-(+)-limonene (R20), 10 μL/L S-(-)-limonene (S10), 20 μL/L S-(-)-limonene (S20). After five days of exposure to the different compounds, some fish from both S-(-)-limonene groups were more static and showed hemorrhagic areas in their mouth, which was half open on the water surface, then some of them even started to die. Therefore, the animals belonging to all groups were anesthetized with 50 μL/L eugenol for 3 min (Cunha et al., 2010) and then euthanized by sectioning of the spinal cord for removal of gill arches. All groups were performed in duplicates (*n*=8, each duplicate).

2.3.2 Experiment #2

Since S-(-)-limonene at 10 or 20 μL/L behaved like an irritant agent to silver catfish, inducing some deaths, it was not appropriate to test the therapeutic potential of this substance in a challenging situation, as *A. hydrophila* infection. Thus, silver catfish (*n*=160) were distributed in 20 plastic boxes (40 L) and divided into 10 groups as follows: control, gentamicin, ethanol, 10 μL/L R-(+)-limonene (R10), 20 μL/L R-(+)-limonene (R20), *A. hydrophila*, gentamicin + *A. hydrophila,* ethanol + *A. hydrophila*, 10 μL/L R-(+)-limonene (R10) + *A. hydrophila,* 20 μL/L R-(+)-limonene (R20) + *A. hydrophila*. After one week of exposure to the different compounds, silver catfish were infected or not with *A. hydrophila*. The strain of *A. hydrophila* (MF 372510) was isolated from a naturally infected juvenile silver catfish and identified through biochemistry and molecular assays (Bandeira Júnior et al., 2017). Silver catfish were inoculated intramuscularly with 100 μL of 0.9% NaCl solution or *A. hydrophila* solution ($4x10^8$ CFU/mL) on the right lateral-dorsal side. At the end of the experimental period (seven days), fish were anesthetized with 50 μL/L eugenol for 3 min (Cunha et al., 2010) and

then euthanized by sectioning the spinal cord for removal of gill arches. All groups were performed in duplicates (*n*=8, each duplicate).

2.4 Treatments

Gentamicin at 10 mg/L (Sutili et al., 2014), ethanol at 90 µL/L (the highest concentration used to dilute the limonene) and 10 or 20 µL/L of R-(+)-limonene or S-(-)-limonene were added to the water only once (at the first day of the treatment). Consequently, these compounds were diluted in each water renewal.

2.5 Morphological analysis

The gills were carefully excised, washed with 0.9% NaCl and fixed in 10% formalin buffer for 24 h. After fixation, tissues were dehydrated in an alcoholic series (70%, 80%, 90% and 100%), then diaphanized with a xylene solution and finally embedded in paraffin at 56-58°C. After inclusion in paraffin, the molds were sectioned at 6 µm using a HM 325 Rotary Microtome (Thermo Scientific, Runcorn, UK) for slides mounting. Such mountings were stained with Masson-Goldner trichrome for morphometric and quantitative analysis of the gills and mucous cells. On the other hand, for ionocyte staining, the mountings were again exposed to an alcoholic series (70%, 80%, 90% and 100%), 1% toluidine blue at 100°C for 30s, and then washed for microscopic analysis. Ionocytes stained using this method can be seen in more detail in Fig. S1. The preparations were analyzed in the composite light microscope Axio Scope.A1 (Zeiss, Jena, Germany) coupled to the digital camera Axiocam 105 color (Zeiss, Jena, Germany). Photomicrographs were recorded at magnification of 20x and 40x.

The morphometric variables examined are illustrated in Fig. 1. Among them are the thickness of filamentous epithelium thickness, conjunctive tissue thickness, lamellar thickness, lamellar length, interlamellar space and mucosal cell size. For this reason, the images were divided into eighty quadrants of 2500 μm^2. A quantitative analysis was performed for the mucous cells and ionocytes. For this purpose, the images were divided into forty-eight quadrants of 1000 μm^2. ImageJ using the Grid plug-in was utilized to analyze these measurements.

2.6 Statistical analysis

The statistical analysis was made using the software Statistica™ (Statsoft, Tulsa, USA). Levene's Test was used to evaluate the data homoscedasticity. Data relating to gills morphometry and quantitative analysis of mucous cells and ionocytes from experiment #1 were standardized, then compared through one-way ANOVA followed by Tukey Multiple Comparisons Test, whereas those from experiment #2 were compared through two-way ANOVA followed by Tukey Multiple Comparisons Test. Results are reported as mean ± standard error. The level of significance considered was 95% ($P < 0.05$).

3 Results

3.1 Experiment #1

3.1.1 Filament and connective tissue morphometry

S20 limonene group (Figs. 2G and 3A) showed an increase in filamentous epithelium thickness compared to the control ($P < 0.05$) (Figs. 2A and 3A), ethanol ($P < 0.05$) (Figs. 2C and 3A), R10 limonene ($P < 0.05$) (Figs. 2D and 3A), R20 limonene ($P < 0.05$) (Figs. 2E and 3A) and S10 limonene groups ($P < 0.05$) (Figs. 2F and 3A). Gentamicin (Figs. 2B and 3A), ethanol (Figs. 2C and 3A), R20 limonene and S10 limonene also revealed higher filamentous epithelium thickness than the control group and R10 ($P < 0.05$) (Fig. 3A). It was observed an inflammatory infiltration into the connective tissue in S20 limonene group (Fig. 2G). Regarding connective tissue thickness, no difference was seen between groups ($P > 0.05$) (Figs. 2A-2G and 3B).

3.1.2 Lamella morphometry

Gentamicin (Figs. 2B and 4A), ethanol (Figs. 2C and 4A), R20 limonene (Figs. 2E and 4A), S10 (Figs. 2F and 4A) and S20 limonene groups (Figs. 2G and 4A) showed an increase in lamellar thickness compared to the control ($P < 0.05$) (Figs. 2A and 4A) and R10 limonene groups ($P < 0.05$) (Figs. 2D and 4A). S10 (Figs. 2F and 4A) and S20 limonene groups (Figs. 2G and 4A) also revealed higher lamellar thickness than gentamicin ($P < 0.05$) (Figs. 2B and 4A), ethanol ($P < 0.05$) (Figs. 2C and 4A), R10 ($P < 0.05$) (Figs. 2D and 4A) and R20 limonene groups ($P < 0.05$) (Figs. 2E and 4A). It was also observed a distal lamellar edema in S10 limonene group (Figs. 2F). S20 limonene group showed basal lamellar edema and lamellar fusion (Figs. 2G).

Lamellar length was lower in S10 (Figs. 2F and 4B) and S20 limonene groups (Figs. 2G and 4B) compared to the control ($P < 0.05$) (Figs. 2A and 4B), gentamicin ($P < 0.05$) (Figs. 2B and 4B), ethanol ($P < 0.05$) (Figs. 2C and 4B), R10 ($P < 0.05$) (Figs. 2D and 4B) and R20 limonene groups ($P < 0.05$) (Figs. 2E and 4B).

Finally, S10 (Figs. 2F and 4C) and S20 limonene groups (Figs. 2G and 4C) revealed a decrease in interlamellar space compared to the control ($P < 0.05$) (Figs. 2A and 4C), gentamicin ($P < 0.05$) (Figs. 2B and 4C), ethanol ($P < 0.05$) (Figs. 2C and 4C), R10 ($P < 0.05$) (Figs. 2D and 4C) and R20 limonene groups ($P < 0.05$) (Figs. 2E and 4C). S20 limonene (Figs. 2G and 4C) exhibited a diminution in interlamellar space compared to S10 limonene group ($P < 0.05$) (Figs. 2F and 4C).

3.1.3 Morphometric and quantitative analysis of mucous cells

S10 (Figs. 2F and 5A) and S20 limonene groups (Figs. 2G and 5A) showed an increased number of mucous cells compared to the control ($P < 0.05$) (Figs. 2A and 5A), gentamicin ($P < 0.05$) (Figs. 2B and 5A), R10 ($P < 0.05$) (Figs. 2D and 5A) and R20 limonene groups ($P < 0.05$) (Figs. 2E and 5A). Gentamicin (Figs. 2B and 5A) and ethanol groups (Figs. 2C and 5A) also revealed higher number of mucous cells than the control ($P < 0.05$) (Figs. 2A and 5A), R10 ($P < 0.05$) (Figs. 2D and 5A) and R20 limonene groups ($P < 0.05$) (Figs. 2E and 5A).

The mucous cells were larger in R20 limonene (Figs. 2E and 5B), S10 (Figs. 2F and 5B) and S20 limonene groups (Figs. 2G and 5B) than in the control ($P < 0.05$) (Figs. 2A and 5B), gentamicin ($P < 0.05$) (Figs. 2B and 5B), ethanol ($P < 0.05$) (Figs. 2C and 5B) and R10 limonene groups ($P < 0.05$) (Figs. 2D and 5B). Moreover, mucous cells size of gentamicin group (Figs. 2B and 5B) was higher than those of the control (Figs. 2A and 5B), ethanol (Figs. 2C and 5B) and R10 limonene groups (Figs. 2D and 5B) ($P < 0.05$).

3.1.4 Quantitative analysis of ionocytes

S10 (Figs. 2F and 6) and S20 limonene groups (Figs. 2G and 6) showed an increased number of ionocytes compared to the control ($P < 0.05$) (Figs. 2A and 6), gentamicin ($P < 0.05$) (Figs. 2B and 6), ethanol ($P < 0.05$) (Figs. 2C and 6), R10 ($P < 0.05$) (Figs. 2D and 6) and R20 limonene groups ($P < 0.05$) (Figs. 2E and 6).

3.2 Experiment #2

3.2.1 Filament and connective tissue morphometry

All groups of *A. hydrophila*-infected silver catfish (Figs. 7F to 7J and 8A) exhibited an increase in the filamentous epithelium thickness compared to the healthy control group ($P < 0.05$) (Figs. 7A and 8A) and their respective uninfected groups ($P < 0.05$) (Figs. 7B to 7E and 8A). Nevertheless, *A. hydrophila*-challenged fish exposed to gentamicin (Figs. 7G and 8A), R10 (Figs. 7I and 8A) and R20 limonene (Figs. 7J and 8A) showed lower filamentous epithelium thickness than non-treated *A. hydrophila*-infected silver catfish ($P < 0.05$) (Figs. 7F and 8A) and those infected fish exposed to ethanol ($P < 0.05$) (Figs. 7H and 8A). An inflammatory infiltration into the connective tissue was observed in non-treated *A. hydrophila*-challenged fish (Fig. 7F) and in those exposed to gentamicin (Fig. 7G). Non-treated *A. hydrophila*-challenged fish (Fig. S2A) and those infected fish exposed to ethanol (Fig. S2B) showed aneurysm.

The connective tissue thickness was increased in non-treated *A. hydrophila*-challenged silver catfish (Figs. 7F and 8B) and in those infected fish exposed to gentamicin (Figs. 7G and 8B) and ethanol (Figs. 7H and 8B) compared to the healthy control group ($P < 0.05$) (Figs. 7A and 8B) and their respective uninfected groups ($P < 0.05$) (Figs. 7B, 7C and 8B). Nevertheless,

A. hydrophila-challenged fish exposed to gentamicin (Figs. 7G and 8B), R10 (Figs. 7I and 8B) and R20 limonene (Figs. 7J and 8B) exhibited lower connective tissue thickness than non-treated infected silver catfish ($P < 0.05$) (Figs. 7F and 8B). *Aeromonas hydrophila*-infected fish exposed to R10 (Figs. 7I and 8B) and R20 limonene (Figs. 7J and 8B) also showed a decrease in connective tissue thickness compared to infected fish exposed to gentamicin ($P < 0.05$) (Figs. 7G and 8B) and ethanol ($P < 0.05$) (Figs. 7H and 8B).

3.2.2 Lamellar morphometry

Ethanol group (Figs. 7C and 9A) showed an increase in lamellar thickness compared to the healthy control ($P < 0.05$) (Figs. 7A and 9A), gentamicin ($P < 0.05$) (Figs. 7B and 9A) and R10 limonene groups ($P < 0.05$) (Figs. 7D and 9A). R20 limonene group (Figs. 7E and 9A) revealed higher lamellar thickness than the healthy control (Figs. 7A and 9A) and R10 limonene groups ($P < 0.05$) (Figs. 7D and 9A). Non-treated *A. hydrophila*-infected silver catfish (Figs. 7F and 9A) and those infected fish exposed to gentamicin (Figs. 7G and 9A), ethanol (Figs. 7H and 9A) and R20 limonene (Figs. 7J and 9A) exhibited an increase in lamellar thickness compared to the healthy control group ($P < 0.05$) (Figs. 7A and 9A). Furthermore, those infected fish exposed to gentamicin (Figs. 7G and 9A) and ethanol (Figs. 7H and 9A) revealed higher lamellar thickness than their respective uninfected groups ($P < 0.05$) (Figs. 7B, 7C and 9A). Lamellar fusion was observed in non-treated *A. hydrophila*-challenged fish (Fig. 7F). Distal lamellar edema, basal lamellar edema, lamellar fusion and blood vessels congestion were also seen in *A. hydrophila*-infected fish exposed to gentamicin (Fig. 7G). *Aeromonas hydrophila*-infected silver catfish exposed to R10 (Figs. 7I and 9A) and R20 limonene (Figs. 7J and 9A) exhibited a decreased lamellar thickness compared to non-treated infected fish ($P < 0.05$) (Figs.

281 7F and 9A) and to infected fish exposed to gentamicin ($P < 0.05$) (Figs. 7G and 9A) and ethanol
282 ($P < 0.05$) (Figs. 7H and 9A).

283 The lamellar length was lower in all groups of infected fish (Figs. 7F to 7J and 9B) than
284 in the healthy control group ($P < 0.05$) (Figs. 7A and 9B) and their respective uninfected groups
285 ($P < 0.05$) (Figs. 7B to 7E and 9B). Nevertheless, infected fish exposed to R20 limonene (Figs.
286 7J and 9B) showed higher lamellar length than non-treated *A. hydrophila*-challenged silver
287 catfish ($P < 0.05$) (Figs. 7F and 9B), infected fish exposed to gentamicin ($P < 0.05$) (Figs. 7G
288 and 9B), ethanol ($P < 0.05$) (Figs. 7H and 9B) and R10 limonene ($P < 0.05$) (Figs. 7I and 9B).
289 On the other hand, infected silver catfish exposed to R10 limonene (Figs. 7I and 9B) exhibited
290 an increase lamellar length compared to non-treated *A. hydrophila*-infected fish ($P < 0.05$) (Figs.
291 7F and 9B), infected fish exposed to gentamicin ($P < 0.05$) (Figs. 7G and 9B) and ethanol (P
292 < 0.05) (Figs. 7H and 9B). *Aeromonas hydrophila*-infected silver catfish exposed to gentamicin
293 (Figs. 7G and 9B) and ethanol (Figs. 7H and 9B) showed higher lamellar length than non-
294 treated *A. hydrophila*-infected silver catfish ($P < 0.05$) (Figs. 7F and 9B). Lamellar hipertrophy
295 was also seen in *A. hydrophila*-challenged fish exposed to gentamicin (Fig. 7G).

296 Finally, interlamellar space was decreased in non-treated *A. hydrophila*-infected silver
297 catfish (Figs. 7F and 9C) and in those infected fish exposed to gentamicin (Figs. 7G and 9C)
298 and ethanol (Figs. 7H and 9C) compared to the healthy control group ($P < 0.05$) (Figs. 7A and
299 9C) and their respective uninfected groups ($P < 0.05$) (Figs. 7B, 7C and 9C). Furthermore,
300 infected fish exposed to R10 (Figs. 7I and 9C) and R20 limonene (Figs. 7J and 9C) showed
301 higher interlamellar space than non-treated *A. hydrophila*-infected silver catfish ($P < 0.05$) (Figs.
302 7F and 9C), infected fish exposed to gentamicin ($P < 0.05$) (Figs. 7G and 9C) and ethanol (P
303 < 0.05) (Figs. 7H and 9C).

304

3.2.3 Morphometric and quantitative analysis of mucous cells

Gentamicin (Figs. 7B and 10A), ethanol (Figs. 7C and 10A) and R20 limonene groups (Figs. 7E and 10A) showed an increased number of mucous cells compared to the healthy control group ($P < 0.05$) (Figs. 7A and 10A) and R10 (Figs. 7D and 10A). Non-treated *A. hydrophila*-infected silver catfish (Figs. 7F and 10A) and infected fish exposed to gentamicin (Figs. 7G and 10A) and ethanol (Figs. 7H and 10A) also exhibited an increased number of mucous cells compared to the healthy control group ($P < 0.05$) (Figs. 7A and 10A). Moreover, infected fish exposed to R10 (Figs. 7I and 10A) and R20 limonene (Figs. 7J and 10A) showed lower number of mucous cells than non-treated *A. hydrophila*-infected silver catfish ($P < 0.05$) (Figs. 7F and 10A), infected fish exposed to gentamicin ($P < 0.05$) (Figs. 7G and 10A) and ethanol ($P < 0.05$) (Figs. 7H and 10A).

Mucous cells were larger in gentamicin (Figs. 7B and 10B), ethanol (Figs. 7C and 10B) and R20 limonene groups (Figs. 7E and 10B) than in the healthy control ($P < 0.05$) (Figs. 7A and 10B) and R10 limonene groups ($P < 0.05$) (Figs. 7D and 10B).All groups of infected fish (Figs. 7F to 7J and 10B) showed larger mucous cells compared to the healthy control group ($P < 0.05$) (Figs. 7A and 10B). *Aeromonas hydrophila*-infected silver catfish exposed to ethanol (Figs. 7H and 10B) revealed higher size of mucous cells than its respective uninfected group ($P < 0.05$) (Figs. 7C and 10B). Moreover, infected fish exposed to gentamicin (Figs. 7G and 10B) showed a decreased size of mucous cells compared to non-treated *A. hydrophila*-infected silver catfish ($P < 0.05$) (Figs. 7F and 10B), infected fish exposed to ethanol ($P < 0.05$) (Figs. 7H and 10B) and R20 limonene ($P < 0.05$) (Figs. 7J and 10B). On the other hand, infected fish exposed to R10 limonene (Figs. 7I and 10B) exhibited lower size of mucous cells compared to non-treated *A. hydrophila*-infected silver catfish ($P < 0.05$) (Figs. 7F and 10B), infected fish exposed

328 to gentamicin (P <0.05) (Figs. 7G and 10B), ethanol (P <0.05) (Figs. 7H and 10B) and R20
329 limonene (P <0.05) (Figs. 7J and 10B).

330

331 **3.2.4 Quantitative analysis of ionocytes**

332 Ionocytes clusters were observed in non-treated *A. hydrophila*-challenged fish (Fig. 7F).
333 Non-treated *A. hydrophila*-infected silver catfish (Figs. 7F and 11) and infected fish exposed to
334 gentamicin (Figs. 7G and 11), ethanol (Figs. 7H and 11) and R20 limonene (Figs. 7J and 11)
335 exhibited higher number of ionocytes than the healthy control group (P <0.05) (Figs. 7A and
336 11). Non-treated *A. hydrophila*-infected fish (Figs. 7F and 11) and infected silver catfish
337 exposed to gentamicin (Figs. 7G and 11) and ethanol (Figs. 7H and 11) also revealed higher
338 number of ionocytes compared to their respective uninfected groups (P <0.05) (Figs. 7B, 7C
339 and 11). Furthermore, infected fish exposed to R10 (Figs. 7I and 11) and R20 limonene (Figs.
340 7J and 11) showed lower number of ionocytes than non-treated *A. hydrophila*-infected silver
341 catfish (P <0.05) (Figs. 7F and 11), infected fish exposed to gentamicin (P <0.05) (Figs. 7G
342 and 11) and ethanol (P <0.05) (Figs. 7H and 11).

343

344 **4 Discussion**

345

346 Essential oils containing monoterpenes in their composition, including limonene,
347 protected fish gills against histopathological changes induced by parasites (Soares et al., 2017a,
348 2017b, 2016) and bacteria (Souza et al., 2016; Thomas et al., 2014). However, the effects of the
349 R-(+)-limonene and S-(-)-limonene on the morphology of the gills of healthy and *A.*
350 *hydrophila*-challenged silver catfish have never been studied. So, for the first time, the current

investigation reveals through histological analysis that differently from R-(+)-limonene, S-(-)-limonene is harmful for the gills of healthy silver catfish. Furthermore, it also shows that R-(+)-limonene has a key role in protecting the gills of these animals against the severe structure alterations caused by *A. hydrophila* infection.

Gills were chosen as a target of this research because they participate in fish respiration, osmoregulation and excretion (Evans et al., 2005), being a frequent focus of histopathological changes during the exposure to toxicants and irritants (Mallatt, 1985) and infections (Mitchell and Rodger, 2011). Histopathological analysis is a valid and rapid tool for locating, describing and even quantifying lesions caused by toxicants in various fish organs, including gills, which are considered sensitive biomarkers for chronic exposure to toxicants; thus, being suggested that modifications in their structure can reflect previous physiological and biochemical changes (Bernet et al., 2001).

There are no data on the absorption of limonene in fish through the gills. However, it was shown that in humans, R-limonene is absorbed up to 70% through inhalation and accumulates in adipose tissues (Falk-Filipsson et al., 1993). Gills are characterized as potential absorption sites for substances, due to high surface area, small diffusion distance and high counter-current flown between water and blood (Hayton and Barron, 1990). Furthermore, it is known that limonene is soluble in water, being stored in the tissues of fish and aquatic organisms (Falk Filipsson et al., 1998). For these reasons, silver catfish were exposed once to the treatments, including gentamicin, ethanol, R-(+)-limonene or S-(-)-limonene, which were added to the water.

Differently from the other constituents, a single exposure to S-(-)-limonene triggered the appearance of hemorrhagic areas in the silver catfish mouth. Skin lesions, as that found in the fish mouth, can be developed in the absence of trauma or pathogens and can also result from

stress and exposure to toxicants (Noga et al., 1998). Therefore, S-(-)-limonene appeared to be the main responsible for such lesions, since no kind of infection was seen in these animals during the current study. Furthermore, among all substances studied in this investigation, S-(-)-limonene seemed to be more irritant than gentamicin and ethanol to the silver catfish gills, inducing severe changes in their architecture.

The gills reaction to irritant and toxic agents includes inflammation, lamellar fusion, excessive mucous production, among others (Flores-Lopes and Thomaz, 2011). S-(-)-limonene seemed to be irritant to silver catfish gills because it caused alterations in most morphometric and quantitative parameters evaluated in the present research through histological analysis, including an increase in the filamentous epithelium thickness, inflammatory cells infiltration into the connective tissue, thus producing an increase in the lamellar thickness with development of lamellar edema and lamellar fusion, leading to a reduction in the interlamellar space. These changes are considered as defense mechanisms of the gills during stress, since they can act promoting an increase in the blood-water barrier, which can reduce and even prevent the water passage through the secondary lamella, resulting or not in lamellar fusion and loss of respiratory surface, leading to fish death by anoxia (Mallatt, 1985). Accordingly, it was reported recently that high concentrations of the essential oil of *Mentha piperita*, that contains limonene in its composition, caused edema and lamellar fusion in the silver catfish gills, and their consequent death (Spanghero et al., 2019).

Additionally, the current study described that S-(-)-limonene also induced an increase in the number of ionocytes as well as in the number and the size of mucous cells. Ionocytes are usually found in the filament, lamellar base or interlamellar regions. They are fundamental to ionoregulation, being the site of Na^+ and Cl^- absorption in freshwater teleosts (Laurent et al., 1985; Laurent and Perry, 1991). Although ionocytes proliferation has been considered to be

399 helpful to maintain fish homeostasis, when in excessive amounts, they can compromise
400 respiration because they increase the blood-water barrier (Laurent and Perry, 1991). On the
401 other hand, mucous cells are also found in the filament. Like ionocytes, their proliferation is
402 considered to be beneficial for fish in order to protect lamellar surfaces against stressful
403 conditions, as infectious agents and toxicants (Mallatt, 1985). Mucous cells are also involved
404 in respiration and osmoregulation, thus, when in excess, they can also compromise these
405 processes (Mallatt, 1985; Powell et al., 1992). The exposure of zebrafish (*Danio rerio*) to high
406 concentrations of perillyl alcohol, a monoterpene found in citrus essential oils, triggered
407 hyperplasia of epithelial cells, edema, raising in the number of ionocytes and the size of mucous
408 cells, promoting an increase in the toxicant-blood diffusion distance, thus reducing the
409 respiratory surface of the gills. According the authors, although it did not happen in their
410 exposure model, further loss of this surface or lamellar fusion could induce fish death through
411 asphyxia due to the collapse and disappearance of the lamella (De Souza et al., 2016).
412 Therefore, such alterations appeared to be the responsible for some S-(-)-limonene-induced
413 deaths of silver catfish observed during the present investigation.

414 A research from our group showed that the chirality of the linalool, a monoterpene
415 isolated from essential oils, influenced its biological activities in silver catfish (Silva et al.,
416 2017). Now, using limonene isomers, the present study found that chirality also interferes on
417 its biological properties. So, it was observed that, differently from S-(-)-limonene, its
418 enantiomer, the R-(+)-limonene form, at both studied concentrations, did not cause fish death.
419 Nevertheless, it is noticeable that there is a difference between both tested concentrations in
420 healthy animals: at the highest concentration (20 μl/L), R-(+)-limonene triggers morphometric
421 and quantitative changes similar to those produced by gentamicin and ethanol exposure, as
422 increased filamentous epithelium thickness and lamellar thickness as well as reduced lamellar
423 length; unlike, at the lowest concentration (10 μL/L), it did not produce any alteration in the

424 parameters evaluated in the gills, maintaining their architecture, thus, being more proper for
425 utilization in healthy silver catfish, causing no toxicity evidenced through histological analysis.

426 Based on the fact that the exposure of silver catfish to S-(-)-limonene caused the
427 appearance of skin lesions and some deaths, this research continued in order to determine the
428 effects of the other treatments, including gentamicin, ethanol and R-(+)-limonene, on *A.*
429 *hydrophila*-induced histopathological changes in the silver catfish gills. Several *in vitro* assays
430 (Ngugi et al., 2017; Parlatan et al., 2009; Pintore et al., 2009; Souza et al., 2016; Thomas et al.,
431 2014) and *in vivo* studies using different fish species (Ngugi et al., 2017; Souza et al., 2016;
432 Thomas et al., 2014) have shown that essential oils containing limonene in their composition
433 have antibacterial properties, being also effective against *A. hydrophila* (Ngugi et al., 2017;
434 Parlatan et al., 2009; Pintore et al., 2009; Souza et al., 2016).

435 *Aeromonas hydrophila* produces important histopathological changes in the fish gills,
436 which can also be considered helpful biomarkers for the evaluation of the general health and
437 stress status of fish (Abdelhamed et al., 2017; Azadbakht et al., 2019; Carraschi et al., 2012;
438 Harikrishnan et al., 2008; Souza et al., 2016). The most frequent gills alterations reported were
439 inflammatory cells infiltration (Abdelhamed et al., 2017; Harikrishnan et al., 2008; Souza et al.,
440 2016), hypertrophy and hyperplasia of the epithelial cells (Azadbakht et al., 2019; Harikrishnan
441 et al., 2008), lamellar fusion (Azadbakht et al., 2019; Carraschi et al., 2012), lifting of the
442 epithelium and edema of lamella with large sub-epithelial space (Azadbakht et al., 2019;
443 Carraschi et al., 2012; Harikrishnan et al., 2008; Souza et al., 2016), hypertrophy (Azadbakht
444 et al., 2019), hyperplasia of the mucosal cells (Carraschi et al., 2012), ionocytes proliferation
445 (Carraschi et al., 2012), hemorrhage with blood congestion (Azadbakht et al., 2019;
446 Harikrishnan et al., 2008; Souza et al., 2016) and aneurysm (Carraschi et al., 2012).

Accordingly, the current research showed that non-treated *A. hydrophila* infection triggered changes in all morphometric and quantitative parameters evaluated in silver catfish gills through histological analysis, including an increase in filamentous epithelium thickness and connective tissue thickness, inflammatory infiltration into the connective tissue, intense lamellar fusion, and finally a reduction in the interlamellar space. Such changes are understood as examples of defense mechanisms and strategies of environmental adaptation when the conditions are harmful and constant (Strzyżewska et al., 2016). It is known that interlamellar epithelium proliferation and lamellar fusion can decrease gill surface area and increase the diffusion barrier for pathogens (Carraschi et al., 2012). The present investigation also revealed that *A. hydrophila* induced an increase in the number of ionocytes and in the number and the size of mucous cells. The abnormal increase of ionocytes in the interlamellar spaces is consistent with altered ion fluxes in the gills during exposure to pathogens (Kumar et al., 2016). Furthermore, mucous cells produce mucus, which can act as a protective barrier against the penetration of pathogenic agents in the respiratory epithelium (Mallatt, 1985). Thus the increase of the mucous cells observed in the current research can result in mucus hypersecretion in order to protect silver catfish gill epithelium against *A. hydrophila*.

The changes observed in non-treated *A. hydrophila* infected fish continued to be evidenced in the silver catfish gills even after their exposure to treatments, as gentamicin and ethanol. Moreover, as a result from the excessive mucus secretion, a breathing difficulty was observed in these animals, characterized by the agglomeration of them on the surface of the water with a half-open mouth. Mucus in excessive amounts does not protect the gill structures from local tissue stress and ends up compromising fish respiration (Mallatt, 1985). The infected silver catfish exposed to gentamicin also exhibited other alterations as lamellar edema, lamellar hypertrophy and blood vessels congestion, resulting in a loss of functional integrity of gill tissue. Lamellar hypertrophy can be related to the need to increase the gas exchange surface

and can result from the disruption of the pillar and pavement cells, causing a disarrangement in the lamellar modelling and a shortening of the lamella (Bernet et al., 2001). Additionally, pillar cells, when disrupted due to the direct effects of pathogens, can cause an increased blood flow inside the lamella and influence the dilatation of the marginal channel, blood vessels congestion and aneurysm (Azadbakht et al., 2019). The present investigation noticed aneurysm, the most severe kind of damage, only in the gills of non-treated *A. hydrophila* infected fish and in infected animals exposed to ethanol. Thus, it seemed that ethanol did not act as antimicrobial agent, since it was not able to prevent *A. hydrophila*-induced morphological changes. Likewise, it was shown that the exposure of parasites-infected tambaqui (*Colossoma macropomum*) to ethanol did not prevent against the pathogens-induced severe alterations in their gills, including hyperplasia and fusion of lamellar gill epithelium, vasodilatation, detachment of the lamellar epithelium and lamellar aneurysm, epithelial breakdown with hemorrhage, congestion, edema and necrosis (Soares et al., 2017b, 2016).

Differently from gentamicin and ethanol, this investigation revealed that R-(+)-limonene protected the silver catfish gills against the *A. hydrophila*-induced structure alterations. R-(+)-limonene, at both concentrations, prevents the increase in filamentous epithelium thickness, connective tissue thickness, and lamellar thickness, as well as the decrease in lamellar length and interlamellar space and the increase in number of mucous cells and ionocytes in the gills of *A. hydrophila*-challenged silver catfish. Accordingly, lime oil nanoemulsion, rich in limonene, avoided the severe damage to the gills of Mozambique tilapia (*Oreochromis mossambicus*) and destruction of primary and secondary lamella triggered by *Pseudomonas aeruginosa* infection (Thomas et al., 2014).

Moreover, it was noticeable that both R-(+)-limonene concentrations also exhibited anti-inflammatory effects, since there was no evidence of edema, blood vessels congestion or

496 inflammatory cells infiltration into the connective tissue. The anti-inflammatory properties of
497 R-(+)-limonene are well described in the literature through *in vitro* (Yoon et al., 2010) and *in*
498 *vivo* studies (Chi et al., 2013) using the lipopolysaccharide-induced inflammation model.
499 Recently, an investigation of our group using the essential oil of *Melaleuca alternifolia*, that
500 contains limonene in its composition, also showed that such essential oil possessed anti-
501 inflammatory effects, since it reduced inflammatory cells infiltration and blood congestion in
502 the gills of *A. hydrophila*-challenged silver catfish (Souza et al., 2016).

503 However, only at the lowest concentration (10 μL/L), R-(+)-limonene restored most
504 morphometric and quantitative parameters, including connective tissue thickness, lamellar
505 thickness, interlamellar space, number of mucous cells and ionocytes, reestablishing the normal
506 gill architecture after *A. hydrophila* infection, which was similar to that found in the healthy
507 silver catfish. Thus, taking into account all functions performed by mucous cells and ionocytes
508 that were mentioned above, it can be suggested that it is probable that R-limonene could be able
509 to maintain the gill function on osmoregulation.

510 Therefore, although several studies found in the literature indicated that *A. hydrophila*
511 isolates from silver catfish are highly sensible to gentamicin (Andrade et al., 2006; Barcellos et
512 al., 2008; Costa et al., 2008), the current research, for the first time, showed that although such
513 substance is widely used in the treatment of *A. hydrophila* infection, it was not able to prevent
514 against *A. hydrophila*-induced inflammation and severe histopathological changes to the gills.
515 The toxic properties of gentamicin are known only on fish kidney. It was exhibited, through
516 histopathological analysis, that it can induce tubular necrosis in Nile tilapia (Augusto et al.,
517 1996). Thus, R-(+)-limonene stands out as a natural alternative to gentamicin in order to control
518 *A. hydrophila*-induced morphological changes because it acted protecting silver catfish gills
519 from such modifications.

Finally, in conclusion, among all substances tested in healthy silver catfish, S-(-)-limonene, at 10 or 20 μL/L, seems to be the most irritant, triggering death, inflammation and altering most morphometric and quantitative parameters studied in their gills through histological analysis. Thus, it is suggested that, in future studies, lower concentrations of S-(-)-limonene should be tested in order to determine its therapeutic potential. Conversely, R-(+)-limonene preserves the structure of the gills, being more proper for utilization in healthy silver catfish, specially at 10 μL/L. Furthermore, R-(+)-limonene at both concentrations (10 or 20 μL/L) exerts an anti-inflammatory effect on the gills of *A. hydrophila*-challenged silver catfish, since no signal of edema, blood vessels congestion or inflammatory cells infiltration into the connective tissue are noted. Besides, R-(+)-limonene at 10 μL/L also appears to be more suitable for treating *A. hydrophila*-induced histopathological alterations, since it reestablishes the normal gill architecture.

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714

715 **Figure captions**

716

717 **Fig. 1. Histological parameters analyzed in the silver catfish gills through light**
718 **microscopy.** Representative image of Masson-Goldner trichrome histological staining in the
719 gill sections of healthy sliver catfish, 600x (bar = 30 μm). Asterisk: mucous cells. Arrow:
720 ionocytes. Abbreviations: FT, filamentous epithelium thickness; CTT, connective tissue
721 thickness; LT, lamellar thickness; LL, lamellar length; IS, interlamellar space.

722

723 **Fig. 2. Effects of R-(+)-limonene and S-(-)-limonene on the histopathological**
724 **findings observed in the healthy silver catfish gills.** Representative images of Masson-
725 Goldner trichrome histological staining in the gill sections of healthy silver catfish exposed to
726 the water (control) (A), gentamicin (B), ethanol (C), 10 μL/L R-(+)-limonene (D), 20 μL/L R-
727 (+)-limonene (E), 10 μL/L S-(-)-limonene (F), 20 μL/L S-(-)-limonene (G), 400x (bar = 40 μm).
728 Asterisk: mucous cells. Arrow: ionocytes. Abbreviations: BLE, basal lamellar edema; DLE,
729 distal lamellar edema; ICI, inflammatory cells infiltration; LF, lamellar fusion.

730

731 **Fig. 3. Effects of R-(+)-limonene and S-(-)-limonene on the morphometric analysis**
732 **of the filament and connective tissue in the healthy silver catfish gills.** Filamentous
733 epithelium thickness (A) and connective tissue thickness (B) in the gills of healthy silver catfish
734 exposed to water (control), gentamicin, ethanol, 10 μL/L R-(+)-limonene, 20 μL/L R-(+)-
735 limonene, 10 μL/L S-(-)-limonene and 20 μL/L S-(-)-limonene. Data are showed as mean ±
736 standard error (*n*=8). One-way Anova and Tukey Test, $p < 0.05$. Distinct letters indicate a
737 significant difference between treatments.

Fig. 4. Effects of R-(+)-limonene and S-(-)-limonene on the morphometric analysis of the lamella in the healthy silver catfish gills. Lamellar thickness (A), lamellar length (B) and interlamellar space (C) in the gills of healthy silver catfish exposed to water (control), gentamicin, ethanol, 10 μL/L R-(+)-limonene, 20 μL/L R-(+)-limonene, 10 μL/L S-(-)-limonene and 20 μL/L S-(-)-limonene. Data are showed as mean ± standard error (*n*=8). One-way Anova and Tukey Test, $p < 0.05$. Distinct letters indicate a significant difference between treatments.

Fig. 5. Effects of R-(+)-limonene and S-(-)-limonene on the morphometric and quantitative analysis of mucous cells in the healthy silver catfish gills. Mucous cells number (A) and mucous cells size (B) in the gills of healthy silver catfish exposed to water (control), gentamicin, ethanol, 10 μL/L R-(+)-limonene, 20 μL/L R-(+)-limonene, 10 μL/L S-(-)-limonene and 20 μL/L S-(-)-limonene. Data are showed as mean ± standard error (*n*=8). One-way Anova and Tukey Test, $p < 0.05$. Distinct letters indicate a significant difference between treatments.

Fig. 6. Effects of R-(+)-limonene and S-(-)-limonene on the quantitative analysis of ionocytes in the healthy silver catfish gills. Ionocytes number in the gills of healthy silver catfish exposed to water (control), gentamicin, ethanol, 10 μL/L R-(+)-limonene, 20 μL/L R-(+)-limonene, 10 μL/L S-(-)-limonene and 20 μL/L S-(-)-limonene. Data are showed as mean ± standard error (*n*=8). One-way Anova and Tukey Test, $p < 0.05$. Distinct letters indicate a significant difference between treatments.

Fig. 7. Effects of R-(+)-limonene on the histopathological findings observed in the gills of *A. hydrophila*-infected silver catfish. Representative images of Masson-Goldner trichrome histological staining in the gill sections of healthy silver catfish exposed to water (healthy control) (A), gentamicin (B), ethanol (C), 10 μL/L R-(+)-limonene (D) and 20 μL/L R-(+)-limonene (E), 200x (bar = 20 μm), and *A. hydrophila*-infected silver catfish exposed to water (infected control) (F), gentamicin (G), ethanol (H), 10 μL/L R-(+)-limonene (I) and 20 μL/L R-(+)-limonene (J), 400x (bar = 40 μm). Asterisk: mucous cells. Arrow: ionocytes. Circle: Ionocytes *clusters*. Abbreviations: BLE, basal lamellar edema; DLE, distal lamellar edema; ICI, inflammatory cells infiltration; LF, lamellar fusion; LH, lamellar hipertrophy; VC, blood vessels congestion.

Fig. 8. Effects of R-(+)-limonene on the morphometric analysis of the filament and connective tissue in the gills of *A. hydrophila*-infected silver catfish. Filamentous epithelium thickness (A) and connective tissue thickness (B) in the gills of healthy and *A. hydrophila*-infected (I) silver catfish exposed to water (healthy and infected controls, respectively), gentamicin, ethanol, 10 μL/L R-(+)-limonene and 20 μL/L R-(+)-limonene. Data are showed as mean ± standard error (n=8). Two-way Anova and Tukey Test, $p < 0.05$. Different lowercase letters indicate significant difference between the healthy groups. Different uppercase letters indicate significant difference between the *A. hydrophila* infected groups. Asterisk (*) indicates a significant difference from the respective healthy group. Number sign (#) indicates a significant difference from the healthy control group.

Fig. 9. Effects of R-(+)-limonene on the morphometric analysis of the lamella in the gills of *A. hydrophila*-infected silver catfish. Lamellar thickness (A), lamellar length (B) and interlamellar space (C) in the gills of healthy and *A. hydrophila*-infected (I) silver catfish exposed to water (healthy and infected controls, respectively), gentamicin, ethanol, 10 μL/L R-(+)-limonene and 20 μL/L R-(+)-limonene. Data are showed as mean ± standard error (*n*=8). Two-way Anova and Tukey Test, $p < 0.05$. Different lowercase letters indicate significant difference between the healthy groups. Different uppercase letters indicate significant difference between the *A. hydrophila* infected groups. Asterisk (*) indicates a significant difference between healthy and *A. hydrophila* infected groups. Number sign (#) indicates a significant difference in relation to the healthy control group.

Fig. 10. Effects of R-(+)-limonene on the morphometric and quantitative analysis of mucous cells in the gills of *A. hydrophila*-infected silver catfish. Mucous cells number (A) and mucous cells size (B) in the gills of healthy and *A. hydrophila*-infected (I) silver catfish exposed to water (healthy and infected controls, respectively), gentamicin, ethanol, 10 μL/L R-(+)-limonene and 20 μL/L R-(+)-limonene. Data are showed as mean ± standard error (*n*=8). Two-way Anova and Tukey Test, $p < 0.05$. Different lowercase letters indicate significant difference between the healthy groups. Different uppercase letters indicate significant difference between the *A. hydrophila* infected groups. Asterisk (*) indicates a significant difference between healthy and *A. hydrophila* infected groups. Number sign (#) indicates a significant difference in relation to the healthy control group.

Fig. 11. Effects of R-(+)-limonene on the quantitative analysis of ionocytes in the gills of *A. hydrophila*-infected silver catfish. Ionocytes number in the gills of healthy and *A.*

808 *hydrophila*-infected (I) silver catfish exposed to water (healthy and infected controls,
809 respectively), gentamicin, ethanol, 10 μL/L R-(+)-limonene and 20 μL/L R-(+)-limonene. Data
810 are showed as mean ± standard error (*n*=8). Two-way Anova and Tukey Test, $p < 0.05$. Different
811 lowercase letters indicate significant difference between the healthy groups. Different
812 uppercase letters indicate significant difference between the *A. hydrophila* infected groups.
813 Asterisk (*) indicates a significant difference between healthy and *A. hydrophila* infected
814 groups. Number sign (#) indicates a significant difference in relation to the healthy control
815 group.

816 **Supplementary figure captions**

817

818 **Fig. S1. Visualization of ionocytes stained with toluidine blue**. Representative
819 images of 1% toluidine blue histological staining in the gill section of healthy silver catfish
820 exposed to water (healthy control) (A) and *A. hydrophila*-infected silver catfish exposed to
821 water (infected control) (B), 400x (bar = 40 μm). Arrow: ionocytes.

822

823 **Fig. S2. Visualization of aneurysms in the gills of *A. hydrophila*-infected silver**
824 **catfish.** Representative images of Masson-Goldner trichrome histological staining in the gill
825 sections of *A. hydrophila*-infected silver catfish exposed to water (infected control) (A) and
826 ethanol (B), 400x (bar = 40 μm). Abbreviations: A, aneurysm.

Fig. 1

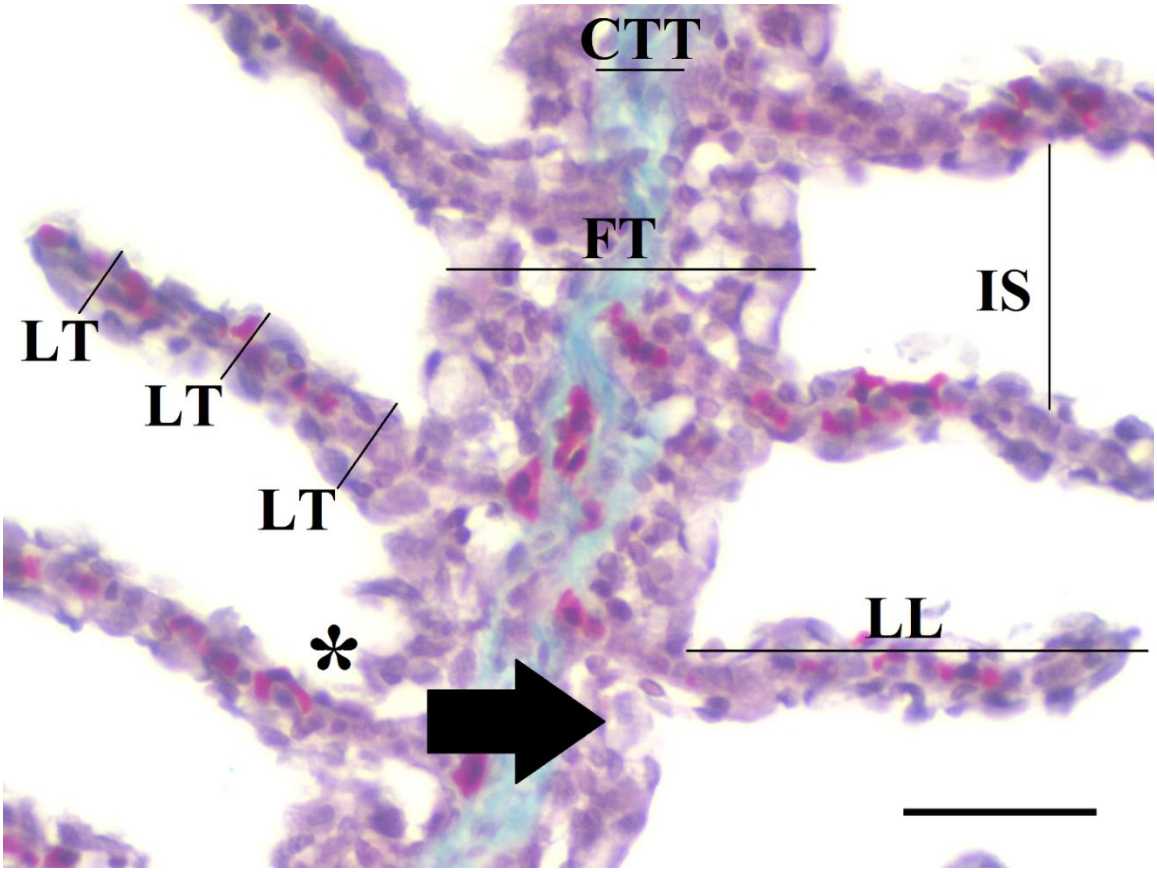


Fig. 2

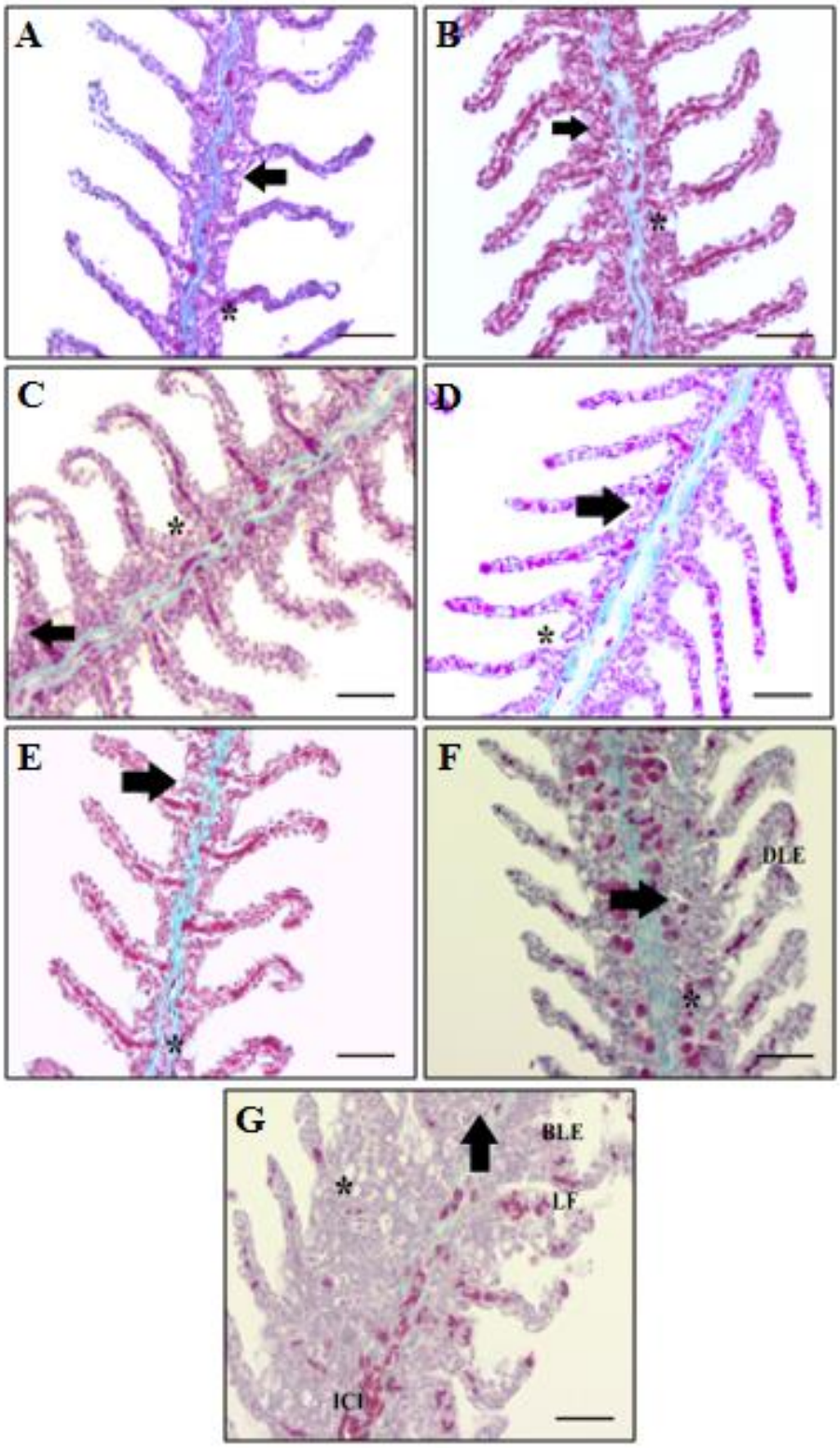


Fig. 3

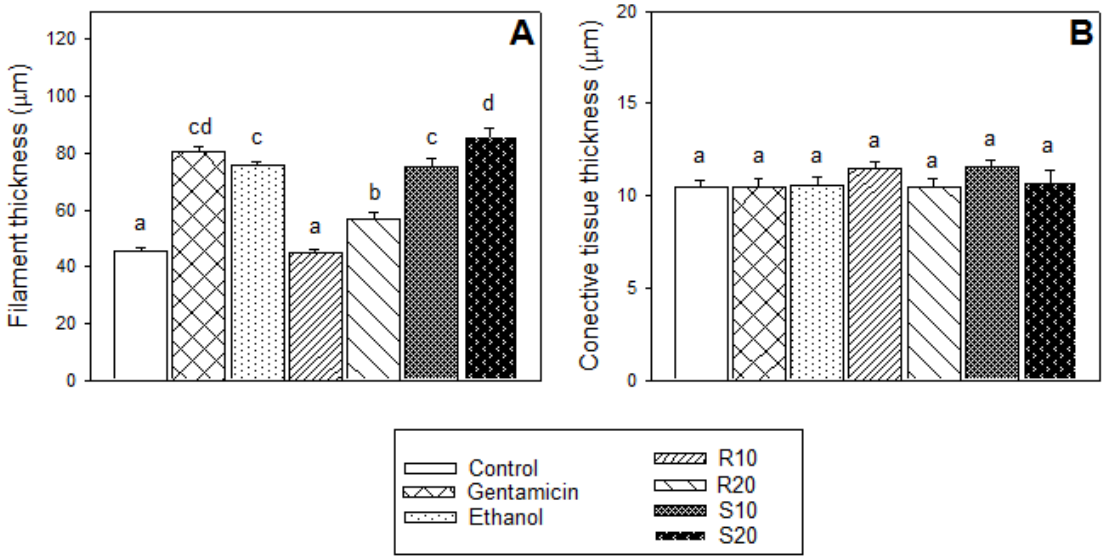

A
B
Filament thickness (µm)
Conective tissue thickness (µm)
Control
Gentamicin
Ethanol
R10
R20
S10
S20

Fig. 4

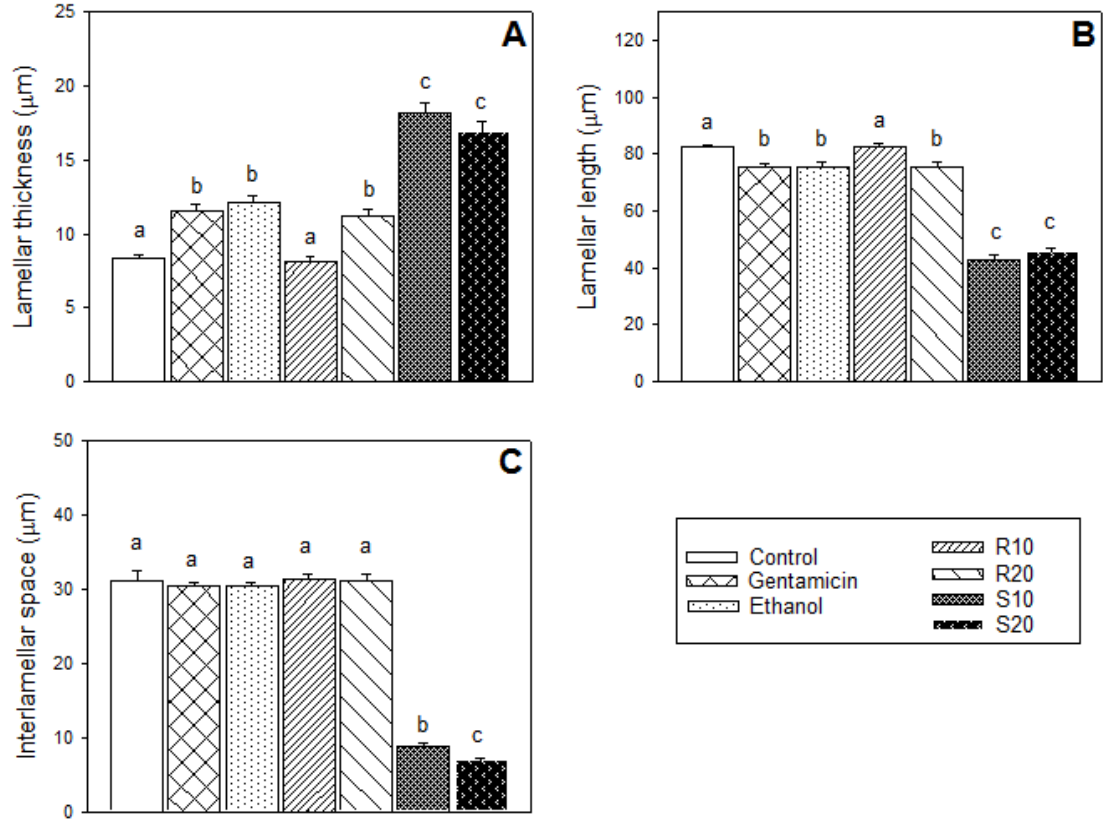

A
B
C
Lamellar thickness (µm)
Lamellar length (µm)
Interlamellar space (µm)
Control
Gentamicin
Ethanol
R10
R20
S10
S20

Fig. 5

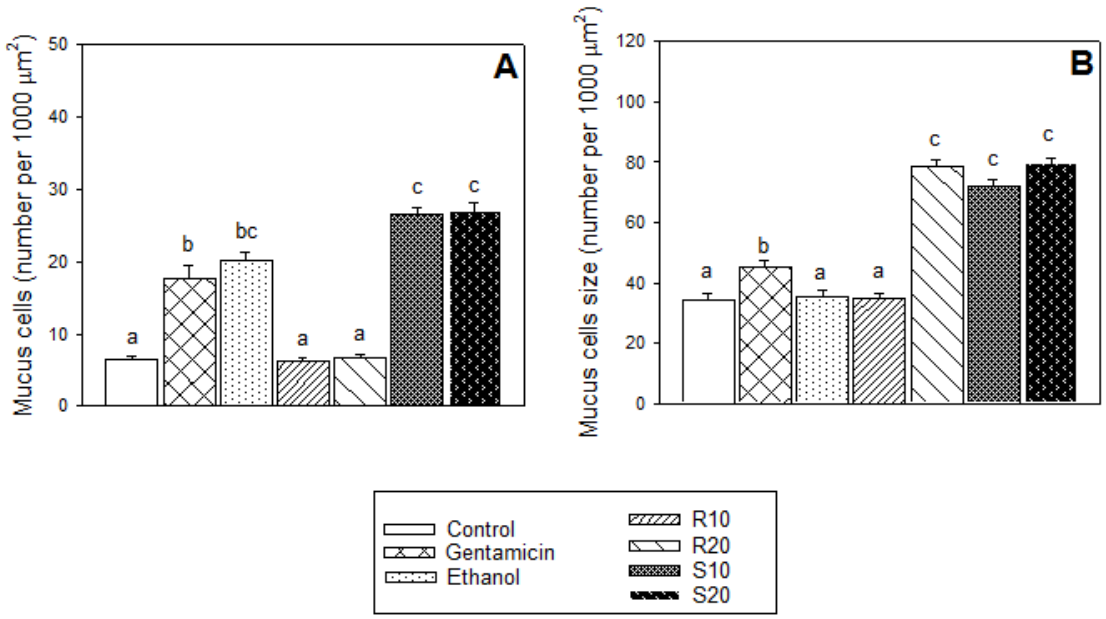

A
B
Mucus cells (number per 1000 µm²)
Mucus cells size (number per 1000 µm²)
a
b
bc
a
a
c
c
a
b
a
a
c
c
c
Control
Gentamicin
Ethanol
R10
R20
S10
S20

Fig. 6

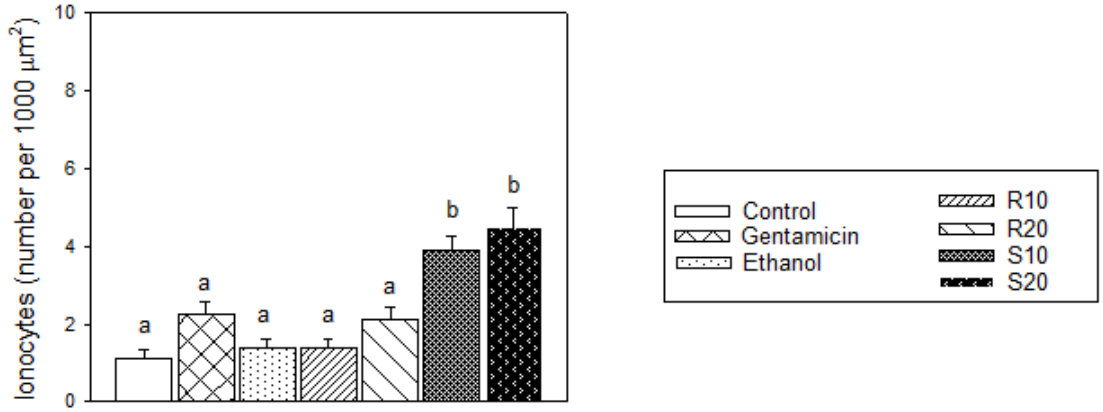

Ionocytes (number per 1000 µm²)
a
a
a
a
a
b
b
Control
Gentamicin
Ethanol
R10
R20
S10
S20

Fig. 7

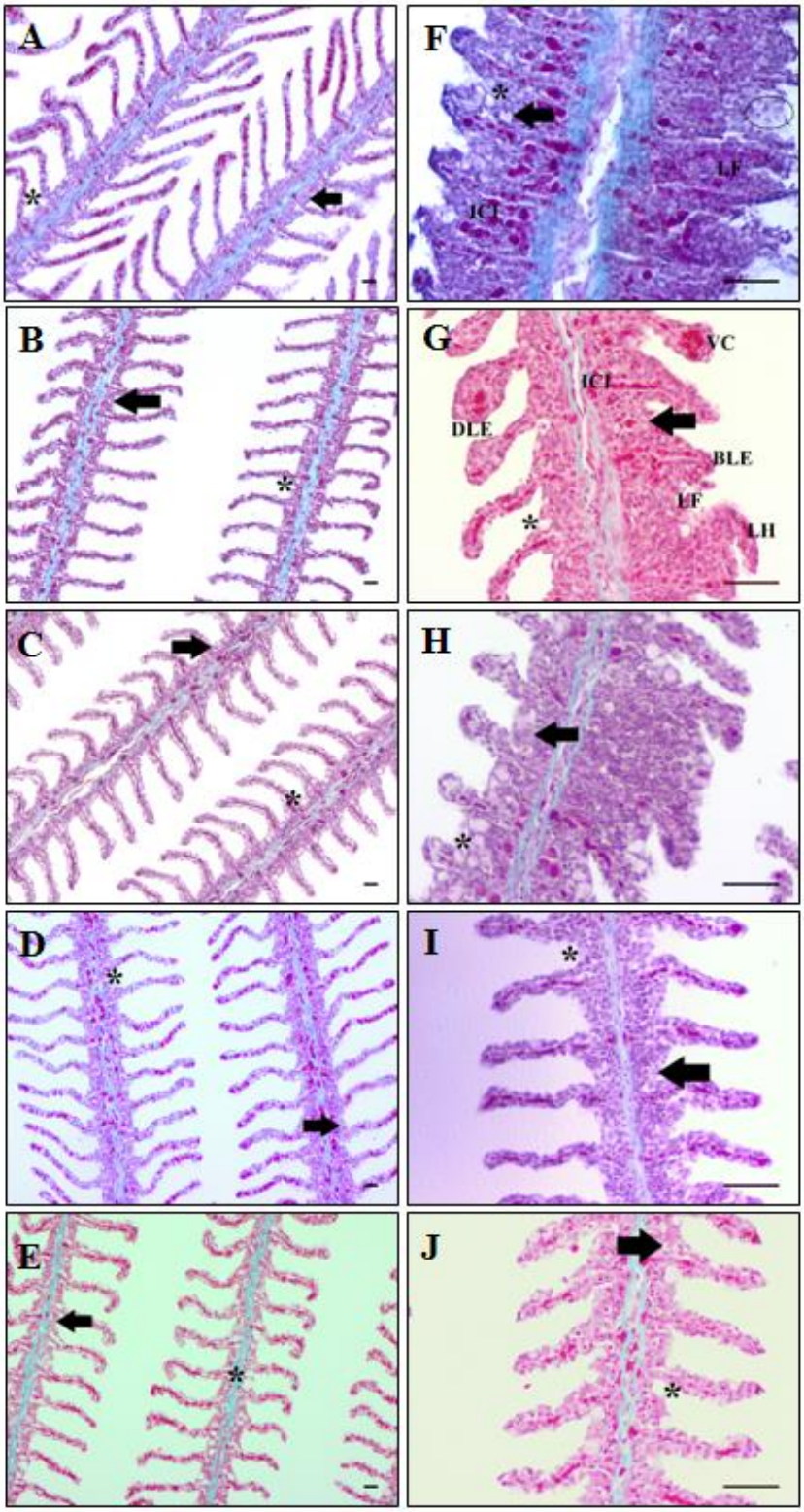


Fig. 8

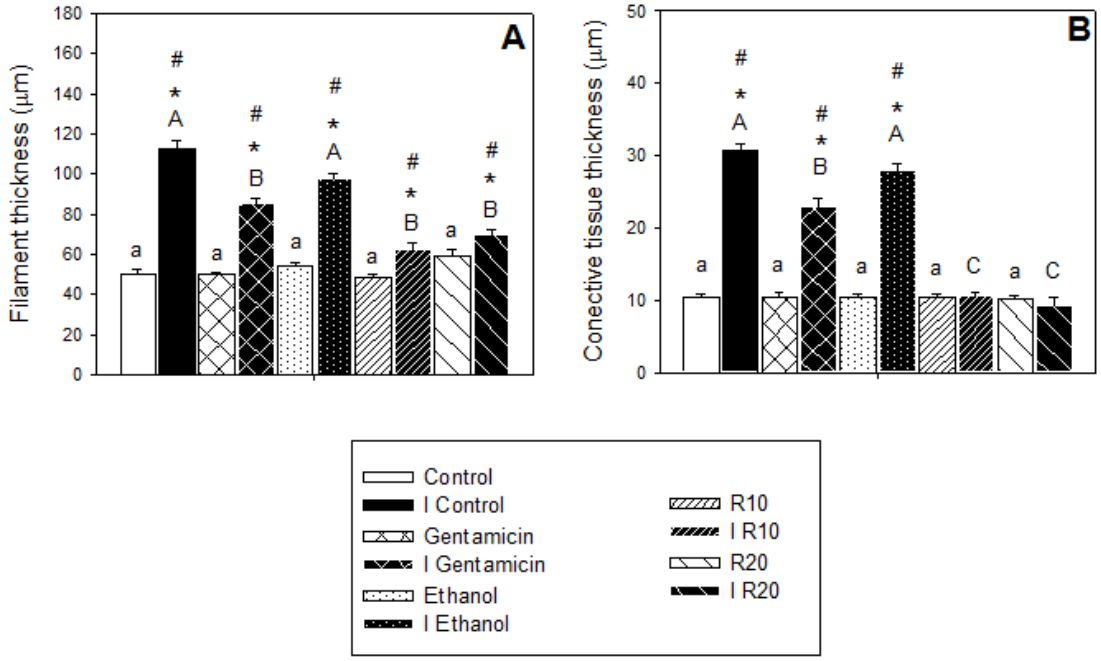

A
B
Filament thickness (µm)
Conective tissue thickness (µm)
Control
I Control
Gentamicin
I Gentamicin
Ethanol
I Ethanol
R10
I R10
R20
I R20

Fig. 9

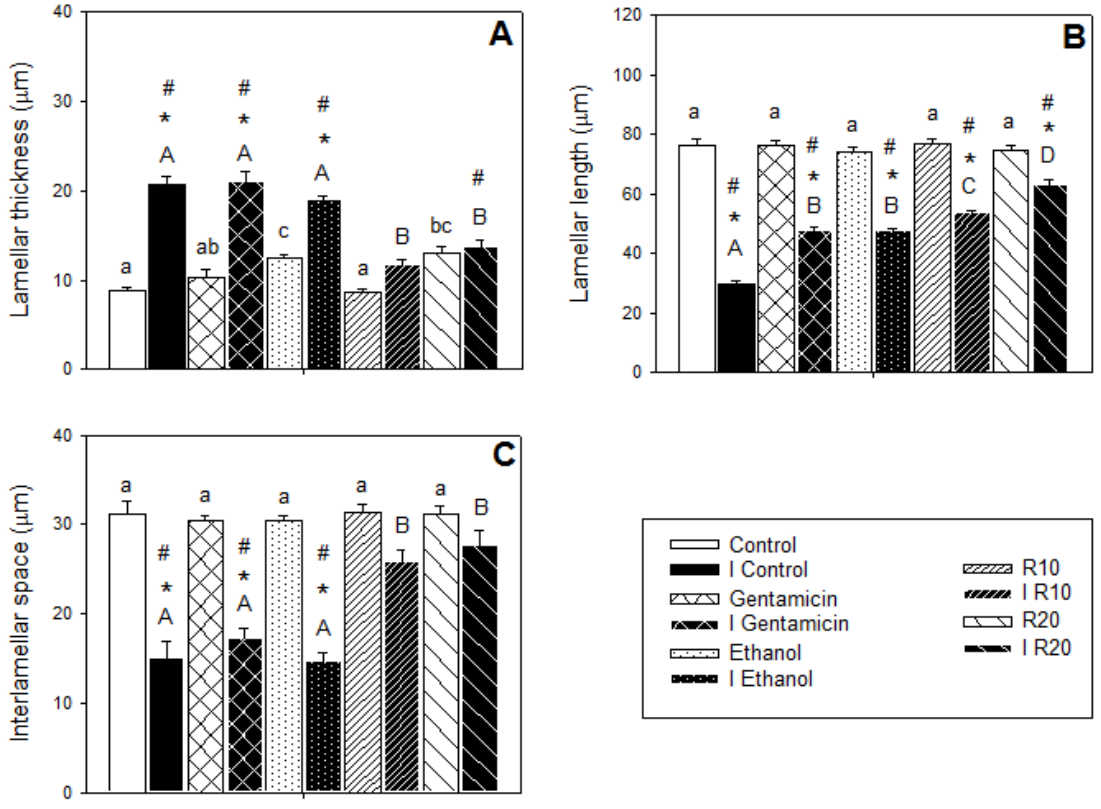

A
B
C
Lamellar thickness (µm)
Lamellar length (µm)
Interlamellar space (µm)
Control
I Control
Gentamicin
I Gentamicin
Ethanol
I Ethanol
R10
I R10
R20
I R20

Fig. 10

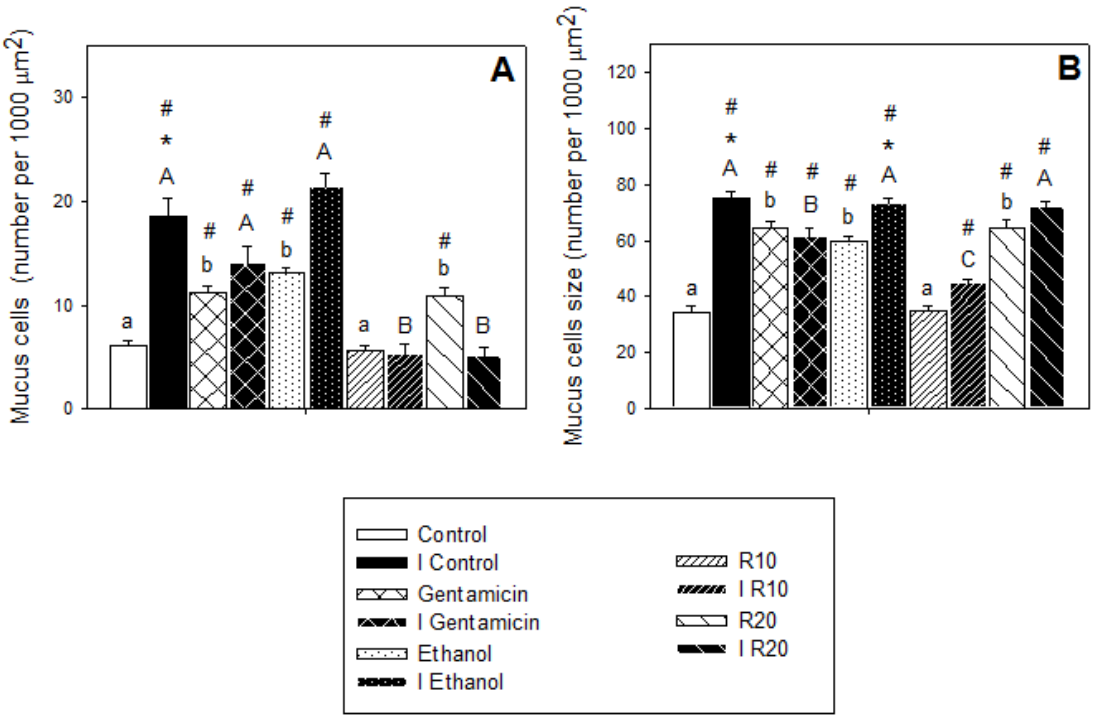

A
B
Mucus cells (number per 1000 µm2)
Mucus cells size (number per 1000 µm2)
Control
I Control
Gentamicin
I Gentamicin
Ethanol
I Ethanol
R10
I R10
R20
I R20

Fig. 11

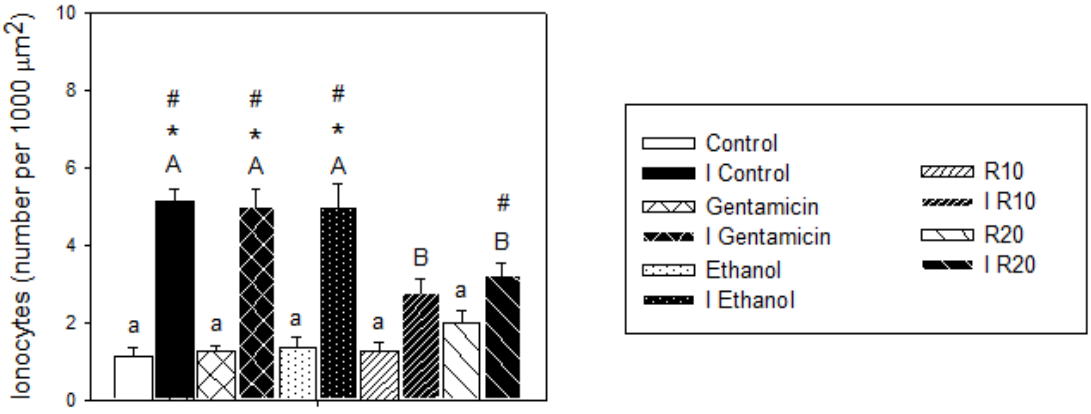

Ionocytes (number per 1000 µm2)
Control
I Control
Gentamicin
I Gentamicin
Ethanol
I Ethanol
R10
I R10
R20
I R20

Fig. S1

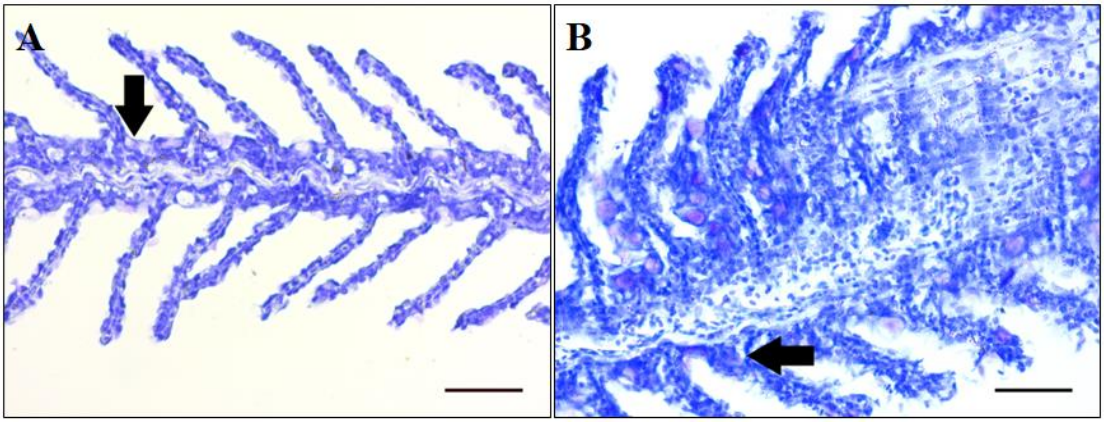
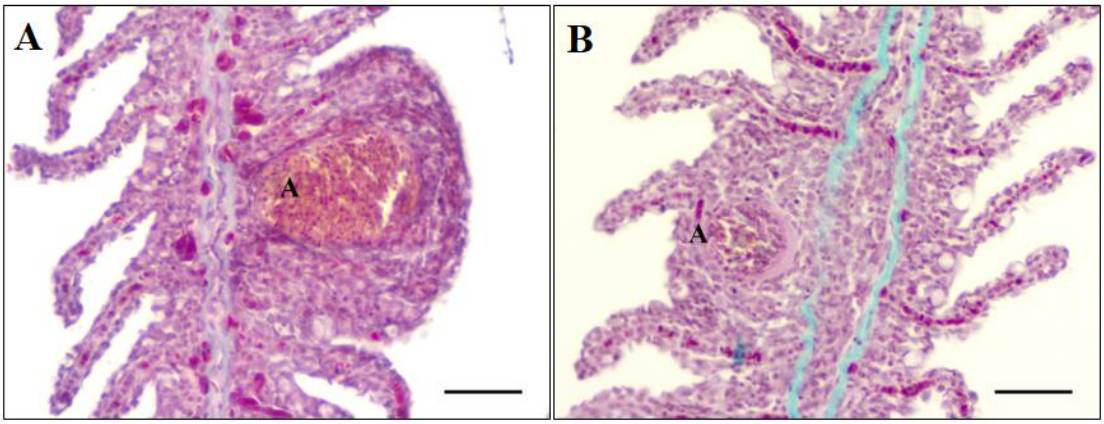


Fig. S2



5 CONCLUSÕES

Os peixes expostos ao S-(-)-limoneno apresentam sinais de inflamação e alterações morfométricas e quantitativas na histologia branquial. O R-(+)-limoneno mantém a estrutura normal das brânquias em bagres prateados saudáveis. Nos peixes desafiados com *Aeromonas hydrophila* e tratados com R-(+)-limoneno, este isômero exerce um efeito anti-inflamatório nas brânquias uma vez que não foi observado edema, congestão dos vasos sanguíneos e infiltração de células inflamatórias no tecido conjuntivo. Assim, sugere-se que o S-(-)-limoneno é irritante para *Rhamdia quelen* e menores concentrações precisam ser testadas para determinar seu potencial protetor. Por outro lado, o R-(+)-limoneno, demonstra ser mais adequado, inclusive no tratamento de alterações histopatológicas induzidas por *A. hydrophila,* pois preserva a arquitetura branquial.

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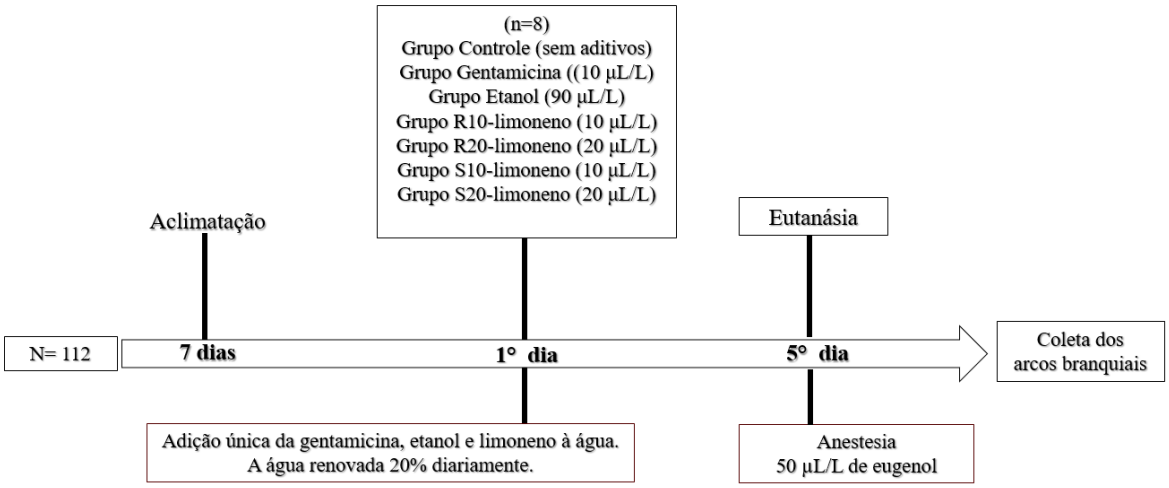
ANEXO A

DELINEAMENTO EXPERIMENTAL 1



ANEXO B

DELINEAMENTO EXPERIMENTAL 2