UNIVERSIDADE ESTADUAL PAULISTA

FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

CÂMPUS DE BOTUCATU

EFEITOS DE *QUILLAJA SAPONARIA*, *YUCCA SCHIDIGERA* E *SACCHAROMYCES CEREVISIAE* NO DESEMPENHO PRODUTIVO E SAÚDE DE TILÁPIAS-DO-NILO (*OREOCHROMIS NILOTICUS*) SUBMETIDAS A DIFERENTES TIPOS DE ESTRESSE

MATHEUS GARDIM GUIMARÃES

Tese apresentada ao Programa de Pós-graduação em Zootecnia como parte dos requisitos para obtenção do título de Doutor

BOTUCATU – SP

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Orientadora: Profª. Ass. Dra. Margaria Maria Barros

Coorientador: Dr. Pedro Luiz Pucci Figueiredo de Carvalho

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UNESP BOTUCATU Seção de Pós-Graduação

ATESTADO DE APROVAÇÃO - DEFESA

Atestamos que **MATHEUS GARDIM GUIMARÃES**, RA nº: ZNP200174, RG nº 45.094.299-5, expedido pela SSP/SP, defendeu, no dia 15/03/2024, a tese intitulada **Efeitos de Quillaja saponaria, Yucca schidigera e Saccharomyces cerevisiae no desempenho produtivo e saúde de tilápias-do-Nilo (Oreochromis niloticus) submetidas a diferentes tipos de estresse**, junto ao Programa de Pós Graduação em Zootecnia, Curso de Doutorado, tendo sido 'APROVADO'.

Atestamos ainda que a obtenção do título dependerá de homologação pelo Órgão Colegiado competente.

Botucatu, 15 de março de 2024

Claudia Cristina Moreci

Cláudia Cristina Moreci
Assistente Administrativo
Seção de Pós-Graduação / FMVZ

BIOGRAFIA DO AUTOR

Matheus Gardim Guimarães, filho de Renato Ferreira Guimarães e Fabiana Gardim Guimarães, nasceu no município de Caieiras, São Paulo, no dia 31 de julho de 1995. Ingressou no curso de graduação em Zootecnia no Universidade Estadual Paulista *"Júlio de Mesquita Filho"*, Câmpus de Botucatu em março de 2013 e concluiu em 01 de dezembro de 2017.

No ano seguinte, ingressou no Programa de Pós-graduação em Zootecnia, Curso de Mestrado, na Universidade Estadual Paulista *"Júlio de Mesquita Filho"*, Câmpus de Botucatu, concentrando seus estudos na área de Nutrição e Saúde de Peixes, sendo que a pesquisa desenvolvida avaliou a capacidade antioxidante e o desempenho produtivo de tilápia-do-Nilo alimentada com dietas suplementadas com farinha de bagaço de uva e resveratrol. Obteve o título de Mestre no dia 29 de maio de 2020.

Em agosto de 2020, ingressou no curso de doutorado em Zootecnia pela mesma instituição e desenvolveu projeto de pesquisa no Laboratório de Nutrição e Saúde de Peixes orientado pela Profª. Ass. Dra. Margarida Maria Barros e coorientado pelo Dr. Pedro Luiz Pucci Figueiredo de Carvalho. A pesquisa desenvolvida investiga os efeitos de *Quillaja saponaria*, *Yucca schidigera* e *Saccharomyces cerevisiae* no desempenho produtivo e saúde de tilápias-do-Nilo (*Oreochromis niloticus*) submetidas a diferentes tipos de estresse.

No dia 15 de março de 2024 obteve o título de Doutor em Zootecnia.

“Não estamos aqui para fazer o fácil; estamos aqui para fazer o difícil. ”

Paulo Cavalcante Muzy

DEDICATÓRIA

Aos meus pais,

Renato Ferreira Guimarães e Fabiana Gardim Guimarães por tudo que fazem, fizeram e se precisar fazem o impossível por mim, por todos os ensinamentos e conselhos e, principalmente, por serem meus melhores amigos, amo vocês mais que uma rua!

À minha esposa,

Michele Alves de Sousa Guimarães, amor da minha vida, minha melhor amiga, companheira, que sempre está lá para dividir tudo que se passa comigo ou com ela, que está sempre comigo para me erguer e deixar meu dia melhor. Te amo demais minha vida!

À

Toda minha família e amigos, que mesmo com a distância de alguns sempre que estamos juntos, é maravilhoso, além de sempre me incentivarem e estarem sempre lá para o que der e vier.

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Agradeço primeiramente a Deus e a Nossa Senhora Aparecida, pela vida e saúde para me levantar e fazer o que é necessário e por sempre me dar forças, paz, sabedoria e direcionamento para conseguir conquistar todos os meus objetivos.

Aos meus pais Renato Ferreira Guimarães e Fabiana Gardim Guimarães, que fizeram e fazem de tudo para que eu consiga seguir no meu objetivo e na minha carreira como pesquisador e, principalmente, em ser profissional, além de me ajudar em seguir na área que amo, a Zootecnia, e mesmo eu tendo a pretensão de realizar o doutorado e sabendo da possibilidade que realizá-lo sem bolsa para me manter em outra cidade e em pleno ápice da pandemia do Covid19, me apoiaram de todas as maneiras. Com certeza eu não estaria onde estou hoje pretendendo ao título de Doutor em Zootecnia sem a amizade que temos e, principalmente, o amor que nos une, essa conquista é por vocês e para vocês. Amo vocês demais! Muito mais que uma rua!!!

À minha esposa, Michele Alves de Sousa Guimarães, amor da minha vida há 14 anos. Quando li os agradecimentos da minha dissertação ainda era minha noiva, e hoje você carrega a materialização do nosso amor no seu ventre, ele que foi tão desejado e já é muito amado. Mesmo tendo passado metade da minha vida ao seu lado, ainda estamos só no começo da nossa história juntos. Eu estar aqui hoje se deve muito a você, por ter encarado a empreitada de sair da casa dos seus pais, e morar comigo durante o decorrer do meu doutorado, além de buscar ter uma vida em Botucatu, com um bom emprego, e boas amizades. Não menos importante, em me apoiar e acalmar em momentos difíceis assim como meus pais faziam e fazem até hoje, como por exemplo nas duas vezes que perdi meu experimento de doutorado em que ambas estavam próximas da metade do período experimental. Espero, e farei de tudo para ter você sempre ao meu lado, portanto, mais uma vez, esta também é uma conquista por você e para você! Te amo demais minha vida!

À minha orientadora, Profª. Ass. Drª. Margarida Maria Barros, por me acolher em seu grupo de pesquisa desde o dia que fui pedir para dar uma palestra no grupo de estudos e posteriormente ao estágio, conseguimos uma iniciação científica (CNPq – PIBIC). Além disso me deu a oportunidade de realizar meu estágio de 5º ano no exterior (Auburn University, USA) e, posteriormente, aceitou me orientar no mestrado. No doutorado, logo abracei a oportunidade de realizar o experimento com empresa, que como dizemos “o prazo é para três anteontem” mas, infelizmente, não deu certo por duas vezes e, mesmo assim, não me deixou abalar e prontamente realizamos as reformas e manutenções necessárias para darmos prosseguimento. Além de tudo isso, me mostrar ainda mais o amor pela pesquisa. Não tenho

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O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001.

RESUMO GERAL

Com a expansão contínua da aquicultura e a adoção dos sistemas intensivos de produção, têm sido observado aumento da exposição dos peixes à fatores estressores, uso indiscriminado de antimicrobianos e comprometimento da saúde, o que pode resultar em perdas econômicas significativas. O uso errôneo de antimicrobianos pode afetar negativamente o ambiente além de selecionar agentes patogênicos resistentes. Na tentativa de contornar tais fatores, produtores buscam alternativas, sendo que os aditivos nutricionais se destacam. Desta forma, a utilização de aditivos eubióticos surge como alternativa promissora para promover o desempenho produtivo, resistência a manejos indispensáveis e respostas imunológicas dos peixes, além de permitir a redução no uso de antimicrobianos. Sendo assim, esta pesquisa teve como objetivos avaliar os efeitos da suplementação dietética de *Quillaja saponaria*, *Yucca schidigera* e *Saccharomyces cerevisiae* no desempenho produtivo, morfometria intestinal, digestibilidade, perfil antioxidante, respostas hematológicas e imunológicas de tilápias-do-Nilo (*Oreochromis niloticus*) submetidas a três desafios: estresse por classificação de tamanho, infeção por *Streptococcus agalactiae*, e infeção por *Streptococcus agalactiae* + 12 horas de hipóxia. Desta forma, a pesquisa foi dividida em três estudos. No primeiro estudo foi avaliada a digestibilidade das rações. Para tal foram utilizados 216 tilápias-do-Nilo machos (~ 80g) distribuídos em 12 tanques-(18 peixes/tanque) e alimentados com quatro dietas experimentais (dieta de controle, dieta suplementada com extrato de *Quillaja saponaria* + *Yucca schidigera*, dieta suplementada com *Saccharomyces cerevisiae* e a combinação de ambos) para coleta de fezes durante período de 9 dias e, posteriormente, cálculado o coeficiente de digestibilidade aparente. Para o segundo estudo, 600 tilápias-do-Nilo machos (~ 8,9 g) foram distribuídos aleatoriamente em 40 tanques de 250 L (15 peixes/tanque) e alimentados com as mesmas dietas anteriormente mencionadas, durante o período de 60 dias. Posteriormente, foram avaliados o desempenho produtivo, a composição bromatológica da carcaça e a morfometria intestinal. Além desses parâmetros foi analisado o perfil hematológico, resposta imune inata, atividade de enzimas do sistema antioxidante e parâmetros bioquímicos. Para tal, foram coletados sangue de dez peixes por tratamento. Os dados obtidos foram considerados antes dos desafios. Também após aos 60 dias, no terceiro estudo, grupos foram submetidos a diferentes tipos de estresse: um grupo de 60 peixes foi submetido a estresse por classificação de tamanho, outro grupo de 72 peixes a infeção por *Streptococcus agalactiae* (BAC) (15 dias) e, um outro grupo de 136 peixes, a infeção por *Streptococcus agalactiae* + 12 horas de hipóxia (BAC + HP) (15 dias). Após os desafios, as análises de perfil hematológico, resposta imune inata, atividade de enzimas do sistema antioxidante e parâmetros bioquímicos foram novamente realizadas, para gerar comparativo com o período sem desafio. Os dados foram submetidos à análise de variância (ANOVA) seguida do teste de Tukey e do teste t. As diferenças foram consideradas

estatisticamente significativas quando $P < 0,05$. Os peixes arraçoados com dietas suplementadas não apresentaram diferenças significativas no desempenho e apresentaram efeitos limitados nos coeficientes de digestibilidade aparente dos nutrientes e da energia. As dietas suplementadas com extrato de *Quillaja saponaria* + *Yucca schidigera* apresentaram maior nível de conteúdo fenólico, capacidade antioxidante e maior sobrevivência após BAC e BAC + HP, e a combinação de *Quillaja saponaria*, *Yucca schidigera* e *Saccharomyces cerevisiae*, o menor. A suplementação de *Saccharomyces cerevisiae* melhorou o comprimento das vilosidades do intestino médio e promoveu menor sobrevivência após BAC e BAC + HP. Os peixes alimentados com dietas suplementadas com *Saccharomyces cerevisiae* apresentaram melhores respostas imunitárias inata e capacidade antioxidante após desafios. Os resultados deste estudo sugerem que a suplementação dietética com *Saccharomyces cerevisiae* determinou melhores resultados gerais para saúde, mas não foi suficiente para evitar a mortalidade por infecção bacteriana. Além disso, mais estudos são necessários para validar a combinação de aditivos eubióticos a base de *Quillaja saponaria*, *Yucca schidigera*, *Saccharomyces cerevisiae* e suas combinações na nutrição dos peixes.

Palavras-chave: Aditivos eubióticos; imunonutrição; *Oreochromis niloticus*; sistemas intensivos de produção; *Streptococcus agalactiae*.

ABSTRACT

With the continuous expansion of aquaculture and the adoption of intensive production systems, there has been an increase in the exposure of fish to stress factors, indiscriminate use of antimicrobials and compromised health, which can result in significant economic losses. The misuse of antimicrobials can negatively affect the environment and select resistant pathogens. In an attempt to overcome these factors, producers are looking for alternatives, with nutritional additives standing out. In this way, the use of eubiotic additives has emerged as a promising alternative to promote growth performance, resistance to essential management and immune responses in fish, as well as allowing a reduction in the use of antimicrobials. The aim of this study was to evaluate the effects of dietary supplementation with *Quillaja saponaria*, *Yucca schidigera* and *Saccharomyces cerevisiae* on growth performance, intestinal morphometry, digestibility, antioxidant profile, hematological and immunological responses of Nile tilapia (*Oreochromis niloticus*) subjected to three challenges: size-sorting-induced stress, *Streptococcus agalactiae* infection, and *Streptococcus agalactiae infection* + 12 hours of hypoxia. The research was divided into three studies. In the first study, the digestibility of the feed was assessed. For this purpose, 216 male Nile tilapia (~ 80g) were distributed in 12 tanks (18 fish/tank) and fed four experimental diets (control diet, diet supplemented with *Quillaja saponaria* + *Yucca schidigera* extract, diet supplemented with *Saccharomyces cerevisiae* and a combination of both) to collect feces over a 9-day period and then calculate the apparent digestibility coefficient. For the second study, 600 male Nile tilapia (~ 8.9 g) were randomly allocated to 40 250 L aquariums (15 fish/aquarium) and fed the same diets as mentioned above for a period of 60 days. Subsequently, growth performance, wholy-body composition and intestinal morphometry were assessed. In addition to these parameters, the hematological profile, innate immune response, antioxidant enzyme activity and biochemical parameters were analyzed. For this purpose, blood was collected from ten fish per treatment. The data obtained was considered before challenges. Also after 60 days, in the third study, groups were subjected to different types of stress: a group of 60 fish were subjected to size-sorting-induced stress, another group of 72 fish to *Streptococcus agalactiae* infection (BAC) (15 days) and another group of 136 fish to *Streptococcus agalactiae infection* + 12 hours of hypoxia (BAC + HP) (15 days). After the challenges, the hematological profile, innate immune response, antioxidant enzyme activity and biochemical parameters were analyzed again in order to generate a comparison with the period without challenge. The data was submitted to analysis of variance (ANOVA) followed by Tukey's test and the t-test. Differences were considered statistically significant when $P < 0.05$. Fish fed supplemented diets showed no significant differences in growth performance and limited effects on the apparent digestibility coefficients of nutrients and energy. Diets supplemented with *Quillaja saponaria* + *Yucca schidigera* extract showed the highest levels of phenolic content,

antioxidant capacity and survival after BAC and BAC + HP, and the combination of *Quillaja saponaria*, *Yucca schidigera* and *Saccharomyces cerevisiae* the lowest. *Saccharomyces cerevisiae* supplementation improved midgut villus length and promoted lower survival after BAC and BAC + HP. Fish fed diets supplemented with *Saccharomyces cerevisiae* showed better innate immune responses and antioxidant capacity after challenges. The results of this study suggest that dietary supplementation with *Saccharomyces cerevisiae* determined better overall health parameters, but it was not sufficient to avoid mortality under bacterial infection. Furthermore, futher studies are necessary to validate the combination of eubiotic additives based on *Quillaja saponaria*, *Yucca schidigera*, *Saccharomyces cerevisiae* and their combinations in fish nutrition.

Keywords: Eubiotic additives; immunonutrition; intensive production systems; *Oreochromis niloticus*; *Streptococcus agalactiae*.

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LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

ACH50	Serum alternative complement activity
ADC	Apparent digestibility coefficients
AGCC	Ácidos graxos de cadeia curta
ATP	Adenosina trifosfato / Adenosine TriPhosphate
BAC	*Streptococcus agalactiae* infection
BAC + HP	*Streptococcus agalactiae* infection + 12 hours hypoxia
BHI	Brain heart infusion
CAM	Complexos de ataque a membrana
CAT	Catalase
CD4	Linfócitos T auxiliares
CD8	Linfócitos T citotóxicos
CE	Crude energy
CHMI	Complexo de histocompatibilidade maior classe I
CHMII	Complexo de histocompatibilidade maior classe II
CJA	Complexo de junções apicais
CL	Crude lipid
CORT	Cortisol
CP	Crude protein
DM	Dry matter
DNA	Ácido desoxirribonucleico
DPPH	2,2-difenil-1-picrilhidrazila
EROs	Espécies reativas de oxigênio
Fe^{2+}	Ferro férrico
Fe^{3+}	Ferro ferroso
FITC-d	Fluoresceína isotiocianato–dextrana
FRAP	Ferric reducing antioxidant power
GLU	Glucose
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPx	Glutationa peroxidase

GR	Glutationa redutase
GSH	Glutationa reduzida
GSSG	Glutationa oxidada
H^+	Hidrogênio
H_2O	Água / Water
H_2O_2	Peróxido de hidrogênio / Hydrogen peroxide
Hb	Hemoglobin
HSP70	Proteínas de choque térmico de 70 quilodaltons
Htc	Hematocrit
IL	Interleucinas
JAs	Junções aderentes
JOs	Junções oclusivas
LD_{50}	Lethal dose 50%
LYZ	Lysozyme
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
MDA	Mmalonaldeído
MDA	Malondialdehyde
MOS	Mananoligossacarídeos / Mannan-oligosaccharides
NADPH	Nicotinamida adenina dinucleótido fosfato
NADPH	Fosfato de dinucleótido de nicotinamida e adenina
NBT	Nitroblue tetrazolium
NH_3	Amoníaco
NO_3^-	Nitrato
O_2	Oxigênio
O_2^-	Ânion superóxido
OH^-	Radical hidroxila / Hydroxyl radical
ON	Óxido nítrico
$ONOO^-$	Peroxinitrito / Peroxynitrite
PBS	Phosphate-buffered saline
pH	Potencial hidrogeniônico

PMAAPs	Padrões moleculares associados a agentes patogênicos
RBC	Red blood cell
RTTs	Receptores tipo toll
SOD	Superóxido dismutase
SOD	Superoxide dismutase
SSIS	Size-sorting induced stress
TBA	Ácido tiobarbitúrico / Thiobarbituric acid
TCA	Ácido tricloroacético / Trichloroacetic acid
TNF	Fator de necrose tumoral
TP	Fenóis totais / Total phenols
β	Beta

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"Effects of *Quillaja saponaria*, *Yucca schidigera* and *Saccharomyces cerevisiae* on growth performance and health of Nile tilapia (*Oreochromis niloticus*) subjected to different types of stress"

CAPÍTULO I

Considerações Iniciais

A piscicultura visa a produção de peixes para o consumo humano, aliada a aquicultura, é a atividade que tem apresentado maior evolução nos últimos anos. O setor é responsável por mais de 50% dos alimentos provenientes do ambiente aquático, ultrapassando a quantidade de alimentos provenientes do extrativismo (FAO, 2021). Devido à crescente demanda de proteína animal oriunda do crescimento populacional, a prática da aquicultura demonstrou evolução contínua, passando de sistemas extensivos de produção, até chegar aos dias atuais com a adoção de sistemas intensivos de produção (Chen *et al*., 2014). Dentre as espécies produzidas em sistemas intensivos de produção, a tilápia-do-Nilo (*Oreochromis niloticus*) se destaca por suas características zootécnicas favoráveis, sendo que a produção em 2020 atingiu 9% da produção global de peixes, configurando como a segunda espécie mais produzida no mundo (El-Sayed, 2019; FAO, 2020). O Brasil atualmente é o 4º maior produtor mundial de tilápia-do-Nilo, sendo que no ano de 2022 foram produzidas mais de 550 mil toneladas desta espécie (Peixe BR, 2023).

O sistema intensivo de produção é caracterizado pela alta taxa de densidade de alojamento, seguido pela deterioração da qualidade de água e consecutivos manejos, sejam eles para classificação e/ou vacinação dos peixes. Consequentemente, torna-se evidente o declínio nas respostas produtivas, o desafio ao bem-estar animal e os surtos de doenças enfrentadas, sejam bacterioses ou viroses (Abdel-Tawwab, 2012; Oliva-Teles, 2012; Yilmaz, 2019).

1. Revisão de Literatura

1.1. Cenário atual da piscicultura intensiva e seus desafios

A intensificação da produção aquícola aumenta a ação de agentes estressores, o que resulta em problemas de saúde para os peixes e perdas econômicas para o produtor. Tais agentes podem demandar respostas fisiológicas além da homeostase, com redirecionamento de energia que, dependendo da origem, percepção ou duração dos fatores estressores, podem debilitar os

peixes e favorecer a infecção por agentes patogênicos (Tort, 2011). Entre as diversas práticas de manejo, há aquelas que são consideradas mais estressantes, como por exemplo, a captura dos peixes, a qual é precedida por perseguição, exposição aérea e lesões na superfície corporal (Arends *et al*., 1999; Hoshiba *et al*., 2009; Fernandes Jr *et al*., 2016; Freitas *et al*., 2022). Somado ao estresse de manejo ocorrem, ainda, os fatores estressores naturais como variações de temperatura, pH, níveis de amônia e oxigênio na água, por exemplo (Tsuzuki *et al*., 2001; Usha *et al*., 2011).

Naturalmente o sistema fisiológico do animal procura se sobressair frente aos agentes estressores, sendo que tais reações são denominadas como respostas ao estresse (Figura 1), sendo divididas em primária, secundária e terciária. A resposta primária, conhecida como reação de alarme é caracterizada pela rápida resposta fisiológica, a qual é ativada pelo sistema nervoso central, resultando na liberação de catecolaminas e corticosteroides, sendo esta seguida por uma segunda resposta fisiológica. Durante a resposta secundária, o organismo se adapta ou compensa as condições alteradas a fim de recuperar a homeostase, sendo definida por múltiplas ações e efeitos desses hormônios no sangue e tecido, incluindo o aumento do débito cardíaco, consumo de oxigênio e mobilização de substratos energéticos e distúrbio do balanço osmótico. Se o estresse é excessivamente severo ou duradouro, a compensação pode não ser estabelecida e o organismo entra em colapso. Esta é a resposta terciária, de natureza crônica, acarretando ao animal a inibição do crescimento, reprodução, resposta imune e redução à tolerância aos agentes estressores subsequentes (Tort, 2011). Devido ao gasto energético e desbalanço osmótico, caudado pelo enfrentamento do estresse, os animais ficam susceptíveis a patógenos oportunistas presentes no meio (Abdel-Latif *et al*., 2020).

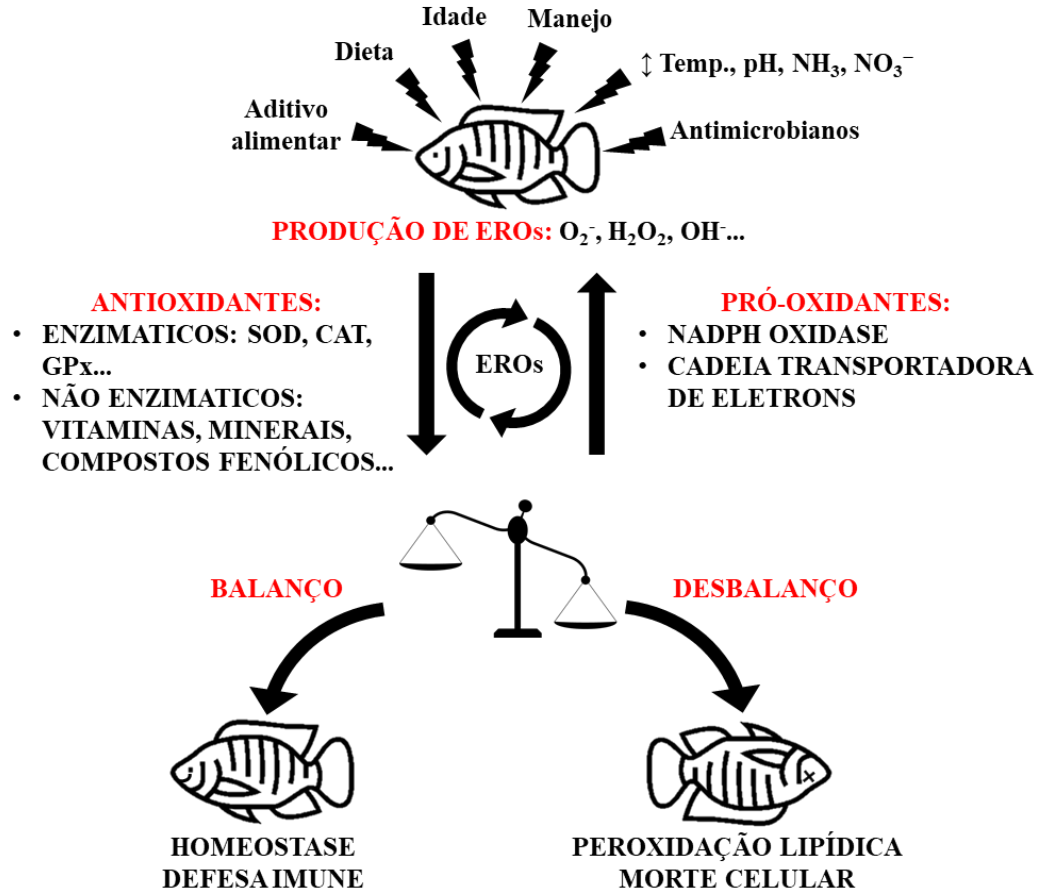

Figura 1: Resposta ao estresse, adaptado Hoseinifar *et al*., 2021.

Um dos principais patógenos comumente encontrados em sistemas intensivos de produção no mundo é a bactéria Gram-positiva *Streptococcus spp.* (Klesius *et al*., 2008; Suanyuk *et al*., 2008; Aly, 2013; Jantrakajorn *et al*., 2014; Hassan *et al*., 2020). As infecções por *Streptococcus spp.* em tilápias-do-Nilo são causadas por bactérias da família *Streptococcaceae*, filo *Firmicutes* e ordem *Lactobacillales*. O gênero *Streptococci* é amplo e possui mais de 50 espécies, dentre elas a *Streptococcus agalactiae*, *Streptococcus iniae*, *Streptococcus dysagalactiae* e *Streptococcus parauberis*, que foram descritas como patógenos que causam infeção em peixes (Curras *et al*., 2002; Eldar; Ghittino, 1999; Soltani *et al*., 2012; Zamri-Saad *et al*., 2010).

A *Streptococcus agalactiae*, pertencente aos *Streptococcus* do grupo B, é uma bactéria Gram-positiva que está presente mundialmente nos sistemas intensivos de produção (Dramsi *et*

al., 2006; Zhu *et al*., 2018). A infecção por *Streptococcus agalactiae* acarreta vários sinais clínicos inespecíficos, que incluem hemorragias nas brânquias, redução no consumo de ração, hemorragias nos olhos, opacidade da córnea, hemorragias na base das nadadeiras e opérculos e lesões hemorrágicas na pele (Bullock, 1981; Yanong & Floyd, 2002; Salvador *et al*., 2005). Outros sinais clínicos incluem escurecimento da pele e natação irregular. Em alguns casos, no entanto, os peixes afetados não apresentavam sinais clínicos antes da morte, e a mortalidade ocorre devido a septicemia (Barham *et al*., 1979; Yanong; Floyd, 2002). A infecção pela bactéria tem provocado perdas econômicas significativas para a piscicultura intensiva, sendo que a prevalência e severidade da infecção dependem de fatores ambientais, como elevada temperatura e baixa qualidade da água, geralmente ocasionados por manejos incorretos e/ou por altas densidades de alojamento (Bromage; Owens, 2002). A bactéria *Streptococcus agalactiae* além de infectar peixes, pode causar diversas doenças infecciosas, incluindo pneumonia, sepse e meningite em humanos e animais terrestres (Lalioui *et al*., 2005; Pereira *et al*., 2010).

Na busca por prevenir e minimizar perdas econômicas ocasionadas por doenças e, consequentemente, declínios na produção, diferentes antimicrobianos, fitoterápicos e outros medicamentos têm sido administrados regularmente por meio de injeções, banhos, ou em alguns casos, via ração (Ding; He, 2010; Rashidian *et al*., 2020). Os antimicrobianos podem ser definidos como substâncias que têm a capacidade de eliminar ou inibir o crescimento de agentes patogênicos, e tais fármacos podem ser oriundos de fontes naturais ou sintéticas (Romero *et al*., 2012). Os antimicrobianos devem ser seguros para o hospedeiro, permitindo seu uso como agentes quimioterápicos para o tratamento de doenças, e seu uso pode ser categorizado como profilático, terapêutico ou metafilático (Cabello, 2006). Nos sistemas intensivos de produção, os antimicrobianos em níveis terapêuticos são frequentemente administrados por curtos períodos, por meio de banhos ou adicionados as rações (Defoirdt *et al*., 2011).

Entretanto, os peixes não metabolizam os antimicrobianos de forma eficaz, fazendo com que grande parte seja eliminado pelas excretas. Estima-se que 75% dos antimicrobianos fornecidos aos peixes sejam excretados na água (Burridge *et al*., 2010). Segundo DEFOIRDT *et al*. (2011), aproximadamente 500 a 600 toneladas de antimicrobianos voltados para o enfrentamento de agentes patogênicos foram utilizadas para a produção aquícola na Tailândia em 1994, e as proporções não seguem padrão, podendo variar de 1 g por tonelada na Noruega a 700 g por tonelada no Vietnã.

Portanto, o uso indiscriminado de tais fármacos pode não só ter efeitos colaterais consideráveis nos sistemas intensivos de produção, suprimindo a imunidade do lote e selecionando cepas mais virulentas de patógenos, mas também ameaça a resistência natural do animal contra patógenos oportunistas (Akanmu, 2018; Dawood; Koshio, 2018; Sarhadi *et al*., 2020). Desta forma, aditivos eubióticos/alternativos, para melhor preparar o animal para possível enfrentamento de doenças, são necessários para garantir a produção saudável de forma sustentável.

Assim, nos últimos anos, tem se empregado o uso de aditivos alimentares conhecidos como eubióticos, os quais além da nutrição, promovem saúde dos animais que consomem este eubiótico, com maior resistência a fatores estressores e patógenos presentes nos sistemas intensivos de produção (Yilmaz, 2020; Yousefi *et al*., 2020).

Os aditivos alimentares foram definidos como ingredientes ou componentes não nutritivos, que ao serem suplementados em formulações, não necessariamente influenciam as propriedades físicas ou químicas da dieta, o ganho de peso dos peixes ou a qualidade do produto final. A natureza química desses aditivos é diversificada e o uso em dietas comerciais para peixes pode variar consideravelmente (NRC, 2011).

Dentre os aditivos eubióticos para rações, estão os prebióticos, probióticos, fitogênicos e ácidos orgânicos, pode-se destacar os prebióticos e fitogênicos, os quais têm por finalidade

promover maior saúde aos peixes submetidos aos sistemas intensivos de produção. Recentemente, esforços vêm sendo envidados na busca e utilização de produtos derivados de plantas em substituição a fármacos em sistemas intensivos de produção de peixes (Bulfon *et al*., 2015). Fitogênicos, e seus respectivos coprodutos contêm biomoléculas/fitomoléculas que podem ser eficazes, como aditivo (eubiótico). Estes compostos podem promover o desempenho, aumento da sobrevivência, das respostas do sistema antioxidante e a imunomodulação do sistema imunológico (Jeney *et al*., 2015; Vicente *et al*., 2018; Xavier *et al*., 2020; Naliato *et al*., 2021).

Isso se deve ao fato de que as plantas possuem metabólitos secundários como os compostos fenólicos, alcaloides, quinonas, terpenóides, lectinas e polipeptídeos, muitos dos quais são alternativas de suporte aos antimicrobianos, produtos químicos, vacinas e outros compostos sintéticos (Harikrishnan *et al*., 2011).

Como exemplo de aditivo eubiótico fitogênico estão a *Quillaja saponaria* (Figura 2) e *Yucca schidigera* (Figura 3) que são fontes de saponinas e polifenóis, além dos polifeniois presentes em sua composição. Outro aditivo eubiótico com grande representatividade são os prebióticos baseados em levedura *Saccharomyces cerevisiae*. Assim, essas classes de aditivos eubióticos se tornam cada vez mais estratégias promissoras para os sistemas intensivos de produção.

A *Quillaja saponaria* é encontrada em áreas áridas do Chile (Francis *et al*., 2002a), e é fonte reconhecida de extratos ricos em saponinas do tipo triterpenica, e também de compostos polifenólicos e polissacarídeos (San Martin *et al*., 2000; Maier *et al*., 2015). A *Yucca schidigera*, é planta nativa do sudoeste dos EUA e do México (Cheeke *et al*., 2006), que nos últimos anos vem sendo utilizada como extratos no setor aquícola devido aos benefícios apresentados, como por exemplo a redução do estresse em peixes (Santacruz-Reyes; Chien, 2010; Abdel-Tawwab *et al*., 2021). Tais resultados se devem não apenas a presença de

saponinas esteroides em sua composição, mas também polifenóis e polissacarídeos, dentre os quais, o yuccaol A, B, C, D e E (trans-3,3',5,5'-tetrahidroxi-4' metoxistilbeno) e o resveratrol (trans-3,4',5 tetraxidroxiestilbeno) (Oleszek *et al*., 2001; Piacente *et al*., 2004; Piacente *et al*., 2005).

O nome saponina deriva do latim sapo, que significa sabão. Isto, por apresentar propriedades surfactantes formando uma espuma estável, semelhante a sabão, ao serem agitados em solução aquosa. Quimicamente, o termo saponina define um grupo de glicosídeos de elevado peso molecular que consistem em uma porção glicana ligada a uma aglicona, também designada genina ou sapogenina (Hostettmann; Marston, 2005). A estrutura química da sapogenina define a classificação das saponinas como saponinas triterpenóides (30 átomos de carbono), que ocorrem principalmente na classe das *Magnoliopsida* (dicotiledôneas) e saponinas esteróides (27 átomos de carbono), que estão quase exclusivamente presentes na classe das *Liliopsida* (monocotiledôneas) (Sparg *et al*., 2004).

Por apresentarem estruturas triterpenóides, substâncias que possuem capacidade em formar complexos insolúveis com lipídios presentes na dieta, pode haver prejuízo na emulsificação de gorduras e na formação de micelas com sais biliares e colesterol no intestino, o que pode aumentar a excreção, de forma que o animal não acumule gordura em sua carcaça (Oakenfull, 1986; Cheeke, 2000). Nas membranas celulares, o colesterol é organizado na forma de estruturais cinéticas ou “pools”. As saponinas, por meio de seus grupos OH, podem interagir com o colesterol e com os fosfolipídios da membrana celular. Além disso, sua estrutura esteroide hidrofóbica pode intercalar no interior hidrofóbico da bicamada lipídica. Ambos os efeitos podem contribuir para alterar a camada lipídica entorno da membrana e ativar receptores, canais ou enzimas específicas, podendo promover maior área de absorção e

resistência a célula (Attele *et al*., 1999; Francis *et al*., 2002b). Além disso, as saponinas têm uma porção antioxidante que permite a eliminação de ânions superóxidos, formando intermediários de hidróxido e, assim, evitar danos moleculares (Sparg *et al*., 2004).

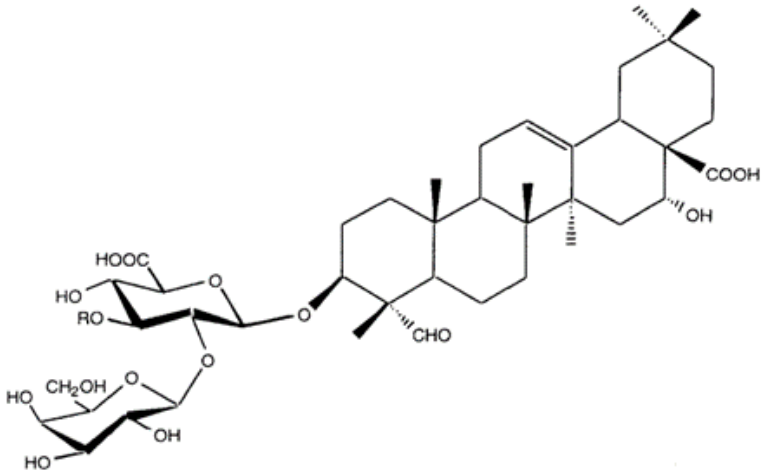

Figura 2: Estrutura de Saponina de *Quillaja saponaria*

(Guo *et al*., 1998).

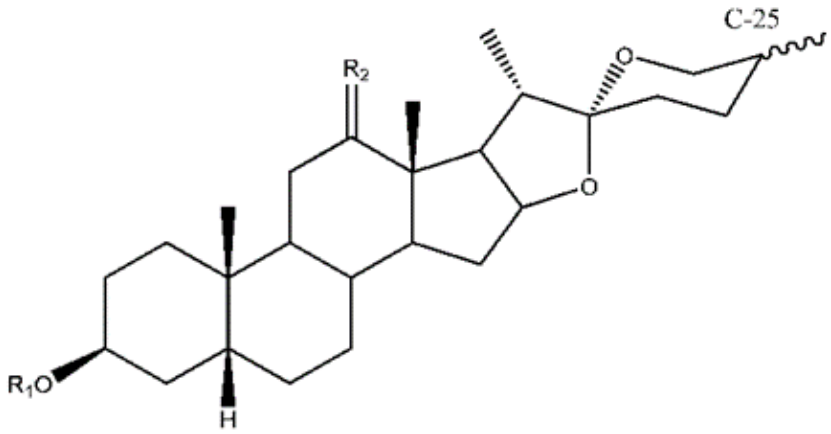

Figura 3: Estrutura de Saponina de *Yucca schidigera*
(Jiménez *et al*., 2021).

GEE *et al*. (1996) enfatizaram que o aumento da permeabilidade aparente das bordas em escova intestinais promovido por baixas doses de saponinas pode ter importantes implicações na absorção de macromoléculas, cuja passagem através do epitélio é normalmente restrita. JOHNSON *et al*. (1986) propuseram que algumas saponinas reagem com o colesterol das membranas do microvilo, causando lesões estruturais, proporcionando o aumento da permeabilidade dos enterócitos, facilitando a absorção de substâncias que normalmente não

seriam absorvidas. Outro fator benéfico à suplementação de saponinas é que possuem a capacidade de promover a secreção de enzimas digestivas como por exemplo a amilase, lipase, lactato desidrogenase e citocromo c oxidase (Güroy *et al*., 2016; Serrano, 2013; Wang *et al*., 2020). SERRANO (2013) observou que o consumo de saponinas por carpas (*Cyprinus carpoio*) propiciou aumento no ganho de peso, taxa de crescimento específico, eficiência proteica e atividade das enzimas intestinais, tais como amilase e tripsina, bem como enzimas do fígado, lactato desidrogenase e citocromo c-oxidase.

CHEN *et al*. (2011) aventaram a hipótese de que peixes alimentados com dietas suplementadas com baixas doses de saponinas podem apresentar melhores resultados para ganho de peso devido a melhor taxa de absorção de nutrientes pelo intestino, induzida pela maior permeabilidade dos enterócitos, consequência da ação das saponinas. Isto se dá por baixas doses (<1000ppm) de extratos de plantas que possuem saponinas e apresentam impacto benéfico no crescimento dos peixes, enquanto doses elevadas contribuem para o desenvolvimento de enterite. Tilápias-do-Nilo alimentadas com dietas suplementadas com 100 mg kg^{-1} de saponinas apresentaram melhores resultados nos parâmetros zootécnicos, comparados à peixes alimentados com dietas suplementadas com 140 e 200 mg kg^{-1} (El-Keredy; Naena, 2020).

A suplementação de 150 mg kg^{-1} de saponina promoveu o crescimento dos peixes e a maior absorção de nutrientes em carpa comum (Francis *et al*., 2002a). Tilápias-do-Nilo alimentadas com dietas suplementadas com 300 mg kg^{-1} de saponina por 14 semanas, apresentaram taxa de crescimento 26% maior em comparação à dieta ausente de suplementação. Os índices hepatossomático e o intestinossomático foram reduzidos de acordo com o aumento do nível de saponina na dieta e, concomitantemente, demonstraram menor excreção de energia pelas fezes, o que indicou o uso mais eficiente do alimento (Francis *et al*., 2001b).

FRANCIS *et al*. (2005) afirmaram que as saponinas agem como promotores de crescimento naturais e que podem ser vastamente utilizadas na aquicultura, pois reduzem a taxa metabólica e promovem o crescimento de peixes, como descrito para a carpa e a tilápia-do-Nilo. A suplementação de 150 mg kg^{-1} de saponinas de *Quillaja saponaria* inibiu a produção de ovos de tilápia-do-Nilo (Francis *et al*., 2002a,b,c) promovendo, assim, o direcionamento do metabolismo energético para a produção de tecido (filé). ANGELES *et al*. (2017) demonstraram que houve maior ganho de peso para a tilápia-do-Nilo alimentada por seis semanas com dietas suplementadas com a associação de 150 mg kg^{-1} de *Quillaja saponaria* e 150 mg kg^{-1} de *Yucca schidigera*. A associação de *Yucca schidigera* e/ou à levedura *Saccharomyces cerevisiae* foi avaliada por ABDEL-TAWWAB *et al*. (2021). Os autores avaliaram as respostas da tilápia-do-Nilo quando os produtos foram colocados na água dos tanques (1 g/m^3) durante oito semanas. Houve efeito positivo para ganho de peso quando da associação de *Yucca schidigera* e levedura. Entretanto, já foi descrito que as saponinas provenientes de *Quillaja saponaria* e *Yucca schidigera* quando em altas concentrações na água podem ser consideradas letais devido a sua caracteristca de danificar o epitélio branquial dos peixes (Makkar *et al*., 2007).

De modo oposto, a suplementação dietética combinada de *Quillaja saponaria* e *Yucca schidigera* determinou efeitos negativos nos parâmetros zootécnicos como, ganho de peso, peso final e taxa de crescimento específico para camarões (Hernández-Acosta *et al*., 2016) e tilápia-do-Nilo (Angeles *et al*., 2017). Estudos demonstraram que a suplementação dietética de 3 g kg^{-1} de saponinas de *Quillaja saponaria*, em dietas à base de farelo de soja, determinaram redução significativa no ganho de peso, e também danos intestinais para truta arco-íris (*Oncorhynchus mykiss*) e salmão (*Salmo salar*) (Bureau *et al*., 1998). Resultado semelhante foi associado a suplementação de 2 g kg^{-1} de saponinas de soja em dietas contendo 33% de concentrado proteico de ervilha para salmões alimentados por 11 semanas (Chikwati *et al*., 2012). O mesmo nível de suplementação quando fornecido durante a primeira fase de alimentação para alevinos

de tilápia-do-Nilo, acarretou alta mortalidade do lote (Makkar *et al*., 2007). Tais efeitos negativos foram também reportados com a suplementação de 3,2 g kg^{-1} saponinas de soja em dietas a base de farinha de peixe (Chen *et al*., 2011).

Outro aditivo funcional com importante relevância no cenário atual são os prebióticos, substâncias não digeríveis que permitem alterações específicas na composição e/ou na atividade da microbiota intestinal, o que pode promover efeito positivo na absorção de nutrientes e na saúde (Ringo *et al*., 2014). Tal composto é utilizado como substrato energético pelas bactérias do intestino, e podem ser considerados como sacarídeos funcionais (Roberfroid, 1993; Song *et al*., 2014). Os prebióticos são aditivos promissores e apresentam efeitos benéficos no desempenho, saúde da microbiota intestinal, melhora da morfologia intestinal, melhora da imunidade e, consequentemente, maior resistência a agentes patogênicos (Ganguly *et al*., 2013; Daniels; Hoseinifar, 2014; Ringo *et al*., 2010, 2014; Song *et al*., 2014; Torrecillas *et al*., 2014; Dawood *et al*., 2015a, b). Além disso, a suplementação de prebióticos pode promover maior produção de metabolitos como o propionato, butirato e ácidos graxos de cadeia curta (AGCC) pela microbiota que, consequentemente, podem ser utilizados pelas células imunitárias do intestino (Bach Knudsen *et al*., 2003). Em geral, estudos demonstraram que a suplementação de prebióticos promovem significativamente o desempenho e a eficiência alimentar de diferentes espécies de peixes (Mahious *et al*., 2006; LV *et al*., 2007; Huang *et al*., 2015).

A parede celular de levedura *Saccharomyces cerevisiae*, que atua na saúde e na redução dos impactos do estresse. A parede celular da levedura é uma rede dinâmica de polissacáridos e glicoproteínas que estão ligados entre si por interações covalentes e não covalentes. A sua composição é constituída principalmente por mananoligossacarídeos (MOS) que estão contidos na camada exterior, β-glucanos e, em menor proporção, por quitina, que compreende a camada interior. O uso de dietas enriquecidas com MOS e β-glucano apresenta relação custo-benefício comprovada, podendo ser utilizado por piscicultores de pequeno e grande porte e pode

determinar vários benefícios, desde o melhor desempenho dos peixes até o aumento das respostas imunológicas (Kim *et al*., 2009; Carbone; Faggio, 2016; Dawood *et al*., 2017a, b; Ji *et al*., 2017; Iswarya *et al*., 2018; Van Doan *et al*., 2020). Os MOS são complexos glucomanano proteicos derivados da parede celular da levedura *Saccharomyces cervesiae*, estão entre os prebióticos mais comuns avaliados nos peixes (Merrifield *et al*., 2010). Dietas suplementadas com MOS (1 g kg^{-1} e 1,5 g kg^{-1}) promoveram a eficiência alimentar e o ganho de peso de carpas quando arraçoadas durante oito semanas (Ebrahimi *et al*., 2012). O mesmo para ganho de peso foi relatado por RODRIGUES-ESTRADA *et al*. (2009) para truta-arco-íris suplementadas por 4 g kg^{-1}. Entretanto, não foram relatadas diferenças em relação ao grupo controle quando salmões-do-Atlântico foram alimentados com dietas suplementadas com 10 g kg^{-1} durante quatro meses (Grisdale-Helland *et al*., 2008).

Tilápias-do-Nilo alimentadas com dietas suplementadas com β-glucano, apresentaram maior ganho de peso, melhor morfologia intestinal, melhor resposta imunológica e, consequentemente, maior resistência quando submetidas a estresse por densidade (Welker *et al*., 2012; Dawood *et al*., 2020). A melhora no ganho de peso foi igualmente observada para *Pseudosciaena crocea* quando arraçoados com dietas suplementadas por 90 mg kg^{-1} de β-glucano (Ai *et al*., 2007). Por outro lado, a suplementação de três níveis de β-glucano, baixo (38 g kg^{-1}), médio (52 g kg^{-1}) e alto (82 g kg^{-1}), em dietas para *Oncorhynchus mykiss* proporcionaram redução na taxa de crescimento específico (Sealey *et al*., 2008). Concomitantemente, estudos anteriores demonstraram que a suplementação de β-glucano ou mananoligossacarídeos não determinaram efeitos positivos no ganho de peso de tilápias-do-Nilo (Whittington *et al*., 2005; Sado *et al*., 2008; Shelby *et al*., 2009; Barros *et al*., 2014).

2. Sistema antioxidante em peixes

Todos os organismos aeróbicos, incluindo aqueles que vivem em ambientes aquáticos, necessitam de oxigênio (O_2), sendo que tal molécula desempenha papel vital em vários

processos metabólicos e também na produção de energia (Ahmad, 1995). Quando ocorre a redução parcial do oxigênio são gerados intermediários reativos, denominados de radicais livres e espécies reativas de oxigênio (EROs) (Figura 4). Dentre as EROs, as mais relevantes e ativas do ponto de vista fisiológico são o radical ânion superóxido (O_2^-), radical hidroxila (OH^-) e o peróxido de hidrogênio (H_2O_2) (Cadenas, 1995; Schieber; Chande, 2014; Halliwell; Gutteridge, 2015). As EROs primordialmente formadas são, em geral, o radical ânion superóxido (O_2^-), sendo que sua produção ocorre principalmente nas mitocôndrias pela cadeia respiratória, com função de gerar energia na forma de adenosina trifosfato. O O_2^- gerado nas células desencadeia diversas reações químicas, afetando funções biológicas. A eliminação do O_2^- é de responsabilidade da superóxido dismutase (SOD), amplamente encontrada tanto nas mitocôndrias (Mn^{2+}SOD) quanto no citosol (Cu^{2+}SOD e Zn^{2+}SOD) (Cao *et al*., 2019). Esta enzima catalisa a dissociação de duas moléculas de O_2^- em H_2O_2. Na ausência da SOD, esse radical livre leva à formação do radical hidroxila (OH^-) (Halliwell; Gutteridge, 2015; LI *et al*., 2016). O OH^- é caracterizado por apresentar meia vida extremamente curta e é considerado a EROs mais reativa e prejudicial. Uma vez que não existe antioxidante capaz de prevenir a ação do OH^-, somente é possível inibir a sua formação ou reparar os danos resultantes da sua ação (Schieber; Chande, 2014).

Por outro lado, o H_2O_2 é produzido abundantemente na matriz mitocondrial durante o processo de redução do O_2^-. O peróxido de hidrogênio pode ser parcialmente eliminado por diversas enzimas, incluindo a catalase (CAT) que é encontrada tanto nas mitocôndrias quanto nos peroxissomos, a qual catalisa a decomposição do H_2O_2 em H_2O e O_2, mantendo o equilíbrio de formação e eliminação de EROs, essencial para o funcionamento da imunidade inata (Devi *et al*., 2019). A glutationa peroxidase (GPx) também age na decomposição do H_2O_2 em H_2O, além de ser restabelecida à sua forma reduzida pela ação da glutationa redutase (GR), via nicotinamida adenina dinucleótido fosfato (NADPH), e também desempenha papel importante

na prevenção da peroxidação lipídica da membrana celular (Monteiro *et al*., 2006; Fukai, Ushio-Fukai, 2011).

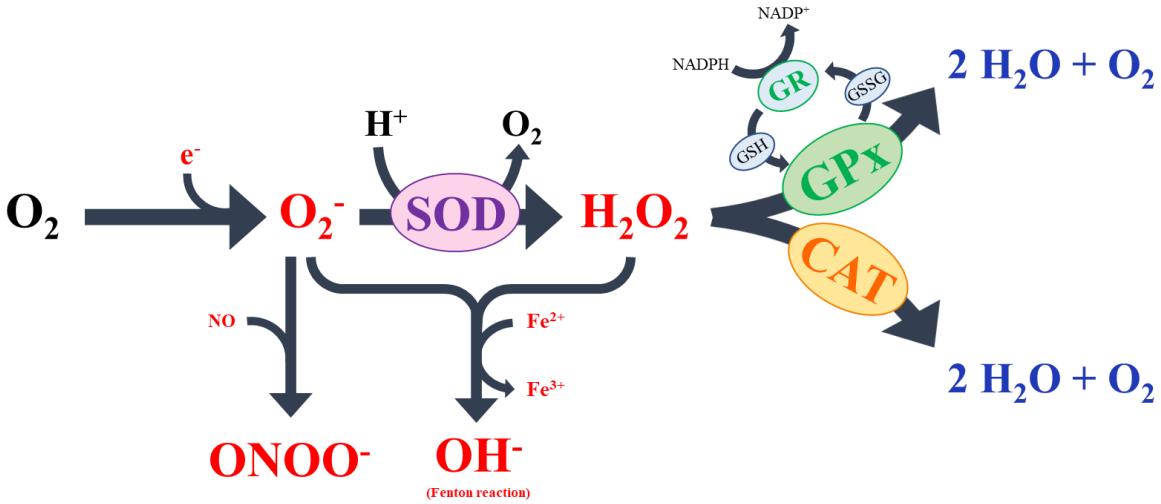

Figura 4: Sistema antioxidante adaptado de Fukai, Ushio-Fukai, 2011.

Devido à sua natureza, o H_2O_2 apresenta meia vida mais longa em comparação com a maioria das EROs e é capaz de atravessar facilmente membranas, podendo ser liberado no citoplasma celular. Na presença de íons metálicos com valência variável, o H_2O_2 pode ser convertido no altamente reativo OH^- (Dong *et al*., 2017). Em circunstâncias fisiológicas normais, as EROs desempenham diversas funções, como na defesa do animal (por exemplo, defesa contra microrganismos patogênicos) e nas múltiplas vias de sinalização celular (Martínez-Álvarez *et al*., 2005). Em concentrações baixas a moderadas, as EROs não causam danos, mas em concentrações elevadas induzem modificações negativas nos componentes celulares, como lipídios, proteínas e DNA. Especificamente, as EROs podem induzir a peroxidação lipídica de ácidos graxos poliinsaturados, levando ao rompimento da bicamada lipídica da membrana e à alteração da estrutura e permeabilidade da membrana (Hematyar *et al*., 2019). Por fim, para evitar o desequilíbrio na produção de EROs, os organismos aeróbicos podem reduzir a formação de EROs ou reduzir a alta reatividade desses compostos por meio de

componentes antioxidantes, produzidos pela célula (fonte endógena) ou obtidos por meio da dieta (fonte exógena) (Wang *et al*., 2013; Franco; Martínez-Pinilla, 2017).

Como exemplo de fonte exógena de antioxidantes, as saponinas presentes na *Quillaja saponaria* e *Yucca schidigera* apresentam tais características, devido ao elevado teor de polifenóis, como por exemplo os flavan-3-ols, que tem como característica estabilizar e eliminar a produção excessiva de EROs, prevenindo possíveis danos a célula dos peixes, como por exemplo, a prevenção da peroxidação lipídica, aumento da atividade da SOD, CAT, GPx e mieloperoxidase (Martínez-Álvarez *et al*., 2005; Cigerci *et al*., 2009; Bae *et al*., 2020; Elbialy *et al*., 2020; Abdel-Tawwab *et al*., 2021; Dawood *et al*., 2021; Peng *et al*., 2022). ANGELES *et al*. (2017) encontraram melhores taxas de sobrevivência para tilápia-do-Nilo arraçoada com dietas suplementadas com *Quillaja saponaria* e *Yucca schidigera* e desafiadas por hipóxia. Carpas espelho (*Cyprinus carpio*) demonstraram melhor capacidade antioxidante total e menores níveis de malonaldeído (MDA) quando arraçoadas com dietas suplementadas com *Yucca schidigera*, entretanto não foi observada diferença na atividade da SOD (Wang *et al*., 2020). Resultados positivos em relação a atividade da SOD foram observados para tilápia-do-Nilo, sendo que peixes arraçoados com dietas suplementadas pela mistura de *Quillaja saponaria* e *Yucca schidigera* demonstraram atividade 48% menor da SOD em relação ao grupo controle, devido a capacidade antioxidante (Angeles *et al*., 2017).

Outros componentes antioxidantes obtidos por meio da dieta são o MOS e o β-glucano. A suplementação de 45 g kg^{-1} de MOS em dietas para tilápia-do-Nilo determinaram aumento significativo da atividade das enzimas SOD, CAT e GPx, além de reduzir a peroxidação lipídica (Özlüer-Hunt *et al*., 2011). Igualmente, o β-glucano além de apresentar características de modulação e produção de enzimas do sistema antioxidante, pode promover a expressão do gene HSP70, o qual está relacionado ao estresse celular (Salah *et al*., 2017; Pogue *et al*., 2021; Reis *et al*., 2021), como também, pode sequestrar radicais livres (Kofuji *et al*., 2012; Zhang *et al*.,

2022). A suplementação de 4 g kg^{-1} de β-glucano em dietas para tilápias-do-Nilo promoveu maior expressão de genes relacionados as enzimas SOD e CAT e reduziu os níveis de MDA quando os peixes foram submetidos a desafio por intoxicação por Fipronil (El-Murr *et al*., 2019). Quando alimentadas com dietas suplementadas com 5 g kg^{-1} de β-glucano e desafiadas por intoxicação com deltametrina, foi observada redução dos danos ocasionados pelo estresse (Dawood *et al*., 2020).

3. Resposta imunológica em peixes

A função imunológica dos peixes ocorre de duas maneiras, via imunidade inata e via imunidade adaptativa. A primeira linha de defesa dos peixes contra agentes patogênicos são as barreiras físicas como escamas, pele e brânquias, as quais são envoltas por mucosa e contem lisozima, enzima responsável pela degradação da parede peptidioglicano de agentes patogênicos, protegendo o animal contra possíveis infecções (Manning, 1998).

Assumindo a hipótese de que o agente patogênico adentre por tais barreiras, será desencadeada a resposta imune inata, conhecida também como não especifica. Tal resposta é mediada nos primeiros minutos após infecção por duas subdivisões: resposta imune inata celular e resposta imune inata humoral (Alberts *et al*., 2012). Como parte da resposta imune inata humoral, esta representada principalmente pelo sistema complemento, o qual pode ser considerado como uma das respostas não celulares mais eficiente do sistema imune. Tal sistema engloba mais de 35 proteínas que além de contribuírem na resposta imune inata, contribuem com a resposta imune adaptativa. O sistema complemento pode ser ativado por três diferentes vias: clássica (ligação anticorpo – antígeno), lectina (moléculas de lectinas que se ligam a carboidratos da membrana patogênica) e alternativa (reconhecimento de padrões moleculares associados a agentes patogênicos - PMAAPs). Tais vias tem função de lisar os agentes patogênicos por meio da formação de complexos de ataque a membrana (CAM), promover a inflamação no local da infeção e opsonizar os agentes patogênicos por meio de ligações com

imunoglobulinas (via imunidade adaptativa) ou por fragmentos do complemento na superfície patogênica, favorecendo a fagocitose (Abbas *et al*., 2012).

Ao mesmo tempo é desencadeada a resposta imune inata celular, os neutrófilos compõem o grupo das primeiras células de defesa a chegarem ao local da infecção. Estas células são ativadas por meio das PMAAPs, sem o reconhecimento e sem capacidade de gerar memória imunológica, promovendo a liberação de citocinas e lise celular de células infectadas (Greenlee *et al*., 1991). A partir do momento que entram em contato com o agente patogênico, identificam os PMAAPs por meio de suas proteínas transmembranas, conhecidas como receptores tipo toll (RTTs), e então fagocitam o agente patogênico e promovem a explosão respiratória (*Burst*), induzindo a produção de espécies reativas ao oxigênio (EROs) como O_2^-, H_2O_2, OH^- e óxido nítrico (ON) (Akhter *et al*., 2015). Após este processo e a respectiva morte do neutrófilo, ocorre a liberação de citocinas inflamatórias, que sinalizam a localidade da infecção e, por quimiotaxia estimulam a vinda e ação dos então monócitos (Secombes; Fletcher, 1992).

Os monócitos assim que saem da corrente sanguínea e chegam ao local, passam a ser macrófagos, que por sua vez, são as principais células fagocitárias da imunidade inata celular. Diferentemente dos neutrófilos, que morrem devido ao gasto energético após a fagocitose, os macrófagos após englobar o agente patogênico promovem o burst e produção de espécies reativas ao oxigênio e nitrogênio, além da liberação de citocinas inflamatórias, incluindo o fator de necrose tumoral (TNF) e interleucinas (IL-1 - IL-12) (Abbas *et al*., 2012). Com a liberação de citocinas do tipo TNF, estas se ligam a receptores que estimulam a liberação de transferrinas. Estas por sua vez, se ligam a moléculas de ferro (Fe^{3+}) tornando-as indisponíveis para a multiplicação de agentes patogênicos, como bactérias por exemplo (Chen *et al*., 2009). Não se limitando apenas à ação de fagocitar agentes patogênicos, os macrófagos também atuam na reparação tecidual, como regeneração de tecidos e vasos por exemplo (Secombes; Fletcher, 1992).

A resposta imune adaptativa, ou comumente conhecida como resposta imune específica, tem como característica a alta especificidade e a capacidade de gerar memória contra agentes patogênicos (Secombes *et al*., 2008). Os macrófagos que por sua vez fagocitam o agente patogênico, processam e apresentam o antígeno para células dendriticas, como outros macrófagos e linfócitos B, isto através de receptores para antígenos endógenos, conhecidos como complexo de histocompatibilidade maior classe II (CHMII), com a geração de citocinas especificas, estas estimulam linfócitos B a se diferenciarem em plasmócitos, para a produção de anticorpos específicos contra tal patógeno, além de gerar linfócitos B de memória que possuem alta durabilidade para rápida ação contra possíveis reinfeções frente aos mesmos agentes patogênicos que induziram sua produção (Secombes *et al*., 2008). Paralelamente, os linfócitos T auxiliares (CD4), reconhecem este antígeno e liberam citocinas, estimulando a ação de outros macrófagos e linfócitos T citotóxicos (CD8,) que por sua vez reconhecem a célula infectada via complexo de histocompatibilidade maior classe I (CHMI), promovendo a apoptose (Secombes; Fletcher, 1992).Como forma de promover a imunidade dos peixes, de modo que não seja necessário a utilização de antimicrobianos para combater possíveis agentes patogênicos, a utilização de suplementos alimentares com propriedades imunoestimulantes ganhou popularidade nas últimas décadas (Dawood *et al*., 2018; Dawood *et al*., 2019; Van Doan *et al*., 2019a).

A suplementação de saponinas na alimentação de peixes pode promover melhora do sistema imunológico, visto que saponinas provenientes de *Quillaja saponaria* podem desencadear marcadores imunológicos (como células T CD8), estimular a resposta inata e adaptativa para possíveis infecções, induzindo diretamente macrófagos e células dendríticas, e a expressão de diferentes citocinas, mesmo quando os peixes não apresentam sinais de infecções (Welsby *et al*., 2017; Wang *et al*., 2020; Cortés *et al*., 2023).

Tilápias-do-Nilo arraçoadas com dietas suplementadas com 100 mg kg^{-1} de saponinas de *Yucca schidigera* apresentaram maior atividade da lisozima e do burst respiratório e maior resistência a infecção por *Aeromonas hydrophila* (Njagi *et al*., 2017). Para a mesma espécie, também arraçoadas com dietas suplementadas com o mesmo tipo de saponinas, foram relatadas menores taxas de mortalidade quando da infecção por *Pseudomonas aeruginosa* e comparado ao grupo controle (El-Keredy; Naena, 2020). BAE *et al*. (2020) verificaram que linguado verde-oliva (*Paralichthys olivaceus*) arraçoados com dietas suplementadas com 1,5 g kg^{-1} de saponinas de *Yucca schidigera* apresentaram melhores resultados para o burst respiratório e maior taxa de sobrevivência.

Os prebióticos são definidos como fibras indigestas que promovem a população e colonização da microbiota intestinal benéfica, resultando na melhoria do estado geral de saúde (Song *et al*., 2014). Prebióticos, como levedura *Saccharomyces cerevisiae*, que possue MOS e β-glucano em sua composição, são comumente utilizados e cada vez mais aplicados na aquicultura como agentes imunomoduladores (Ringo *et al*., 2010; Ringo *et al*., 2014; Hoseinifar *et al*., 2015; Dawood; Koshio, 2016; Dawood *et al*., 2020). Em geral, estudos demonstraram que os prebióticos modularam as respostas fisiológicas dos peixes contra fatores estressantes (Torrecillas *et al*., 2012). Também possuem papel significativos na eficiência das respostas humorais (Chang *et al*., 2013) e na imunidade inata dos peixes (Serradell *et al*., 2020; Barros *et al*., 2014; Zhang *et al*., 2014).

STAYKOV *et al*. (2007) observaram que dieta suplementadas com 2 g kg^{-1} de MOS promoveu maior atividade da lisozima e do sistema complemento de truta arco-íris. O mesmo parâmetro foi relatado em estudo com tambor vermelho (*Sciaenops ocellatus*) arraçoados com dietas suplementadas com 10 g kg^{-1} durante oito semanas (Zhou *et al*., 2010) e seis semanas (Buentello *et al*., 2010). Entretanto, o mesmo nível foi prejudicial para a atividade da lisozima em salmões do atlântico (Grisdale-Helland *et al*., 2008).

Os β-glucanos em sua forma livre, têm sido utilizados como imunoestimulantes para espécies aquáticas (Dawood *et al*., 2015b; Dawood; Koshio 2016). Esses compostos geralmente influenciam a resposta imunológica, incluindo a produção de radicais oxidativos por neutrófilos no sangue e na produção de ânions superóxidos em macrófagos (Gatlin, 2002). Os β-glucanos são compostos por monômeros de D-glicose, unidos por ligações β-glicosídicas e são notórios pela habilidade em estimular o sistema imunológico (Ringo *et al*., 2011; Volman *et al*., 2008). Estudos anteriores demonstraram que o β-glucano pode se ligar a padrões moleculares associados a agentes patogênicos (PMAAPs) específicos para induzir a resposta imune, além de promover atividades antimicrobianas, antioxidantes e anti-inflamatórias (Dawood *et al*., 2020; Miao *et al*., 2020).

A utilização de β-glucanos na suplementação de dietas tem sido associado ao aumento da imunidade específica e não específica em espécies de peixes, como *Danio rerio* (Rodríguez *et al*., 2009) e *Larimichthys polyactis* (Rodríguez *et al*., 2003). Efeito imunoestimulador do β-glucanos foi relatado para *Sciaenops ocellatus* em experimento conduzido in vitro e in vivo (Yamamoto *et al*., 2018). ENGSTAD *et al*. (1992) constataram maiores níveis de lisozima quando alevinos/jovens de salmão-do-Atlântico foram inoculados com 1 ml de β-glucanos. O mesmo foi observado para rohu (*Labeo rohita*) (Misra *et al*., 2006) e Pseudosciaena crocea (Ai *et al*., 2007).

HASAN *et al*. (2018) avaliaram a suplementação de β-glucanos durante oito semanas para linguado verde-oliva (*Paralichthys olivaceus*), e encontraram maiores expressões de TNF, IL1 e IL6 nos fígados, rins e baços. Melhores níveis de lisozima, imunoglobulina (IgM) e sistema complemento foram encontradas em truta arco-íris alimentadas com dietas suplementadas com 2 g kg^{-1} de β-glucanos (Ghaedi *et al*., 2015). Foi demonstrado que dietas suplementadas com β-glucanos isolado ou com a associação de spirulina, promoveram a recuperação da atividade fagocitária pós inibição da mesma por ação de inseticida (Abu-Elala

et al., 2020; Mokhbatly *et al*., 2020). Dentre a suplementação de dietas com o β-glucanos, VERLHAC *et al*. (1998), ao avaliarem a suplementação de β-glucanos isoladamente e de sua associação com vitamina C, tais compostos promoveram a atividade fagocitária e a ação da lisozima para truta arco-íris.

A suplementação de β-glucanos em dietas para tilápias-do-Nilo promoveram a função imune de modo geral, como no aumento da ativação do sistema complemento (Amphan *et al*., 2019), e quando estas foram desafiadas por infecção por Aeromonas hydrophila demonstraram melhores níveis de lisozima (Lu *et al*., 2019). Dentro do mesmo cenário anterior, tilápias-do-Nilo apresentaram maiores índices de fagocitose, entretanto, tal efeito foi constatado apenas quando os peixes foram alimentados com dietas suplementadas com β-glucanos em semanas alternadas, e não quando alimentados constantemente durante o período de duas ou quatro semanas (Barros *et al*., 2014; Petit, 2019).

Entretanto, OGIER DE BAULNY *et al*. (1996) afirmam que a suplementação de β-glucano em dietas para *Scophthalmus maximus* administradas por longos períodos não determinaram efeito significativo na atividade do sistema complemento e na atividade da lisozima. Da mesma forma, SABIONI *et al*. (2020) não encontraram diferenças significativas na atividade do sistema complemento quando comparados os parâmetros antes e após desafio por infecção por *Aeromonas hydrophila* para pacus (*Piaractus mesopotamicus*). Os autores concluíram que tais resultados se devem ao efeito imunossupressor dos níveis de cortisol encontrado nos peixes. Contudo, a suplementação de 1 g kg^{-1} de β-glucanos em dietas para tilápias-do-Nilo e salmão do atlântico, não promoveram alterações na expressão de genes correlacionadas com o sistema imunológico (Rodríguez *et al*., 2016; Salah *et al*., 2017).

Os efeitos contraditórios sobre a suplementação de β-glucanos em dietas para peixes pode ser justificado pelas diferentes doses, período de alimentação ou fase da vida do peixe. Além disso, os β-glucanos comerciais possuem diferentes métodos de processamento, bem como

fonte (fungos e bactérias), o que pode levar a diferentes atividades e resultados, no que se refere ao sistema imunológico (Ai *et al*., 2007; Bridle *et al*., 2005; Ringo *et al*., 2010).

4. Saúde intestinal em peixes

Como anteriormente citado, as barreiras físicas como escamas, pele e brânquias constituem a primeira linha de defesa dos peixes contra agentes patogênicos (Manning, 1998). Aliado a tais barreiras e apontado como um dos indicadores mais importantes de saúde, o intestino é considerado o maior órgão imunológico dos peixes. A saúde intestinal tem ligação direta com a função fisiológica, pois afeta a taxa de absorção dos nutrientes e energia, como também a função imunológica (Zhang *et al*., 2019), além de ser considerado como tecido linfoide de peixes (Boschi *et al*., 2011).

As células epiteliais do intestino, os enterócitos, possuem a função de barreira física contra agentes patogênicos (Liu *et al*., 2020c). Os enterócitos se unem por meio de junções intercelulares, como por exemplo, o complexo de junções apicais (CJA) (Tsukita *et al*., 2001). A integridade e o desempenho de tal barreira dependem do CJA, que é composto por junções oclusivas (JOs), ou como conhecidas do inglês *tight junctions*, e as junções aderentes (JAs), os desmossomos (Gonzalez-Mariscal *et al*., 2003; Wheelock; Johnson, 2003). Os complexos constituídos de proteínas das JOs são bem conhecidos por incluir as zônulas de oclusão (ZOs), que possuem proteínas como claudinas e ocludinas (Chen *et al*., 2017; Schneeberger, 2004; Tsukita *et al*., 2001), enquanto as JAs consistem em proteínas de transmembranas, como as caderinas (Ogita; Takai, 2010; Wheelock; Johnson, 2003). Essas junções desempenham papel fundamental na regulação da permeabilidade do intestino dos peixes, logo, é necessário a avaliação da morfologia intestinal para detectar possíveis lesões e implicações (Niklasson *et al*., 2011; Chamorro *et al*., 2019). Portanto, a saúde dos peixes está diretamente ligada à saúde intestinal, pois o aumento da permeabilidade intestinal, seja devido a ação de componentes nutricionais ou estresse, pode suprimir a absorção de nutrientes e, possivelmente, reduzir o

ganho de peso, além de proporcionar maior risco de translocação de toxinas, parasitas e agentes patogênicas (Sundh *et al*., 2010; Grenier; Applegate, 2013; Shi *et al*., 2017).

Como método para a avaliação da saúde intestinal dos animais, a fluoresceína isotiocianato–dextrana (FITC-d) tem sido utilizada como indicador para a avaliação da permeabilidade intestinal (Napolitano *et al*., 1996). Os dextranos são polissacáridos não digeríveis com tamanhos moleculares que variam entre 3-kDa e 2000-kDa (Wang *et al*., 2015; Woting; Blaut, 2018). O FITC-d com tamanho molecular de 4-kDa é o mais utilizado atualmente em aves, por ser suficientemente grande e não atravessar a barreira epitelial intestinal em grandes quantidades após a administração oral, a menos que a barreira intestinal esteja comprometida (Gilani *et al*., 2017b). Em contrapartida, as moléculas menores (<300 Da) podem atravessar passivamente as JOs (Sun *et al*., 1998). No entanto, a inflamação ou lesão do epitélio intestinal podem induzir a disfunção da barreira, resultando em distúrbios que aumentam a migração da molécula FITC-d para a camada serosa do intestino e, subsequentemente, na circulação (Yan *et al*., 2009; Condette *et al*., 2014). Como agente auxiliador da saúde intestinal, DAWOOD *et al*., (2021) observaram que suplementação dietética de saponinas para carpa comum promoveu a integridade da parede intestinal, melhorando as alturas e espessuras das vilosidades.

O intestino dos peixes contém vários tipos de células imunocompetentes que são responsáveis pela eliminação dos agentes patogênicos (Balcázar *et al*., 2006; Nie *et al*., 2017). A microbiota intestinal dos peixes também auxilia na proteção contra a colonização e multiplicação de agentes patogênicos (Gómez; Balcázar, 2008). Em geral, a ingestão dietética de prebióticos promove maior número de bactérias benéficas (*Lactobacillus spp.* e *Bifidobacterium spp.*) e menor número de bactérias patogênicas (*Vibrio spp.* ou *Aeromonas spp.*) (Ringo *et al*., 2010; Song *et al*., 2014; Torrecillas *et al*., 2014). Os β-glucanos são

benéficos tanto por seus efeitos nas células intestinais dos peixes, quanto pelas interações com as bactérias benéficas (Murphy *et al*., 2020).

Os β-glucanos ao chegarem no intestino dos peixes via ração, estimulam os enterócitos epiteliais a sintetizarem apolipoproteína A-IV (APOA4), a qual está relacionada com o metabolismo dos carboidratos e lipídeos, que provavelmente captura o β-glucano e o conduz para a circulação. A actina está permanentemente presente nas microvilosidades intestinais, que juntamente com a transgelina participam na captação do β-glucano dependente da actina (Kiron *et al*., 2016). Além disso, a presença de RTTs nos enterócitos pode promover o reconhecimento do β –glucano (Lauriano *et al*., 2016). Quando os β-glucanos adentram na circulação são reconhecidos por certos receptores, como as lectinas encontradas nas células imunes inatas e, juntamente com a tirosina quinase, geram a transdução de sinal intracelular pelas vias das proteínas quinases ativadas por mitógenos e TNF (Kiron *et al*., 2016; Pietretti *et al*., 2013; Angulo *et al*., 2018).

Tanto as saponinas, como o MOS e β-glucano são comumente utilizados de forma isolada, ou associados com outros produtos, na suplementação de dietas para peixes, pois podem promover o desempenho, resistência a doenças e absorção de nutrientes. Contudo, a associação de saponinas, polifenóis, MOS e β-glucano ainda não foi documentada até a presente data. Portanto, o presente estudo tem por objetivo avaliar os efeitos da suplementação dietética dos aditivos eubióticos à base da mistura de diferentes fontes de saponinas e polifenóis e à base de parede celular de levedura, de forma isolada e conjunta nos parâmetros zootécnicos, no coeficiente de digestibilidade aparente de nutrientes, energia e minerais, e na morfologia intestinal, como também, na capacidade antioxidante, perfil hematológico, no estado imunológico de tilápias-do-Nilo frente a estresse por classificação de tamanho, infecção por *Streptococcus agalactiae* e infecção por *Streptococcus agalactiae* + 12 horas de hipóxia.

O Capítulo II, denominado “Effects of *Quillaja saponaria*, *Yucca schidigera* and *Saccharomyces cerevisiae* on growth performance and health of Nile tilapia (*Oreochromis niloticus*) subjected to different types of stress” foi redigido de acordo com as normas para publicação do periódico Aquaculture (fator de impacto 5.135).

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CAPÍULO II

Effects of *Quillaja saponaria, Yucca schidigera* and *Saccharomyces cerevisiae* on the growth performance and health of Nile tilapia (*Oreochromis niloticus*) subjected to different types of stress

ABSTRACT: This research aims to evaluate the effects of *Quillaja saponaria* + *Yucca schidigera* extract, *Saccharomyces cerevisiae* and their combination on the growth performance, intestinal morphometry, intestinal permeability, digestibility, antioxidant profile, hematological and immunological parameters of Nile tilapia (*Oreochromis niloticus*) subjected to three different challenges: size sorting induced stress (SSIS), bacterial challenge with *Streptococcus agalactiae* infection (BAC), and bacterial challenge with *Streptococcus agalactiae* infection + 12 hours of hypoxia (BAC + HP). For this purpose, the research was divided into three studies. In the first study, digestibility trial, 216 male Nile tilapia (~ 80g) were distributed randomly into 12 tanks (18 fish/tank with 250 L) and fed four experimental diets (control diet, *Quillaja saponaria* + *Yucca schidigera* extract - QY, *Saccharomyces cerevisiae* – SC, and combination of both - QYSC) to feces collection and the apparent digestibility coefficient of diets determination. For study second, 600 male Nile tilapia (~ 8.9 g) were randomly distributed randomly into 40 tanks (250 L) with 15 fish/tank and fed the same diets as aforementioned for 60-day feeding period. Then, growth performance, whole body composition and intestinal morphometry were evaluated. Ten fish per treatment were collected for hematological profile, innate immune response, antioxidant enzyme activity and biochemical parameters analysis. The data obtained was considered before stress parameters. Then, for the third study, stresses, one group with 60 fish was subjected to SSIS, the second group with 72 fish was subjected to BAC (15 days), and the third group with 136 fish was subjected to BAC + HP (15 days). After the challenges, the same aforementioned analyses were performed, generating after stress data. Fish fed supplemented diets showed no significant differences in growth performance and limited effects on the apparent digestibility coefficients of nutrients and energy. QY supplemented diets showed the highest levels of phenolic content, antioxidant capacity and survival after BAC and BAC + HP, and QYSC supplemented diets the lowest. SC improved mid intestine villus length and promote lower survival after BAC and BAC + HP. Fish fed SC showed better innate immune responses and antioxidant capacity after challenges. Results of the present study suggest that dietary supplementation of SC determined better overall health results, but it was not sufficient to avoid mortality under bacterial infection. Therefore, futher studies are necessary to validate the combination of eubiotic additives based on *Quillaja saponaria*, *Yucca schidigera*, *Saccharomyces cerevisiae* and their combinations in fish nutrition.

Keywords: feed additives; fish resistance; immunenutrition; *Oreochromis niloticus*; *Streptococcus agalactiae*; stress response.

1. *Introduction*

Nile tilapia (*Oreochromis niloticus*) is one of the most important fish species in the world aquaculture industry, occupying the 4th position among other cultured fish species for human consumption. In Brazil, Nile tilapia is the main species commercially produced, due to its rapid growth, adaptability, and resistance to different environmental conditions (Al-Deriny *et al*., 2020).

Due to its rusticity and capacity to cope with stress, inherent in the intensive culture systems, Nile tilapia has been stocked in high densities to meet the demand of Brazilian and global market. Aiming to increase production per square meter and ensure greater yield in less time (Dawood, 2020), aquaculture industry have been adopting procedures that tend to harm fish immunological system, which may be associated to reduced growth rates and disease-related losses in intensive systems (Mohammadi *et al*., 2020).In intensive systems, fish are subjected to multifactorial stressors, which can lead to increased infection by opportunistic pathogenic bacteria and, consequently, significant losses in production (Abdel-Latif *et al*., 2020). One of the bacteria that has been described as a main problem in fish production is *Streptococcus agalactiae*, present worldwide (Dramsi *et al*., 2006; Zhu *et al*., 2018), and its severity depends on environmental factors, such as high temperatures and poor water quality, generally caused by incorrect management and/or high stocking densities (Shoemaker & Klesius, 1997; Bromage & Owens, 2002).

In addition to the negative health effects, intensive systems can have adverse impacts on intestinal function, impairing the absorption of nutritional components present in the feed, due to the reduction in the capacity for digestion and absorption (Hegazi & Hasanein, 2010; Yilmaz, 2019). Thus, functional additives have been proposed as nutritional strategies to mitigate the consequences of the stressful effects intrinsic to intensive production systems (Rajabiesterabadi *et al*., 2020; Yilmaz, 2020; Yousefi *et al*., 2020).

Studies have been carried out to elucidate the possible effects of feed additives on the antioxidant enzymes activity and innate immune system, which can be initiated from the absorption on gastrointestinal tract of fish (Hoseinifar *et al*., 2017; Dawood *et al*., 2020a). The intestine can be considered one of the main entries for pathogens, particularly when fish are not prepared to face adverse conditions that the intensive system imposes (Standen *et al*., 2016). To ensure the health and well-being of Nile tilapia, efforts have been made by animal nutrition companies to offer products that improve the resistance and health of fish under intensive production conditions. Among many additives, two classes of eubiotics can be highlighted, phytogenic and prebiotic, as an example of phytogenic, we can highlight the extract of *Quillaja saponaria* and *Yucca schidigera* (source of saponins and polyphenols), as an example of prebiotic, we can also highlight the yeast *Saccharomyces cerevisiae*.

Quilaja saponaria and *Yucca schidigera*, which have been described to act on digestion and absorption of nutrients, mainly due to the presence of saponins and polyphenols in their composition. Saponins can be classified as bioactive compounds related to the change in the permeability of the intestinal mucosa, inhibiting the transport of some nutrients and facilitating the absorption of others (Hauptli & Lovatto, 2006). In addition, *Quillaja saponaria* and *Yucca schidigera* have antioxidant characteristics, due to their high content of polyphenols such as flavan-3-ols, which have the characteristic of stabilizing and eliminating the excessive production of reactive oxygen species, preventing possible damage to fish cells, such as lipid peroxidation, increasing activity of antioxidant enzymes (Martínez-álvarez *et al*, 2005; Cigerci *et al*., 2009; Elbialy *et al*., 2020; Abdel-Tawwab *et al*., 2021; Dawood *et al*., 2021). An increase in weight gain, specific growth rate, protein efficiency, and the activity of intestinal enzymes such as amylase and trypsin and liver enzymes such as lactate dehydrogenase and cytochrome c-oxidase were also described for carp (*Cyprinus carpoio*) by Serrano (2013). A higher growth

rate was described for Nile tilapia fed diets supplemented with 300 mg kg^{-1} of saponin for 14 weeks, along with better feed efficiency (Francis *et al*., 2001b).

Saccharomyces cerevisiae contains mannanoligosaccharides (MOS) and β-glucan, which have been shown to be effective in promoting greater fish resistance under stress conditions, especially when affected by bacterial pathogens, through the stimulation of innate immune responses (Meena *et al*., 2013; Petit & Wiegertjes, 2016; Pilarski *et al*., 2017). The supplementation with MOS (4.5 g kg^{-1}) presented greater antioxidant enzymes activities and reduced lipid peroxidation for Nile tilapia (Özlüer-Hunt *et al*., 2011). Studies have shown that Nile tilapia fed β-glucan supplemented diets presented greater weight gain, as well as better intestinal morphology and immune parameters, and greater resistance when subjected to high-density stress (Welker *et al*., 2012; Barros *et al*., 2014; Dawood *et al*., 2020).

Therefore, this research aims to evaluate the effects of *Quillaja saponaria* + *Yucca schidigera* extract (QY), *Saccharomyces cerevisiae* (SC) and combination of both (QYSC) on growth performance, intestinal morphometry, intestinal permeability, digestibility, antioxidant profile, hematological and immunological parameters of Nile tilapia (*Oreochromis niloticus*) subjected to three types of stress: size sorting induced stress (SSIS), bacterial challenge with *Streptococcus agalactiae* infection (BAC), and bacterial challenge with *Streptococcus agalactiae* infection + 12 hours of hypoxia (BAC + HP).

2. *Material and methods*

This research consisted of three studies. Study I was related to digestibility, study II to growth performance, and study III, to stress. The digestibility trial was set to determine the apparent digestibility coefficients (ADC) of dry matter (DM), crude protein (CP) crude energy (CE), ash and minerals. Twelve groups of 18 fish (~80 g) were fed four experimental diets (control diet, QY, SC and QYSC) containing a non-digestible marker (chromium oxide 3, 1 g kg^{-1}). For growth performance assay a group of 600 male Nile tilapia (~8.9 g) were randomly

distributed in 40 250-L tank (15 fish/tank) and fed the same four experimental diets for 60 days. Then, fish were subjected to tree types of stress. One group was subjected to size-sorting induced stress (SSIS), another group to bacterial challenge with *Streptococcus agalactiae* infection (BAC), and a third group to bacterial challenge with *Streptococcus agalactiae* infection + 12 hours of hypoxia (BAC + HP), aiming to better understand the putative nutritional effect of QY, SC and QYSC on Nile tilapia health. All experimental procedures were approved by the Animal Ethics Committee of the Veterinary and Animal Science College, São Paulo State University (protocol CEUA 0009/2021).

2.1. *Experimental diets and feeding management*

For digestibility and growth trials, four isonitrogenous and isoenergetic diets were formulated to contain 29% of digestible protein and 3200 $kcal^{-1}$ of digestible energy. The additives were added according to the manufacturers' recommendations, as follows: QY (0.05% with 4% of phytoactives); SC (0.3% - MOS and β-glucan) and QY (0.05%) + SC (0.3%). For digestibility trial, all experimental diets contained 0.1% of chromium oxide as a non-digestible marker. Such diets were formulated according to the nutritional requirements established by NRC (2011) and Furuya *et al*. (2010). For feed processing, all ingredients were finely ground (0.5 mm), weighed and homogenized. Water (70ºC) was added to the mixture in the proportion of 25% of the total weight, and then submitted to the extrusion process in simple screw equipment (EX LABORATÓRIO®, Exteec, Ribeirão Preto, SP, Brasil). After processing, diets were dried in a forced circulation oven at 55°C for 24 hours. Diets were stored in a cold chamber (4ºC). Ingredients and proximate composition of the experimental diets are presented in Table 1.

Fish were fed five times a day until apparent satiation (8:30 am; 11:30 am; 2:00 pm; 4:00 pm and 6:00 pm) adjusting the amount of feed according to consumption and leftovers in the tanks. The water quality parameters were monitored using an YSI PRO 1020® multi-probe

system (YSI Environmental, Yellow Springs, OH, USA) once a day: temperature (27 ± 0.6 °C); pH (6.42 ± 0.28); and dissolved oxygen (5.87 ± 0.85 mg L^{-1}). The ammonia concentration (0.05 ± 0.02) was determined using a commercial test kit (Alcon®, Camboriú, SC, Brazil).

2.2. *Quantification of total phenols (TP), DPPH and FRAP in diets*

The TP content was determined according to Singleton and Rossi (1965). Extracts were prepared using acidified methanol solution. Folin-Ciocalteu reagent and 20% sodium carbonate were added to the mixture. The results were calculated with a gallic acid calibration curve and absorbance was measured at 725 nm.

The superoxide radical scavenge activity of diets was determined according to Brand-Williams *et al*. (1995) with slight modifications. Briefly, dried sample (500 mg) were weighed into light-protected scales, extracted with ethanol, and mixed with a 40 μM DPPH ethanolic solution. Trolox was used as the reference standard to convert the inhibitory capacity of each sample to antioxidant power in Trolox equivalence. The ferric reducing power (FRAP) was determined according to Benzie & Strain (1996) and spectrophotometrically measured at 594 nm.

2.3. *Digestibility trial (Study I)*

The digestibility trial was performed using 12 250-L tank equipped with a net cage for feeding procedure and four conical 300 L tanks with a detachable plastic vail for feces collection. The feeding system was connected to the same thermo-regulated recirculating water system, while the feces collection system was individually heated.

A group of 216 male Nile tilapia fingerlings was obtained from Caunesp (Centro de Aquicultura da Unesp, Jaboticabal, SP, Brazil), and transferred to AquaNutri Laboratory facilities (FMVZ, Botucatu, SP, Brazil). Fish were kept in wet laboratory conditions and fed basal diet with no QY, SC and QYSC supplementation for quarantine period.

Nile tilapia juveniles with a mean weight of 80 g were allocated into 12 feeding tanks (18 fish/tank) compose by three replicates. During the first week, fish adapted to the experimental diets and system, and then feces were collected as follows: after the last meal, net cages from four groups of fish were transferred to the feces collection tanks. In the subsequent morning, feces were collected, and the net cages returned to feeding tanks. After that, feces samples were dried, weighed, and frozen at - 20 °C until further analysis. The same procedure was done with the remaining groups on the following day. The procedure was repeated, at least three times, until enough fecal material was collected, which was completed after 9 days of collection. The apparent digestibility coefficients (ADC) of dry matter (DM), crude protein (CP), and crude energy (CE) were determined according to Nose (1960):

$$ADC = 100 - [100 \times (\%\ Cr_2O_3 \text{ in diet} / \%\ Cr_2O_3 \text{ in feces}) \times (\%\ \text{energy or nutrient in feces} / \%\ \text{energy or nutrient in diet})]$$

2.4. *Growth performance (Study II)*

A group of 600 male Nile tilapia fingerlings was obtained from a commercial fish farm (Piscicultura S3, Registro, SP, Brazil), and transferred to AquaNutri Laboratory facilities (FMVZ, Botucatu, SP, Brazil). Fish were kept in wet laboratory conditions and fed basal diet with no QY, SC and QYSC supplementation for quarantine period.

Fish were then randomly sampled (8.93 ± 0.14 g), stocked in fiber glass tanks (250 L, 15 fish/tank) and hand-fed experimental diets five times a day for 60 days to apparent satiation, in a completely randomized design consisting of four treatments (control diet, *Quillaja saponaria* + *Yucca schidigera* extract - QY, *Saccharomyces cerevisiae* – SC, and combination of both - QYSC) and ten replicates. At the end of the 60 days, the analysis of growth performance was performed, consisting of Initial body weight (IBW), final body weight (FBW), weight gain (WG), daily growth index (DGI), feed Intake (FI), feed conversion rate (FCR), specific growth rate (SGR), protein efficiency ratio (PER) and survival rate (SUR).

The recirculated system was supplied with 6 L min^{-1} dechlorinated tap water passing through a biological filter to reduce impurities and ammonia concentration. The photoperiod was maintained at a 12 hours light:12 hours dark schedule.

2.5. *Intestinal permeability*

In order to evaluate the intestinal permeability, two groups from growth performance trial starved for 24 hours and after being anesthetized with benzocaine (100 mg L^{-1}), one group of 32 fish (8 fish / treatment) received fluorescein isothyocianate (FITC)-dextran (IMUNOVA, Curitiba, PR, BR) through oral gavage diluted in deionized water (5 mg dosage) and the control group with eight fish received deionized water following the same procedure. The first group was kept in separate tanks for 4 hours to allow intestinal marker absorption. Then, blood was collected from the caudal vein, immediately centrifuged (5 min, 5000 rpm, 4 °C) and the serum was aliquoted and kept at - 80 °C until further analysis.

2.6. *Intestinal morphometry*

Eight fish per treatment were euthanized at day 60 of feeding trial for intestinal samples collection. The proximal, mid, and distal segments of the intestine were sampled, preserved separately in Bouin's solution for 24 hours, and then transferred to a 70% ethanol solution. Transverse slices of the different segments were processed to 5 mm slides and stained with haematoxylin and eosin after being cut into segments of about 1 cm long and embedded in paraffin. A software analyzer system (AmScope / MU300) was used to further evaluate the electronic pictures after the slides had been examed using a microscope to measure the villus height (μm).

2.7. *Chemical Bromatological Analyzes*

Samples of diets, feces and whole body composition were ground and analyzed in triplicate following standard methods (AOAC, 2000) for dry matter (DM), crude protein (CP),

crude lipid (CL), gross energy (CE). Gross energy content was measured in a calorimeter (C200, IKA, Staufen, Germany), and chromium oxide 3 concentration in diets and feces was determined according to Bremer Neto *et al*. (2005). The mineral profile of the experimental diets and feces was assessed after nitro/perchloric digestion, being the chromium oxide, calcium, cupper, iron, manganese, zinc, selenium and phosphorus contents quantified using atomic absorption in flame technique (Shimadzu, 2002) and visible spectrophotometry (Markzenk, 1976). The apparent digestibility coefficients of nutrients and mineral availability were determined according to Pezzato *et al*. (2004).

2.8. *Challenges (Study III)*

In brief, after 60 days of feeding period fish (ten fish/ treatment) were sampled (benzocaine, 100 mg L^{-1}) for hematological profile, antioxidant enzyme activity, immunological parameters. These data were considered before stress. After that, three other groups were established from the remaining fish: one group was subjected to size-sorting induced stress (SSIS; 15 fish/ treatment), another group to bacterial challenge with *Streptococcus agalactiae* infection (BAC; 24 fish/ treatment), and the other group to bacterial challenge with *Streptococcus agalactiae* infection along with a 12 hours-hypoxia at day 14 (BAC + HP; 34 fish/ treatment). Then, fish (ten fish/ treatment) from each stress were sampled, and the same hematological profile, immunological parameters and antioxidant enzyme activity analyses were determined. These data were considered after stress. Both data, before and after, were compared aiming to understand if fish fed QY, SC and QYSC were nutritionally better prepared to cope with stress.

2.8.1. Size-sorting-induced stress (SSIS)

A group of 60 fish (76.74 ± 7.20 g) was submitted to SSIS as described by Freitas *et al*. (2022). In brief, groups of 15 fish/ treatment were randomly collected and confined into an experimental net cage at a high stock density (140 kg m^{-3}) for 15 min, simulating the harvesting

process. Each net cage (treatment) was kept inside an isolated 250L tank. Then, fish were transferred to a fiberglass table and sorted into three sizes i.e., small, medium, and large, as it is routinely carried out in a Nile tilapia fish farm. This practice lasted one minute per treatment and then fish were immediately subjected to the sampling process. For such, ten fish/treatment were randomly sampled, and the same hematological profile, immunological parameters and antioxidant enzyme activity were evaluated, and the stress profile determined.

2.8.2. Bacterial challenge with Streptococcus agalactiae infection (BAC)

Before challenge, the lethal dose 50% (LD_{50}) of *Streptococcus agalactiae* (GenBank accession n°. KU605571) in Nile tilapia was experimentally determined by intracoelomic cavity injection to verify the optimum bacterial concentration to perform the bacterial challenge according to Vaneci-Silva *et al*. (2022). In sum, *Streptococcus agalactiae* was cultured in brain heart infusion (BHI) (28 °C for 48 hours), inoculated in BHI broth, and incubated for 48 hours at 28 °C. The bacteria were then washed twice with phosphate-buffered saline solution (PBS, pH 7.4), and centrifuged (3000 × g, 4 °C for 10 min, until optical density reach absorbance of 600nm (OD600). The concentration as determined in LD_{50} at 0 (PBS negative control) and 0.600 corresponding to 1 x 10^6 colony forming units (CFU mL^{-1}).

The LD_{50} trial was conducted with four groups of 24 fish (64.58 ± 15.15 g). Fish were anaesthetized in 100 mg L^{-1} benzocaine solution, after that fish were infected intraperitoneally with 0.01mL/g of *Streptococcus agalactiae* with graded concentration levels, while another group of ten fish were injected with PBS as a negative control. Mortality was recorded for 15 days.

After LD_{50} determination (26.05 × 10^6 CFU/mL^{-1}), 96 fish (24 fish/treatment) were anaesthetized in 100 mg L^{-1} benzocaine solution, and then fish were infected intraperitoneally. Fish were randomly stocked into 40, 40 L tanks and mortality was recorded for 15 days, and then fish were collected for further analyses.

2.8.3. Bacterial challenge with Streptococcus agalactiae infection + 12 hours hypoxia (BAC + HP)

A group of 148 fish (34 fish/ treatment) was submitted to the same procedure described in 2.8.2. along with a 12 hours-hypoxia at day 15. Which was caused by turning off the recirculation and aeration system before samples collections.

2.8.4. Hematological profile

Red blood cell count (RBC) was determined by dilution and enumeration using a hemocytometer. Hemoglobin (Hb) was determined by the cyanmethaemoglobin colorimetric method using a commercial kit (Gold Analisa Diagnóstica, Belo Horizonte MG, Brazil) according to Collier (1944). Hematocrit (Htc) was measured by the microhematocrit method (Goldenfarb *et al*., 1971). The mean corpuscular volume (MCV) and the mean corpuscular hemoglobin concentration (MCHC) were calculated as MCV = (10 × Htc)/RBC and MCHC = (100 × Hb)/Htc (Wintrobe, 1934). The concentration of serum glucose (GLU) was determined by spectrophotometry using commercial kits (Lagoa Santa, Minas Gerais, Brazil) and plasma cortisol (CORT) using a commercial kit (DGR Cortisol ELISA, Frauenbergstr, GERMANY) according to the manufacturers' recommendations.

2.8.5. Immunological parameters

Lysozyme activity in serum (LYZ) was defined as the amount of enzyme producing a decrease in absorbance of 0.001/min at 530 nm (Ellis, 1990). Leukocytes respiratory burst activity is the colorimetric determination of the reactive oxygen species produced by the leukocytes respiratory burst, which promotes reduction of nitroblue tetrazolium (NBT) into dark blue precipitate inside the phagocyte in fish blood, called formazan granules, determined according to Anderson & Siwicki (1995), with modifications. The serum alternative complement activity (ACH50) represents the volume of serum necessary to produce lysis of 50% of the target cells under standard conditions according to Castro *et al*. (2008).

2.8.6. Liver antioxidant enzyme activity

Liver samples were thawed overnight prior to analyses. One gram of liver samples was homogenized in 5 mL of 0.05 M phosphate buffer (pH 7.0) and the homogenate centrifuged (2800 g; 20 min; 4 °C). The supernatant was separated and immediately analyzed for enzyme activity and protein content. Superoxide dismutase (SOD; EC 1.15.1.1) activity was assayed by the nitroblue tetrazolium (NBT) assay which monitors the reduction of NBT spectrophotometrically at 560 nm (Beauchaump & Fridovich, 1971). Catalase (CAT; EC 1.11.1.6) activity was assayed according to Sinha (1972), in which dichromate in acetic acid is reduced to chromic acetate in the presence of H_2O_2 when heated, forming perchromic acid as an unstable intermediate. The absorbance reading was performed in spectrophotometer at 610 nm. Glutathione peroxidase (GPx; EC 1.11.1.9) activity was assayed according to Flohè & Günzler (1984) with absorbance reading at 320 nm. Antioxidant enzyme activities were expressed as $U.mg^{-1}$ of protein with total protein concentration determined by Bradford method (Bradford, 1976) using coomassie brilliant blue G-250 as dye and bovine serum albumin as standard with absorbance reading at 595 nm.

2.8.7. Liver lipid peroxidation (MDA)

Concentration of malonaldehyde (MDA) was determined according to Buege & Aust (1978). An aliquot of supernatant from the homogenate (200 μL) was mixed with 500 μL of a solution containing 15% (w/v) trichloroacetic acid (TCA), 0.375% (w/v) thiobarbituric acid (TBA), 80% (v/v) hydrochloric acid 0.25 N and 0.01% (w/v) butylated hydroxytoluene. The mixture was heated to 100 °C for 15 min. Then, after being cooled to room temperature and centrifuged at 1500 g for 10 min, absorbance was measured at 535 nm in the supernatant. Concentration was expressed as nmol MDA per gram of tissue, calculated from a calibration curve.

2.8.8. Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's test to determine possible significant differences among treatments. All analyses were conducted using Minitab® 16.1.1.0 software. Differences among treatments were considered significant at $P < 0.05$. Each data was expressed as mean ± SD. The Student t-test was used to compare values when the data presented non-parametric behavior, and to compare control diet with QY, SC and QYSC diets.

3. *Results*

3.1. *Phenolic content and antioxidant capacity of experimental diets*

The results for analyzed phenolic content and antioxidant capacity of experimental diets are shown in Table 1. Diets supplemented with QY showed higher total phenols and FRAP. The combination QYSC presented the lowest content levels, followed by the control diet. Moreover, diets showed no statistical differences for DDPH ($P < 0.05$).

3.2. *Apparent digestibility coefficient*

There was no effect of the additivies on dry matter, crude protein, crude lipid and crude energy of the diets ($P > 0.05$). Results are shown in Table 2. QY and SC determined greater calcium digestibility ($P < 0.05$). Although it resulted in greater phosphorous digestibility for QY, SC and QYSC ($P < 0.05$). The lowest iron digestibility was determined for fish fed SC supplemented diets followed by the control diet ($P < 0.05$). QYSC supplemented diets determined the highest selenium digestibility, and QY supplemented diets the lowest, following by QY ($P < 0.05$). For both zinc and copper QYSC lead to the highest digestibility coefficients ($P < 0.05$). Control diet showed the highest manganese digestibility when compared with the supplemented diets ($P < 0.05$).

3.3. *Growth performance and whole body composition*

Growth performance parameters were not significantly affected by either QY, SC or QYSC after 60 days (Table 3) ($P > 0.05$). By comparing treatments, all of them (but SC) showed an increase on dry matter content ($P < 0.05$). Fish fed the control diet showed higher content for lipid, energy and protein ($P < 0.05$).

3.4. *Intestinal permeability*

There was a significant difference on the intestinal permeability. QYSC supplemented diets determined the highest permeability (Table 4) ($P < 0.05$).

3.5. *Intestinal morphometry*

The results of microscopic morphometry indices are shown in Table 5. There was no difference for proximal and distal intestine portions ($P > 0.05$). As to the villus length on mid intestine segment it was observed that SC determined a significant improvement, followed by QYSC, which did not differ from QY and control diet ($P < 0.05$).

3.6. *Hematological parameters: SSIS*

By comparing all treatments after SSIS, fish fed SC supplemented diet showed the highest number of RBC ($P < 0.05$); the highest Htc for fish fed QY and SC ($P < 0.05$), and the highest MCHC ($P < 0.05$) for fish fed the control diet. SSIS determined the highest value ($P < 0.05$) for Htc and the lowest A:G ratio and MCHC, regardless the treatment. The highest value for RBC after SSIS was observed for fish fed QY ($P < 0.05$), and the highest MCV was observed for fish fed QY and QYSC ($P < 0.05$). SSIS determined the highest values for serum glucose, regardless the treatment. As to plasma cortisol, fish fed all treatments (but SC) showed the highest level after SSIS. All results are shown in Table 6 and 7.

3.7. *Hematological parameters: BAC*

Bacterial infection determined a decrease ($P < 0.05$) for RBC, Hb, Htc and A:G ratio. As to MCV, a decrease was observed after BAC ($P < 0.05$) for fish fed QYSC. A significant increase on GLU and CORT was observed for fish fed QYSC supplemented diet (Table 6 and 7).

3.8. *Hematological parameters: BAC + HP*

BAC + HP determined a decrease ($P < 0.05$) on RBC, Hb, and Htc. As to A:G ratio a decrease was observed only for fish fed QY supplemented diet. Values are shown on Table 6**.**

3.9. *Immunological parameters: SSIS*

Fish fed QY and SC supplemented diet showed the highest ($P < 0.05$) LYZ activity (5 min) and serum alternative complement activity (ACH50) after SSIS. The highest LYZ activity (10 min) ($P < 0.05$) was observed for fish fed control diet and SC. Results are shown in Figure 1 – 4.

3.10. *Immunological parameters: BAC*

BAC led to an increase ($P < 0.05$) on LYZ activity (5 min) for fish fed QY and SC. The same result was observed for LYZ (10 min) along with fish fed the control diet (Figure 1 and 2). The lowest mortality rate (Figure 5) was observed for fish fed QY supplemented diet.

3.11. *Immunological parameters: BAC + HP*

BAC + HP led to an increase ($P < 0.05$) on LYZ activity (10 min) for fish fed SC. For serum alternative complement activity (ACH50) all treatments presented low levels after BAC + HP. The lowest mortality rate (Figure 5) was observed for fish fed QY supplemented diet.

3.12. *Antioxidant enzyme activity: SSIS*

By comparing the treatments, the supplementation of QY, SC, and QYSC did not affect SOD and GPx, either before or after SSIS. There was also no effect for lipid peroxidation (MDA) ($P > 0.05$). However, the control group showed the highest catalase (CAT) activity compared to with supplemented diet-groups before SSIS, and by comparing values before and after SSIS a significant decrease in CAT activity was observed ($P < 0.05$). Fish fed QYSC supplemented diet showed significant increase in GPx activity after SSIS challenge ($P < 0.05$). Results are shown in Figure 6 - 9.

3.13. *Antioxidant enzyme activity: BAC*

Comparing all treatments before BAC, it was observed that fish fed the control diet showed the highest CAT activity ($P < 0.05$). Bacterial infection determined a significant reduction on SOD and CAT activity for fish fed all diets ($P < 0.05$) (Figure 6 - 9).

3.14. *Antioxidant enzyme activity: BAC + HP*

Comparing all treatments, it was observed an increase on CAT activity for fish fed the control diet before BAC + HP. Such challenge determined a significant decrease on SOD activity for all fish groups ($P < 0.05$). As to CAT it was observed a significant decrease for fish fed the control group and QY supplemented diet ($P < 0.05$). Fish fed all treatments, but SC supplemented diet showed an increase on GPx activity ($P < 0.05$) after challenge ($P < 0.05$). Related to MDA, fish fed the control diet and QY supplemented diet showed higher lipid peroxidation after stress ($P < 0.05$). Results are shown in Figure 6 - 9.

4. *Discussion*

This research aims to evaluate the effects of *Quillaja saponaria* + *Yucca schidigera* extract (QY), *Saccharomyces cerevisiae* (SC), and combination of both (QYSC) on digestibility, growth performance, intestinal morphometry, antioxidant profile, hematological and immunological parameters of Nile tilapia (*Oreochromis niloticus*) subjected to three different challenges.

The highest dietary antioxidant capacity observed for QY is probably related to the phenolic compounds, such as saponins, and for SC due to the presence of β-glucan. On the other hand, the interaction between saponins and yeast cell membrane resulted in the lowest antioxidant capacity. Researchers have shown that the cell membrane sterol and saponins naturally combine into a complex that may result in a rupture, leading to a modification of the cell wall structure with a pores inside the cell, leading to the inhibition of yeast fermentation (Simons *et al*., 2006; Augustin *et al*., 2011; Cabib & Arroyo, 2013; Alcázar *et al*., 2017). Glycosidic part of saponins may react with the β-glucans in the cell wall (Alcázar *et al*., 2017), leading to a decrease in β-glucan concentration (Berlowska *et al*., 2015). This possible interaction could also explain the lowest survival rate observed for fish fed the combination of both additivies and infected with *Streptococcus agalactie*. Moreover, studies have demonstrated that β-glucan can also act as an antioxidant, due to its hydroxyl scavenging activity (Kofuji *et al*., 2012; Zhang *et al*., 2022). However, such study showed also that this activity varied according to the concentration and source of β-glucan. Therefore, further studies are needed to better understand why both additivies together decrease the dietary antioxidant capacity.

The apparent digestibility coefficient for dry matter, crude protein, crude lipid and crude energy were not affected neither by the additivies, nor by the combination. Johnson *et al*. (1986)

showed, in vitro, that some saponins could increase the permeability of the small intestinal mucosal cells, by inhibiting active nutrient transport, and facilitating the uptake of materials to which the gut would normally be impermeable. On the other hand, studies, in vivo, have shown that saponin supplementation could alter the intestinal mucosa permeability, preventing the transport of some nutrients and boosting the absorption of others (Gee *et al*., 1996; Hauptli & Lovatto, 2006). The lack of saponins positive effect on protein digestibility was also described by Chikwati (2007) for Atlantic salmon (*Salmo salar*) fed diets supplemented with saponin (1 or 2 g kg^{-1}). Moreover, β-glucan could also improve digestibility and utilization of nutrients due to the energy benefits obtained through β-glucan suplementation, by the manipulation of intestinal microbiota, and due to the production of digestive enzymes by beneficial bacteria, as previously described (Misra *et al*., 2006; Andrews *et al*., 2009; Shelby *et al*., 2009; Wu *et al*., 2014). Ours results did not corroborate this hypothesis, probably because of concentration and types of saponins and β-glucan.

However, minerals availability was affected by the additivies. Overall, results showed that the combination of the additivies improved minerals availability. Studies related to dietary saponin and β-glucan on minerals availability are scarce. A study carried out with humans showed that dietary saponins could reduce the absorption of minerals by forming insoluble complexes with minerals, such as zinc and iron (Samtiya *et al*., 2020). However, prebiotics could improve calcium absortion and cause bone mineralization and mineral bioavailability in rats (Cashman, 2003).

The intestinal morphometry could be affected either by saponins or β-glucan. The former could increase the height and branching of intestinal villi and the thickness of the intestinal mucosa, thus enhancing feed efficiency (Dawood *et al*., 2021). In addition, β-glucan could reduce the intestinal inflammation caused by stress, improving intestinal immunity (El-Murr *et al*., 2019). In the present study, a positive effect could be attributed to the modification of the

intestinal structure caused by SC supplementation, which showed a significant improvement in villus length of mid intestine followed by QYSC supplemented diets. Such improvement was previously attributed for Nile tilapia fed dietary β-glucan, 1 g kg^{-1} (Welker *et al*., 2012) and 5g kg^{-1} (Dawood *et al*., 2020a). In contrast, studies have reported no significant effects on intestinal morphometry for mirror carp fed 1 g kg^{-1}, 10 kg^{-1} and 20 kg^{-1} β-glucan-supplemented diet (Ringo *et al*., 2010; Kuhlwein *et al*., 2014; Torrecillas *et al*., 2014), 4.5 g kg^{-1} for hybrid tilapia (Nile Tilapia × Blue Tilapia *Oreochromis aureus*) (Genc *et al*., 2007), 4 g kg^{-1} for European Bass (Pryor *et al*., 2003) and 3 g kg^{-1} for Gulf Sturgeon (*Acipenser oxyrinchus desotoi*) (Torrecillas *et al*., 2007) fed MOS-supplemented diet.

Therefore, it was expected that fish fed QY supplemented diets could have shown an improvement on intestinal morphometry, due to saponin, but it did not occur. It may have occurred due to the low concentration of saponin or the fish size, which was around 70g, thus impairing the measurement of crypt depth. A FITC-d methodology was used to assess intestinal permeability (Napolitano *et al*., 1996). Although there were differences on intestinal morphometry for mid intestine, the intestinal permeability was not different using FITC-d as a marker. Studies have shown opposite results, in which saponin supplementation could change intestinal permeability, preventing the passage of some nutrients and boosting the absorption of others (Gee *et al*., 1996; Hauptli & Lovatto, 2006).

In the present study, the additives throughout 60 days had no impact on growth performance. Similar results have been reported for fish fed saponins supplemented diets (Tidwell *et al*., 1992; Krogdahl *et al*., 1995; Twibell & Wilson, 2004; Penn *et al*., 2005; Penn *et al*., 2012; Couto *et al*., 2015), and for fish fed β-glucan supplemented diets (Whittington *et al*., 2005; Chotikachinda *et al*., 2008; Adloo *et al*., 2015; Domenico *et al*., 2017; Tayyab *et al*., 2019).

However, QY and QYSC supplemented diets significantly affected whole body composition for dry matter and ash followed by SC for the later. Fish fed control diet showed the highest results for crude lipid, crude energy and crude protein content. Those lower effects for fish fed QY supplemented diets could be attributed to saponins structures, which could form insoluble complexes with lipids present in diet, impairing the emulsification of fats and the formation of micelles, thus increasing excretion, resulting in a lower fat content in fish body (Oakenfull, 1986; Cheeke, 2000). Furthermore, results for fish fed SC and QYSC supplemented diets could be attributed to saponin and to β-glucan, which could act on lipolytic pathways modulating lipid metabolism (Lopes *et al*., 2022).

Health parameters such as, hematological profile, innate immune response, and antioxidant capacity were evaluated to determine the impacts of QY, SC and QYSC on Nile tilapia health status. Although fish were submitted to SSIS and BAC challenges the hematological profile remained within the normal range for Nile tilapia (Barros *et al*., 2009; Teixeira *et al*., 2012; Barros *et al*., 2015; Damasceno *et al*., 2016; Araújo *et al*., 2017; Vicente *et al*., 2018; Xavier *et al*., 2020; Freitas *et al*., 2022; Carvalho *et al*., 2023), showing that fish were well prepared to cope with stress. Overall, the stress condition can be confirmed by the high levels of glucose and cortisol after SSIS. In general, SSIS involves capture, densification, and hypoxia, size sorting, and restocking. Such stress represents one of the most harmful scenarios for fish when compared to low temperature, transport, and thermal/hypoxia (Barton, 2002; Conte, 2004; Freitas *et al*., 2022).

Although no changes were observed in fish subjected to BAC for hematological profile, it can be hypothesized that these results are due to the samples being taken on day 15 post-infection (Barros *et al*., 2015; Guimarães *et al*., 2016; Orsi *et al*., 2017; Naliato *et al*., 2021). Similar results were reported by Sousa *et al*. (2020) for Nile tilapia infected with *Streptococcus agalactiae* and sampled on day 20 post-infection.

Furthermore, glucose levels in fish increase during stress, probably due to catecholamines on glycogen stored in the liver and other tissues (Pottinger, 1998). In agreement with our findings, β-glucan reduced cortisol levels of pacu (*Piaractus mesopotamicus*) when submitted to handling and transportation challenges (Sabioni *et al*., 2020; Mello *et al*., 2019). On the other hand, Angeles *et al*. (2017) and Abozeid *et al*. (2021) demonstrated that Nile tilapia glucose decreased significantly compared with the control group for those that received *Quillaja saponaria* and/or *Yucca schidigera*. The authors concluded that these feed additives could be effective in stabilizing the metabolic response for homeostasis.

Streptococcus agalactiae infection plus 12 hours of hypoxia on day 15 was very harmful. Hematological profile showed that all fish were anemic, which can be confirmed by levels of RBC, Hb, and Htc below for the normal range for healthy Nile tilapia (Hrubec & Smith, 2010). Therefore, none of the treatments could keep erythropoiesis under this stressful condition.

The immune responses to all types of challenges in the present study were almost the same. Fish-fed diets supplemented with QY and SC showed an increase in lysozyme (5 min) after SSIS and BAC challenges, and the same diets were responsible for the higher values of alternative complement activity after SSIS. Considering it was an acute stress, BAC + HP determined the lowest values for alternative complement activity, but fish fed SC supplemented diets excelled and presented highest values for lysozyme after challenge.

Lysozymes and alternative complement activity are crucial innate humoral immune components that prevent the invasion of harmful microorganisms. The enhanced lysozyme and alternative complement activities in our study, may have been induced by the QY and SC main components, such as saponin, MOS and β-glucan, respectively. Both can directly activate macrophages, therefore increasing the secretion of lysozyme by macrophages (Jang *et al*., 1995; Robertsen, 1999, Gatlin, 2002; Gantner *et al*., 2003; Herre *et al*., 2004; Sahoo *et al*., 2005; Meena *et al*., 2013).

Saponin is one of the main components of QY diet, this component had the potential to induce an antibody-mediated humoral immune response as reported in other animals (Yuan *et al*., 2020), and it has been shown that saponin improved the production of IFN-γ/IL-4, lymphocyte proliferation, and high specific antibody response (Ma *et al*., 2019). Many of these responses are probably triggered by their interactions with antigen-presenting cells (Barr *et al*., 1998). In parallel, β-glucan, one of the components of SC diet, have the ability to binds to cells, it activates macrophages directly (Sakai, 1999) and then all immune mechanisms, such as phagocytosis, IL1β, IL6 release, granulocyte-macrophage colony-stimulating factor (GM-CSF) and interferons are activated. These cytokines stimulate the production of new white blood cells, thus increasing β-glucan receptors (Meena *et al*., 2013).

Corroborating with our results related to lysozyme activity, Ghaedi *et al*. (2015) reported that rainbow trout fed 2 g kg^{-1} β-glucan improved lysozyme, alternative complement activity, and other immunological defenses as immunoglobulin M and total immunoglobulin. The same was observed for Nile tilapia fed 1 g kg^{-1} β-glucan for 3 weeks (El-Boshy *et al*., 2010). Olive flounder fed β-glucooligosaccharides showed higher expressions of TNFα, IL1β, and IL6 in the liver, kidney, and spleen (Hasan *et al*., 2018). In contrast, Nile Tilapia which were given the same concentration of β-glucan for 4 weeks showed no improvements in immune variables except for the respiratory burst (Welker *et al*., 2012).

Before challenges, fish had health parameters within the expected ranges for the species. Therefore, it may be suggested that before the three challenges there were no challenges imposed. The reduced activity of CAT before challenges might be due to the insufficient reactive oxygen species, since QY and SC are composed by substances that are considered antioxidants (Kofuji *et al*., 2012; Dawood *et al*., 2020a; Elbialy *et al*., 2020; Wang *et al*., 2020; Dawood *et al*., 2021; Abdel-Tawwab *et al*., 2021; Zhang *et al*., 2022), those components may

have neutralized the excess reactive oxygen species, as superoxide anion and hydrogen peroxide, which is supported by the CAT values found for fish fed control diet.

In the present study there were slight alterations on antioxidant enzymes activity for fish submitted to SSIS and BAC. Once more, BAC + HP were the most harmful challenge due to values found in the present study. Nevertheless, fish fed SC supplemented diets remained within the same values as non-challenged group for CAT, GPx and MDA parameters. Furthermore, fish fed QYSC supplemented diets showed similar values for CAT and MDA. Those results may be due to the properties mentioned above, and the anti-stress response can be explained by the fact that β-glucan promotes the innate response and leukocyte mobilization (Rauta *et al*., 2014).

In agreement with our findings, Angeles *et al*., (2017), found that Nile tilapia fed *Quillaja saponaria* and *Yucca schidigera* combination (150 mg kg^{-1} of each) presented 48% lower SOD activity compared with fish fed control diet. Differently from our findings, Elbialy *et al*. (2020), Wang *et al*. (2020) and Dawood *et al*. (2021), showed an increase in SOD and CAT activity after fish fed *Yucca schidigera* supplemented diets. In addition, β-glucan supplemented diets (4 g kg^{-1}) for Nile tilapia promoted greater gene expression related to the enzymes SOD and CAT, and reduced MDA levels when fish were challenged with Fipronil intoxication (El-Murr *et al*., 2019).

The cumulative mortality from BAC and BAC+ HP challenges was recorded till day 15. Fish fed QY supplemented diets demonstrated the lowest cumulative mortality levels for both challenges. These results can be explained by the ability of saponins to trigger the induction of immune markers (such as CD8 T cells), stimulate innate and adaptive cells through the immune system for possible infections, directly inducing macrophages and dendritic cells, and the expression of different cytokines, even when the fish were not infected (Welsby *et al*., 2017; Cortés *et al*., 2023). Corresponding results were found for Nile tilapia fed supplemented diets

supplemented with *Quillaja saponaria* and *Yucca schidigera* combination, there was an increased survival rates by 22% after a hypoxia challenge when compared with control group (Angeles *et al*., 2017), and lower mortality rates when infected with *Pseudomonas aeruginosa* (El-Keredy & Naena, 2020). Nevertheless, Nile tilapia fed β-glucan supplemented diets increased survival rates (Whittington *et al*., 2005), even when the same fish species was submitted to hypoxia challenge (Souza *et al*., 2020). The same survival rates were found for different fish species fed β-glucan supplemented diets (Sealey *et al*., 2008; Domenico *et al*., 2017).

To our knowledge, this is the first research evaluating the association of a *Quillaja saponaria* + *Yucca schidigera* extract (QY) and *Saccharomyces cerevisiae* (SC) on Nile tilapia diet. The results of this study demonstrate that QY, SC and QYSC have no deleterious effect on growth performance after the 60-day feeding period. Even so, further studies should be developed to evaluate the capacity of this combination under different types of challenge as high or low temperatures, transport and the infection with different pathogenic agents. The expression of immune and antioxidant enzymes correlated genes might also provide some insight into the effects of those additivies on fish health. Additionally, the evaluation of humoral immune system activity is also recommended, as the evaluation of T and B lymphocytes.

5. *Conclusion*

Results of the present study suggested that a dietary supplementation of *Saccharomyces cerevisiae* (SC) determined better overall health results, but it was not enough to avoid mortality under bacterial infection. Moreover, further studies are necessary to validate the combination of *Quillaja saponaria* + *Yucca schidigera* extract and *Saccharomyces cerevisiae* on fish nutrition.

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Table 1
Formulation and analyzed proximate composition of experimental diets.

Ingredients (%)	**Control**	**QY**	**SC**	**QYSC**
Corn	34.86	34.81	34.56	34.51
Soybean meal	29.30	29.30	29.30	29.30
Meat and bone meal	10	10	10	10
Feather meal	8	8	8	8
Blood meal	5	5	5	5
Poultry by-product meal	5	5	5	5
Wheat middling	5	5	5	5
Soybean oil	2	2	2	2
DL – Methionine	0.13	0.13	0.13	0.13
L - Tryptophan	0.1	0.1	0.1	0.1
Vitamin C[1]	0.09	0.09	0.09	0.09
Premix Vit/Min[2]	0.4	0.4	0.4	0.4
BHT[3]	0.02	0.02	0.02	0.02
Salt	0.1	0.1	0.1	0.1
Quillaja saponaria + Yucca schidigera extract (QY)	0	0.05	0	0.05
Saccharomyces cerevisiae (SC)	0	0	0.3	0.3
Analyzed proximate				
Dry matter (%)	94.29	94.22	94.98	95.00
Crude Energy (kcal/kg^{-1})	4670.70	4682.66	4646.24	4675.79
Crude protein (%)	36.82	36.95	37.46	36.94
Crude fiber (%)	4.10	4.75	4.00	4.34
Crude lipid (%)	7.00	7.76	7.99	7.40

Analyzed phenolic content and antioxidant capacity of experimental diets					***P* value**
Total phenols (mg g^{-1})	56.30 ± 0.30 **b**	65.60 ± 0.37 **a**	65.15 ± 0.89 **a**	51.59 ± 0.39 **c**	0.000
DPPH (ug/g Trolox)	3.44 ± 0.36	3.53 ± 0.00	3.36 ± 0.19	3.17 ± 0.40	0.485
FRAP (mmol Fe kg^{-1})	4.93 ± 0.08 **c**	5.92 ± 0.05 **a**	5.18 ± 0.04 **b**	4.35 ± 0.04 **d**	0.000

[1]Vitamin C – Stay – C® 35 – DSM. Nutritional Products. Switzerland.

[2]Vitamin and Mineral Premix – Acquamel® – Mcassab®. Brazil. (kg of product): vitamin A = 1.750.000 IU; vitamin D_3 = 375.000 IU; vitamin E = 20.000 IU; vitamin K_3 = 500 mg; vitamin B_1 = 2.000 mg; vitamin B_2 = 2.500 mg; vitamin B_6 = 2.500 mg; vitamin B_{12} = 5.000 mg; vitamin B_3 = 8.750 mg; vitamin B_5 = 7.500 mg; folic acid = 625 mg; calcium pantothenate= 12.000 mg; biotin = 50 mg; vitamin C = 37.500 mg; inositol = 12.500 mg; Fe = 15.000 mg; Cu = 4 mg; Mn = 3.750 mg; Zn = 17.500 mg; Co = 50 mg; I = 100 mg; Se = 75 mg.

[3]Butylated hydroxytoluene – antioxidant.

Values presented as means ± SD. Means in the same line with different letters are significantly different Tukey test ($P < 0.05$).

Table 2
Apparent digestibility coefficients (%) of the experimental diets for nutrient, energy and minerals.

Diets	Control	QY	SC	QYSC	*P* value
Dry matter	68.37 ± 0.14	68.71 ± 0.22	68.59 ± 0.21	68.91 ± 0.23	0.060
Crude protein	68.43 ± 1.22	68.14 ± 0.07	69.05 ± 0.95	68.43 ± 0.88	0.656
Crude lipid	63.81 ± 0.90	65.12 ± 3.07	59.67 ± 2.51	60.76 ± 4.69	0.189
Crude energy	74.09 ± 1.22	75.38 ± 1.72	75.69 ± 1.14	75.03 ± 0.90	0.493
Apparent digestibility coefficients (%) of the experimental diets for minerals.					
Calcium	89.83 ± 0.85 **b**	92.41 ± 0.52 **a**	92.73 ± 0.24 **a**	89.96 ± 0.59 **b**	0.000
Phosphorus	89.44 ± 0.32 **b**	91.20 ± 0.51 **a**	91.24 ± 0.50 **a**	91.70 ± 0.51 **a**	0.002
Iron	89.92 ± 0.19 **b**	91.65 ± 0.11 **a**	67.25 ± 0.87 **c**	91.63 ± 0.14 **a**	0.000
Manganese	93.15 ± 0.33 **a**	90.87 ± 0.15 **c**	91.64 ± 0.39 **b**	90.27 ± 0.16 **c**	0.000
Zinc	89.17 ± 0.04 **c**	90.69 ± 0.03 **b**	90.47 ± 0.01 **b**	91.84 ± 0.18 **a**	0.000
Selenium	95.64 ± 0.15 **b**	95.36 ± 0.03 **c**	95.01 ± 0.05 **d**	95.90 ± 0.08 **a**	0.000
Copper	62.27 ± 1.31 **c**	67.18 ± 1.14 **b**	64.29 ± 1.90 **bc**	73.93 ± 0.15 **a**	0.000

Values presented as means ± SD. Means in the same line with different letters are significantly different Tukey test ($P < 0.05$).

Table 3
Growth performance and whole-body composition of Nile tilapia fed diets for 60 days.

	Diets					
	Control	**QY**	**SC**	**QYSC**	***PSD***	***P* value**
IBW (g)	8.93	8.93	8.91	8.96	0.14	0.884
FBW (g)	75.32	77.52	77.37	76.75	7.33	0.904
WG (g)	65.66	68.26	67.72	67.30	7.36	0.873
DGI	3.56	3.64	3.64	3.61	0.22	0.863
SGR (%)	3.54	3.60	3.60	3.60	0.15	0.829
FI (g)	95.56	95.15	96.26	95.26	7.38	0.987
FCR	1.43	1.40	1.43	1.42	0.09	0.780
FE	0.69	0.72	0.70	0.71	0.05	0.544
PER	1.86	1.94	1.88	1.91	0.13	0.534
SUR (%)	95.56	96.67	92.67	95.33	5.69	0.458
Whole-body composition						
Dry matter (%)	99.43 **a**	99.26 **a**	98.94 **b**	99.25 **a**	0.11	0.004
Crude protein (%)	42.90 **a**	41.53 **ab**	41.32 **b**	41.33 **b**	0.55	0.023
Crude lipid (%)	41.54 **a**	39.47 **b**	39.95 **b**	40.35 **b**	0.34	0.000
Crude energy (%)	6372 **a**	6091 **c**	5950 **d**	6112 **b**	0.08	0.000
Ash (%)	7.18 **b**	10.41 **a**	10.02 **a**	9.73 **a**	0.26	0.000

Initial body weight (IBW) = initial mean weight (g/fish); Final body weight (FBW) = final mean weight (g/fish); Weight gain (WG) = final weight − initial weight; Daily growth index (DGI) = (((final weight)$^{1/3}$ – (initial weight)$^{1/3}$)/(number of days)) × 100; Feed Intake (FI) = dry feed intake (g/fish); Feed conversion rate (FCR) = feed intake / weight gain; Specific growth rate (SGR) = (Ln of final weight − Ln of initial weight) × 100 / experimental period; Protein efficiency ratio (PER) = [weigh gain (g)/(dry matter crude protein intake (g)]; Survival rate (SUR) = (initial fish number – final fish number) / initial fish number x 100 (%);
Values are means ± *SD* of ten replicates.
Letters compare fish between treatments by Tukey test ($P < 0.05$).

Table 4
Intestinal permeability of Nile tilapia fed diets for 60 days.

	Diets					
FITC-d	**Control FITC**	**Control**	**QY**	**SC**	**QYSC**	***P* value**
	0.00 ± 0.00 **b**	0.19 ± 0.18 **ab**	0.21 ± 0.18 **ab**	0.10 ± 0.13 **ab**	0.26 ± 0.20 **a**	0.037

Values presented as means ± SD. Means in the same line with different letters are significantly different Tukey test ($P < 0.05$).

Table 5
Intestinal morphometry of Nile tilapia fed diets for 60 days.

	Diets				
Villus height (µm)	**Control**	**QY**	**SC**	**QYSC**	***P* value**
Proximal intestine	178.00 ± 33.30	173.60 ± 31.80	201.80 ± 28.90	191.50 ± 34.80	0.454
Mid intestine	113.40 ± 35.50 **b**	116.58 ± 8.59 **b**	166.60 ± 38.30 **a**	115.90 ± 33.30 **ab**	0.028
Distal intestine	57.22 ± 12.50	93.40 ± 21.61	80.20 ± 27.30	72.47 ± 10.91	0.151

Values presented as means ± SD – maximum of 167 measurements. Means in the same line with different letters are significantly different Tukey test ($P < 0.05$).

Table 6
Hematological parameters of Nile tilapia subjected to different challenges[1].

		Diets					
		Control	**QY**	**SC**	**QYSC**	***PSD***	***P* value**
RBC[2] (10^6 μL^{-1})	Before	2.14 **Xα**	2.15 **Xα**	2.07 **BXα**	2.16 **Xα**	0.24	0.860
	SSIS	2.08 **b**	2.29 **ab**	2.51 **aA**	2.21 **b**	0.23	0.003
	***p* value**	0.688	0.155	0.000	0.657		
	BAC	1.65 **Y**	1.75 **Y**	1.76 **Y**	1.83 **Y**	0.26	0.718
	***p* value**	0.009	0.012	0.045	0.009		
	BAC + HP	1.49 **β**	1.38 **β**	1.51 **β**	1.54 **β**	0.18	0.327
	***p* value**	0.000	0.000	0.000	0.000		
Hb[2] (g dL^{-1})	Before	8.99 **Xα**	8.73 **Xα**	8.40 **Xα**	8.66 **Xα**	0.71	0.339
	SSIS	8.34	8.52	8.66	8.56	0.78	0.876
	***p* value**	0.112	0.507	0.356	0.814		
	BAC	6.64 **Y**	6.53 **Y**	6.74 **Y**	6.24 **Y**	0.94	0.817
	***p* value**	0.000	0.005	0.007	0.000		
	BAC + HP	6.21 **β**	6.11 **β**	6.12 **β**	5.91 **β**	0.61	0.787
	***p* value**	0.000	0.000	0.000	0.000		
Htc[2] (%)	Before	31.90 **BXα**	32.10 **BXα**	31.60 **BXα**	32.65 **BXα**	2.73	0.852
	SSIS	34.85 **bA**	38.90 **aA**	39.05 **aA**	36.20 **abA**	2.72	0.002
	***p* value**	0.020	0.000	0.000	0.001		
	BAC	24.42 **Y**	23.67 **Y**	24.00 **Y**	23.42 **Y**	3.60	0.967
	***p* value**	0.000	0.005	0.007	0.000		
	BAC + HP	21.75 **β**	20.81 **β**	21.56 **β**	22.81 **β**	3.10	0.641
	***p* value**	0.000	0.000	0.000	0.000		
MCV[2] (fL)	Before	150.53	150.12 **B**	152.95	152.06 **BX**	13.91	0.965
	SSIS	170.91	169.70 **A**	165.82	165.08 **A**	19.12	0.356
	***p* value**	0.068	0.002	0.608	0.045		
	BAC	136.39	136.90	136.99	128.44 **Y**	18.49	0.822
	***p* value**	0.106	0.280	0.124	0.003		
	BAC + HP	148.10	153.40	145.10	139.60	30.02	0.841
	***p* value**	0.856	0.788	0.514	0.221		
MCHC[2] (g dL^{-1})	Before	28.26 **A**	27.23 **A**	26.71 **A**	26.52 **A**	1.94	0.202
	SSIS	23.47 **aB**	21.95 **bB**	22.25 **abB**	23.64 **abB**	1.40	0.022
	***p* value**	0.000	0.000	0.000	0.004		
	BAC	27.22	27.84	28.40	26.66	2.81	0.731
	***p* value**	0.408	0.698	0.308	0.879		
	BAC + HP	27.06	29.80	28.91	26.32	4.72	0.447
	***p* value**	0.522	0.185	0.314	0.900		

[1]SSIS: size-sorting-induced stress; BAC: bacterial challenge with *Streptococcus agalactiae* infection; BAC + HP: bacterial challenge with *Streptococcus agalactiae* infection + 12 hours hypoxia.
[2]RBC: Red blood cell count; Hb: Hemoglobin; Htc: Hematocrit; MCV: Mean corpuscular volume; MCHC: Mean corpuscular hemoglobin concentration. A and B uppercase letters compare the hematological response of fish in the same treatment before and after SSIS, x and y compare before and after BAC and α and β before and after BAC + HP by T test ($P < 0.05$). Lowercase letters compares diets among treatment by Tukey test ($P < 0.05$). Means and *SD* – maximum of ten replicates.

Table 7: Blood glucose and plasma cortisol of Nile tilapia subjected to different challenges[1].

		Diets					
		Control	**QY**	**SC**	**QYSC**	***PSD***	***P* value**
Glucose (mg dL^{-1})	Before	38.60 **B**	42.10 **B**	40.40 **B**	50.01 **BY**	10.95	0.138
	SSIS	84.92 **A**	85.96 **A**	61.83 **A**	76.60 **A**	18.95	0.158
	***p* value**	0.003	0.001	0.030	0.012		
	BAC	46.18	45.06	57.69	66.25 **X**	13.37	0.044
	***p* value**	0.146	0.611	0.083	0.032		
	BAC + HP	46.52	42.47	41.07	45.83	14.64	0.856
	***p* value**	0.207	0.957	0.922	0.498		
CORT (ng/mL)	Before	134.30 **B**	118.90 **B**	153.60	111.50 **BY**	68.94	0.545
	SSIS	239.80 **A**	272.30 **A**	222.60	234.00 **A**	127.06	0.838
	***p* value**	0.046	0.006	0.100	0.007		
	BAC	189.10	161.80	162.20	177.80 **X**	65.10	0.557
	***p* value**	0.214	0.239	0.752	0.048		
	BAC + HP	120.41	127.80	126.00	133.00	41.14	0.838
	***p* value**	0.582	0.767	0.224	0.204		

[1]SSIS: size-sorting-induced stress; BAC: bacterial challenge with *Streptococcus agalactiae* infection; BAC + HP: bacterial challenge with *Streptococcus agalactiae* infection + 12 hours hypoxia.
Glucose: Blood glucose. Means and *SD* – maximum of ten replicates.
CORT: Plasma cortisol Means and *SD* – maximum of ten replicates.
A and B uppercase letters compare the hematological response of fish in the same treatment before and after SSIS, x and y compare before and after BAC by T test ($P < 0.05$).

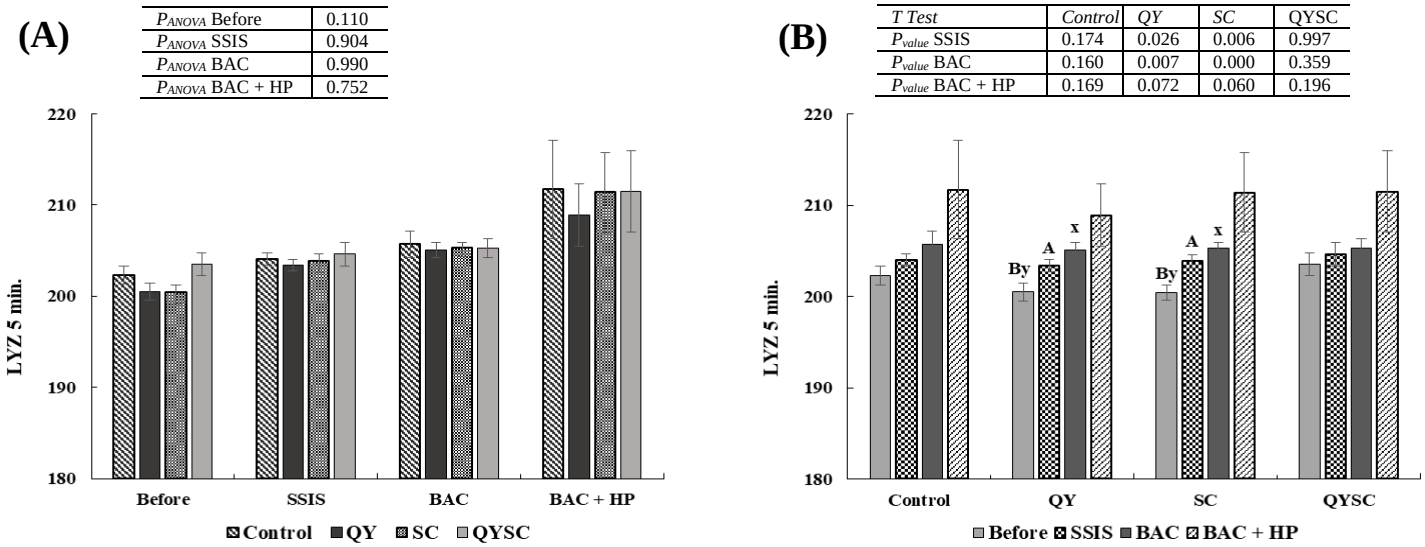


Figure 1. LYZ 5 min.: Serum lysozyme activity after five minutes, values are means ± standard error (n = 10). (A) Compare LYZ 5 min. among treatments (Tukey test). (B) Letters compare LYZ 5 min. between the moments, before and after stress (T test). SSIS: size-sorting-induced stress; BAC: bacterial challenge with *Streptococcus agalactiae* infection; BAC + HP: bacterial challenge with *Streptococcus agalactiae* infection + 12 hours hypoxia.

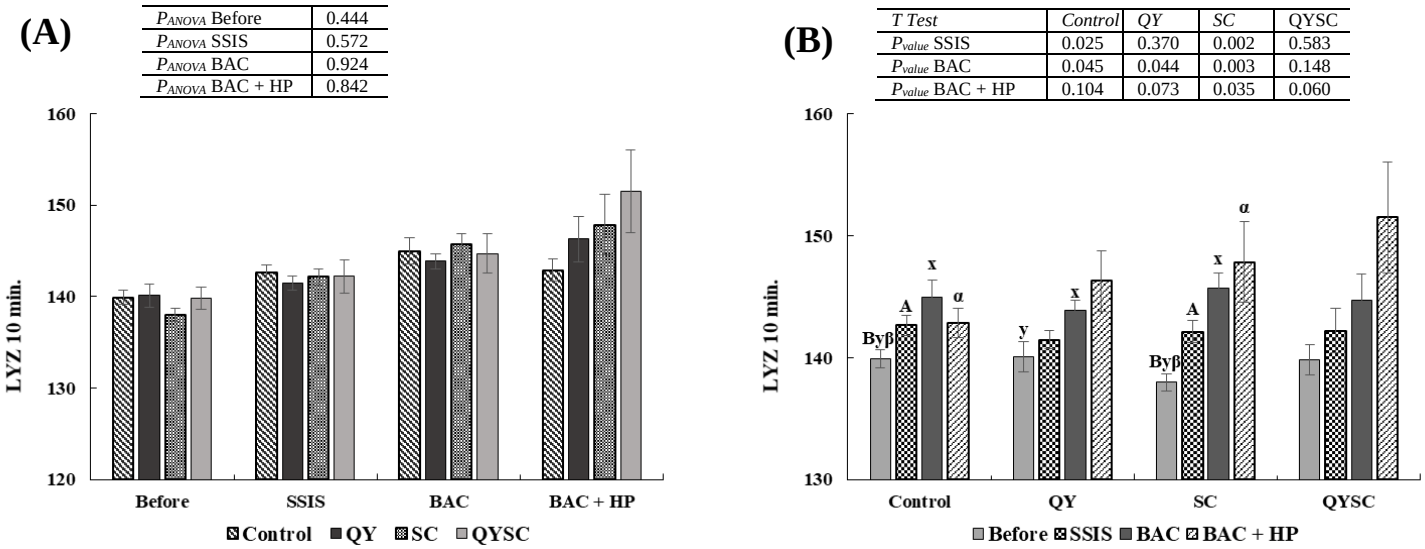


Figure 2. LYZ 10 min.: Serum lysozyme activity after ten minutes, values are means ± standard error (n = 10). (A) Compare LYZ 10 min. among treatments (Tukey test). (B) Letters compare LYZ 10 min. between the moments, before and after stress (T test). SSIS: size-sorting-induced stress; BAC: bacterial challenge with *Streptococcus agalactiae* infection; BAC + HP: bacterial challenge with *Streptococcus agalactiae* infection + 12 hours hypoxia.

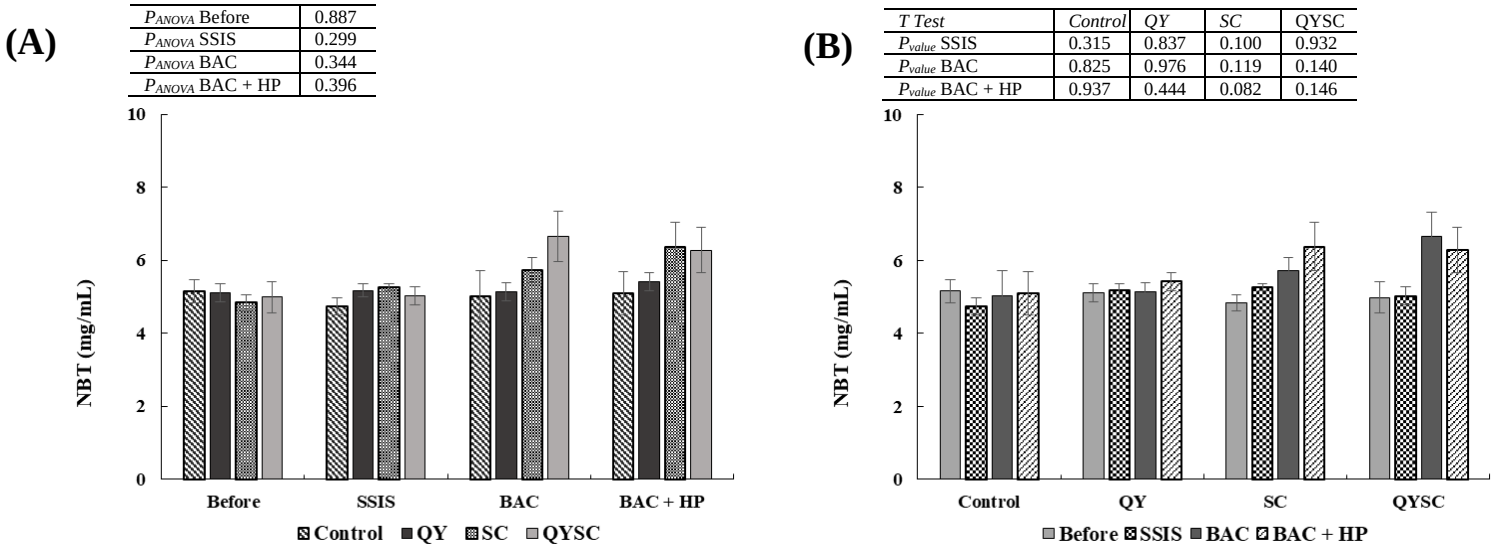


Figure 3. NBT: Nitro blue tetrazolium in blood, values are means ± standard error (n = 10). (A) Compare NBT among treatments (Tukey test). (B) Letters compare NBT between the moments, before and after stress (T test). SSIS: size-sorting-induced stress; BAC: bacterial challenge with *Streptococcus agalactiae* infection; BAC + HP: bacterial challenge with *Streptococcus agalactiae* infection + 12 hours hypoxia.

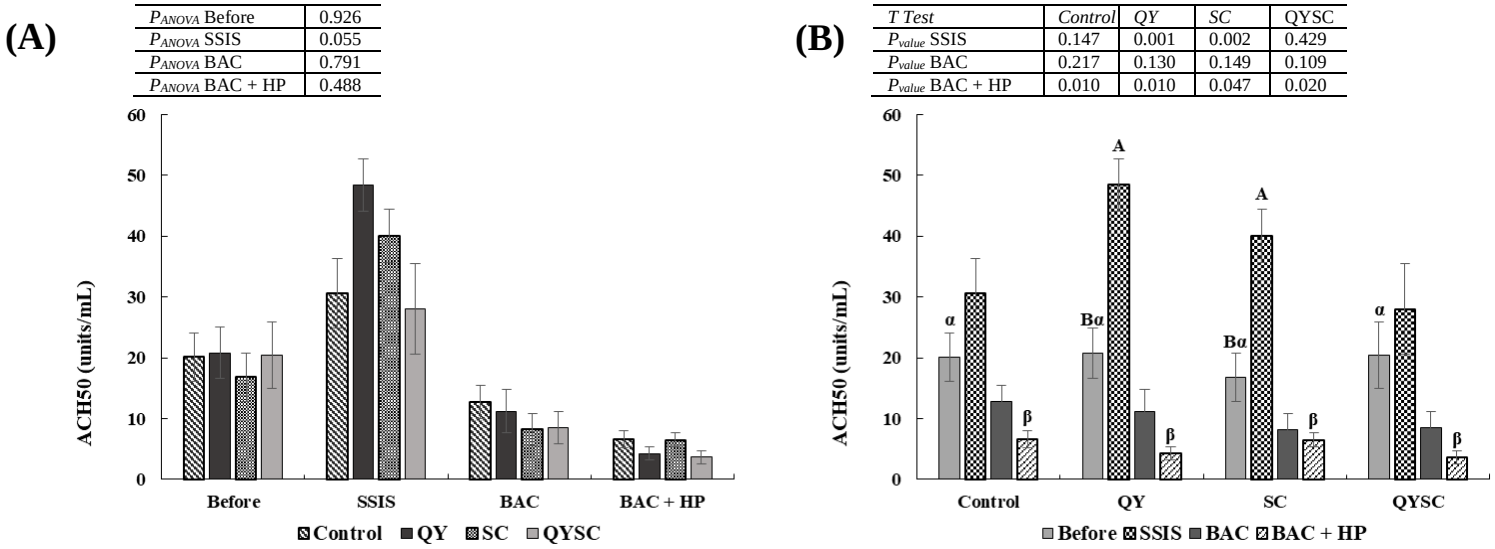


Figure 4. ACH50: Serum alternative complement activity, values are means ± standard error (n = 10). (A) Compare ACH50 among treatments (Tukey test). (B) Letters compare ACH50 between the moments, before and after stress (T test). SSIS: size-sorting-induced stress; BAC: bacterial challenge with *Streptococcus agalactiae* infection; BAC + HP: bacterial challenge with *Streptococcus agalactiae* infection + 12 hours hypoxia.

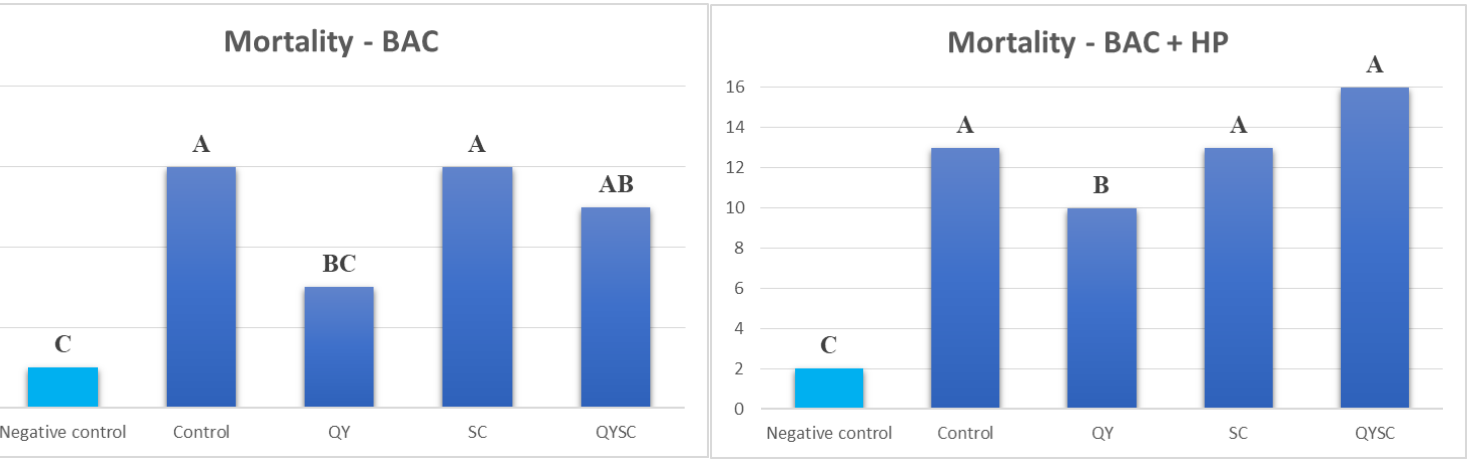


Figure 5: Mortality of BAC: bacterial challenge with *Streptococcus agalactiae* infection; BAC + HP: bacterial challenge with *Streptococcus agalactiae* infection + 12 hours hypoxia.

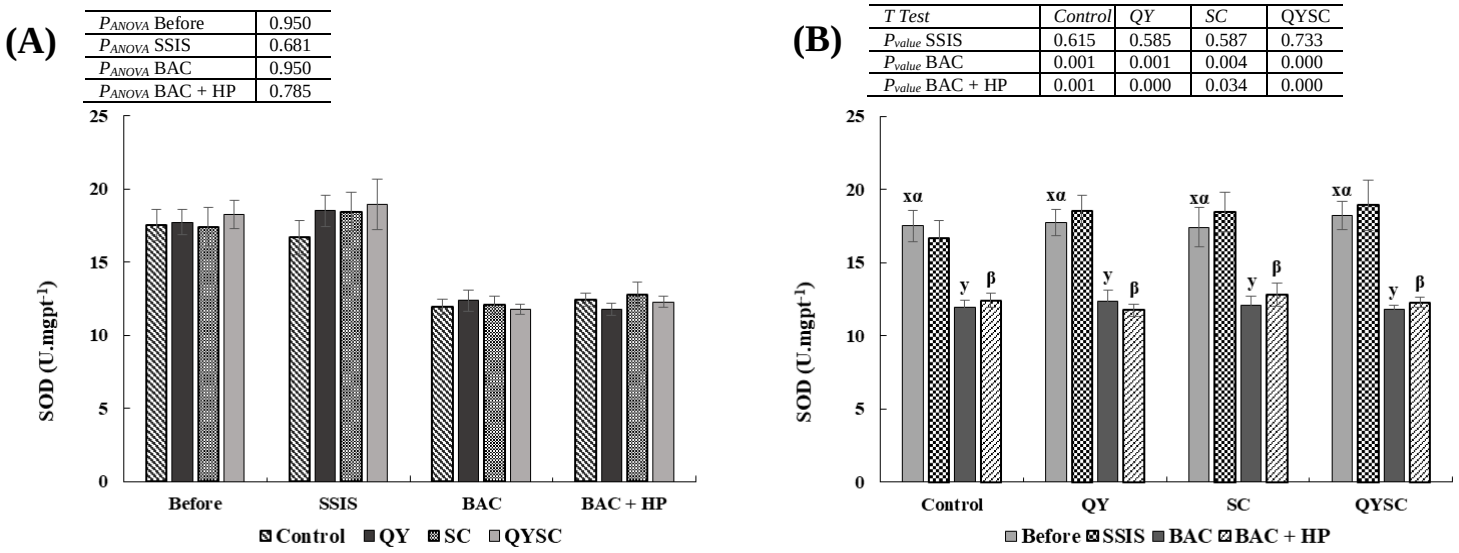


Figure 6. SOD: Superoxide dismutase activity, values are means ± standard error (n = 10). (A) Compare SOD among treatments (Tukey test). (B) Letters compare SOD between the moments, before and after stress (T test). SSIS: size-sorting-induced stress; BAC: bacterial challenge with *Streptococcus agalactiae* infection; BAC + HP: bacterial challenge with *Streptococcus agalactiae* infection + 12 hours hypoxia.

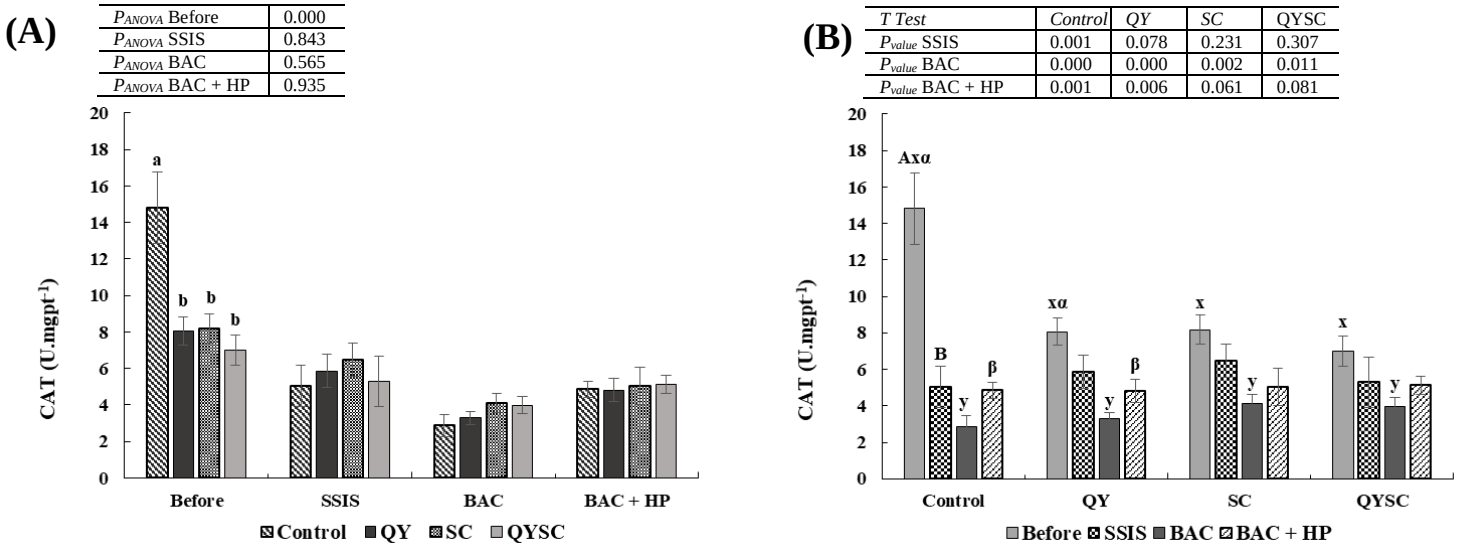
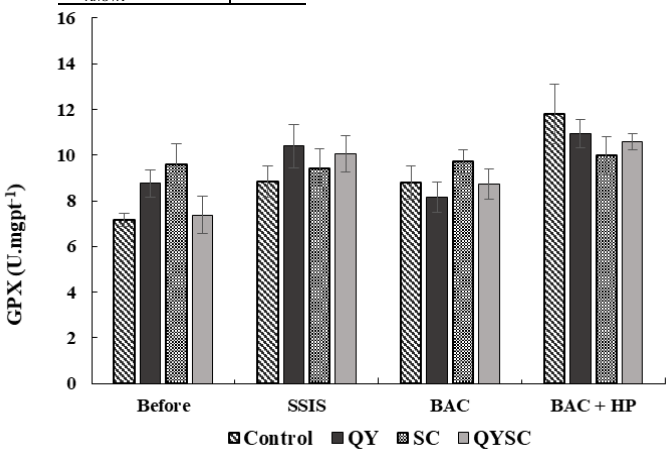


Figure 7. CAT: Catalase activity, values are means ± standard error (n = 10). (A) Letters compare CAT among treatments (Tukey test). (B) Letters compare CAT between the moments, before and after stress (T test). SSIS: size-sorting-induced stress; BAC: bacterial challenge with *Streptococcus agalactiae* infection; BAC + HP: bacterial challenge with *Streptococcus agalactiae* infection + 12 hours hypoxia.

(A)

P_{ANOVA} Before	0.059
P_{ANOVA} SSIS	0.601
P_{ANOVA} BAC	0.609
P_{ANOVA} BAC + HP	0.864



(B)

T Test	*Control*	*QY*	*SC*	QYSC
P_{value} SSIS	0.055	0.170	0.882	0.033
P_{value} BAC	0.149	0.573	0.901	0.274
P_{value} BAC + HP	0.026	0.038	0.783	0.005

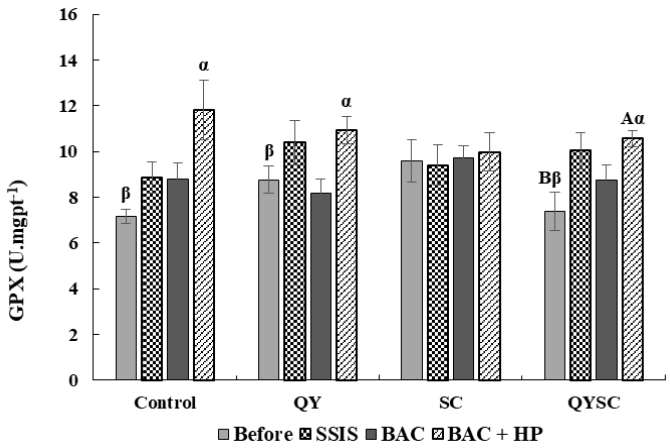
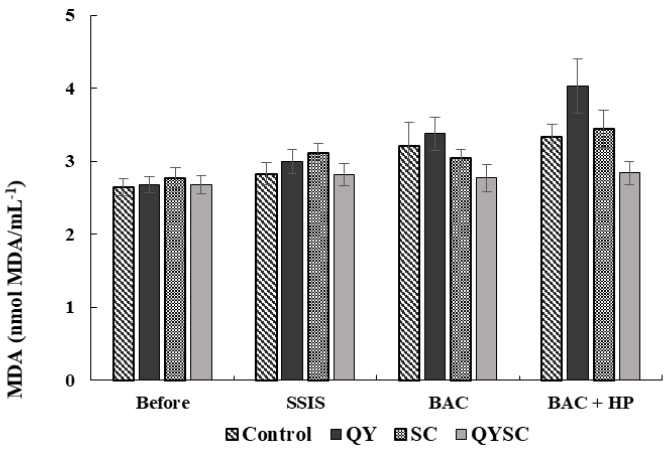


Figure 8. GPx: Glutathione peroxidase activity, values are means ± standard error (n = 10). (A) Compare GPx among treatments (Tukey test). (B) Letters compare GPx between the moments, before and after stress (T test). SSIS: size-sorting-induced stress; BAC: bacterial challenge with *Streptococcus agalactiae* infection; BAC + HP: bacterial challenge with *Streptococcus agalactiae* infection + 12 hours hypoxia.

(A)

P_{ANOVA} Before	0.921
P_{ANOVA} SSIS	0.504
P_{ANOVA} BAC	0.516
P_{ANOVA} BAC + HP	0.087



(B)

T Test	*Control*	*QY*	*SC*	QYSC
P_{value} SSIS	0.340	0.140	0.073	0.513
P_{value} BAC	0.248	0.072	0.235	0.749
P_{value} BAC + HP	0.011	0.017	0.059	0.543

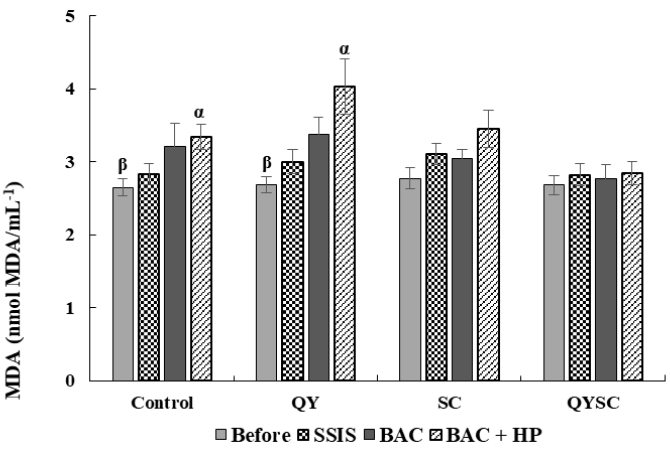


Figure 9. MDA: Liver lipid peroxidation, values are means ± standard error (n = 10). (A) Compare MDA among treatments (Tukey test). (B) Letters compare MDA between the moments, before and after stress (T test). SSIS: size-sorting-induced stress; BAC: bacterial challenge with *Streptococcus agalactiae* infection; BAC + HP: bacterial challenge with *Streptococcus agalactiae* infection + 12 hours hypoxia.

CAPÍTULO III

IMPLICAÇÕES

A intensificação da produção aquícola necessita cada vez mais de tecnologias para se sobressair e atender a demanda. Contudo, a produção passou nos últimos anos de extensiva para intensivas, necessitando que os animais ganhem mais peso, consumindo menos alimento e em menor tempo. Com isso, os desafios encontrados na produção estão paralelamente associados a tal intensificação. Cada vez mais registra-se o surgimento de novas cepas patogênicas, em grande parte pelo mal uso de antimicrobianos, em que ao invés de serem empregados como tratamento, vem sendo utilizados de modo profilático.

Tais antimicrobianos, além de não serem administrados da maneira correta, não são 100% absorvidos pelo organismo dos peixes. Mais além, grande parte da produção piscícola é realizada em águas públicas, que além de possuírem sua biodiversidade natural, são águas destinadas ao consumo humano e animal. Portanto, é necessário a conscientização da aplicação destes antimicrobianos, além da busca por alternativas naturais que possuem a finalidade de promover saúde aos animais. O uso de aditivos com propriedades funcionais vem ganhando destaque, por não apresentarem riscos à saúde pública, além de serem bem aceitos pelos animais que os consomem, que por usa vez, dependendo do caso podem melhorar as respostas zootécnicas.

Os aditivos eubióticos utilizados no presente estudo cumprem, em parte, seu propósito inicial. Mas precisam de novos testes com, talvez, períodos mais longos de suplementação para serem melhor caracterizados e ter funções atribuídas, como por exemplo na melhora do coeficiente de digestibilidade aparente. O tratamento com *Quillaja saponaria* e *Yucca schidigera*, as quais possuem características antioxidantes, não se mostrou eficiente. Entretanto, possivelmente apresente função na imunidade adaptativa, por proporcionar maiores sobrevivências dos animais submetidos aos estresses bacterianos. Já o aditivo à base de *Saccharomyces cerevisiae* corrobora os resultados descritos na literatura para um de seus componentes como o MOS e β-glucano. Por outro lado, a associação dos dois aditivos precisa

ser melhor estudada, por apresentar resultados divergentes do esperado, *in vitro* e *in vivo*, para com ambos os aditivos eubióticos. Entretanto, vale ressaltar que nenhum parâmetro de saúde relacionado aos aditivos determinou resultados inferiores aos dos animais alimentados com a ração controle.