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Vitor Fernandes Silva

Suplementação de minerais em nanopartículas em dietas de peixes

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Tese submetida ao Programa de Pós-Graduação em Aquicultura da Universidade Federal de Santa Catarina como requisito parcial para a obtenção do título de Doutor em Aquicultura e Recursos Pesqueiros.

Orientador: Prof. José Luiz Pedreira Mouriño, Dr.
Coorientador : Prof. Fernando Yugo Yamamoto, Dr.

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O presente trabalho em nível de Doutorado foi avaliado e aprovado, em 07 de maio de 2024 pela banca examinadora composta pelos seguintes membros:

Profª. Débora Machado Fracalossi, Drª.
Universidade Federal de Santa Catarina

Prof. Pedro Luiz Pucci Figueiredo de Carvalho, Dr.
Texas A&M University

Profª. Luara Lucena Cassiano, Drª.
Instituto de Pesca/SP

Prof. José Luiz Pedreira Mouriño, Dr.
Universidade Federal de Santa Catarina

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Prof. José Luiz Pedreira Mouriño, Dr.
Orientador

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RESUMO

A nutrição é um dos principais componentes da produção aquícola comercial. Microelementos como zinco, ferro e cobre estão entre os nutrientes essenciais, e normalmente são incluídos na ração em concentrações acima das concentrações exigidas, devido a baixa biodisponibilidade das fontes de minerais inorgânicas. Nanopartículas possuem alta área superficial específica, o que aumenta sua biodisponibilidade, dessa forma, é possível utilizar menores concentrações na ração. O objetivo deste estudo foi comparar o crescimento e saúde de peixes alimentados com dietas suplementadas com minerais normalmente utilizados pela indústria ou em nanopartículas. Dois experimentos foram executados, no primeiro experimento, os produtos (tratamentos) a base de zinco: óxido de zinco (zinco inorgânico), bisglicinato de zinco (zinco orgânico), zinco em nanopartículas (nanozinco inorgânico) e zinco orgânico em nanopartículas (nanozinco orgânico) foram suplementados na dieta de tilápia na concentração de 15 mg por kg de ração por 60 dias. Após este período, os parâmetros de desempenho e hematoimunológicos, atividade de enzimas do sistema antioxidante, a concentração de zinco no músculo e a sobrevivência após infecção com *Streptococcus agalactiae* foram avaliados. Após o desafio bacteriano, a CHCM aumentou significativamente nas tilápias tratados com zinco orgânico, nanozinco inorgânico e nanozinco orgânico, enquanto no grupo controle (zinco inorgânico), a CHCM permaneceu inalterada. Em relação às células de defesa, o nanozinco inorgânico aumentou o número de basófilos em relação ao zinco orgânico. A contagem de linfócitos aumentou após o desafio apenas nos tratamentos com zinco orgânico (bruto e nanopartículas). Os neutrófilos diminuíram nos tratamentos controle (zinco inorgânico) e nanozinco inorgânico após o desafio. No segundo experimento as seguintes combinações de nanopartículas de ferro (IronNP) e nanopartículas de cobre (CopperNP) foram avaliadas por 9 semanas na dieta do bagre do canal: somente CopperNP; somente IronNP e; CopperNP + IronNP, em comparação a uma dieta controle suplementada com formas inorgânicas de ferro e cobre ($FeSO_4$ and $CuSO_4$). Foram avaliados parâmetros de crescimento e hematológicos, análise proximal dos peixes inteiros, microbiota intestinal e infecção experimental contra *Edwardsiella ictaluri*. Não foram detectadas diferenças para parâmetros de crescimento e sobrevivência após infecção experimental. O hematócrito e RBC dos peixes alimentados com o tratamento contendo nanopartículas de cobre foi significativamente menor do que o grupo controle. Maior abundância relativa de bactérias ácido láticas foi observada na digesta de peixes alimentados com dietas contendo nanopartículas de cobre. Em conclusão, independentemente do tamanho, o zinco orgânico, aumenta a contagem de neutrófilos e linfócitos após a infecção bacteriana, já na forma inorgânica, diminui o número de neutrófilos após infecção com *Streptococcus agalactiae*. Independentemente do tamanho, a suplementação com ferro é necessária para a produção de células vermelhas. Cobre em nanopartículas aumenta a abundância relativa de bactérias gram-positivas no trato gastrointestinal do bagre do canal. No geral, pode-se concluir que nanopartículas de zinco, cobre e ferro não afetam os parâmetros de crescimento e os parâmetros hematoimunológicos de peixes. Estudos futuros devem avaliar as implicações das nanopartículas de cobre na dieta sobre a microbiota intestinal.

Palavras-chave: aquicultura; nanotecnologia; nutrição de peixes; suplementação mineral; piscicultura.

ABSTRACT

Nutrition is one of the main components of commercial aquaculture production. Microelements such as zinc, iron, and copper are among the essential nutrients and are typically included in feed above the required concentrations due to the low bioavailability of inorganic mineral sources. Nanoparticles have a high specific surface area, which increases their bioavailability, so it is possible to use lower concentrations in the feed. The aim of this study was to evaluate the growth and health of fish fed diets supplemented with minerals normally used by industry and nanoparticles. Two trials were carried out. In the first experiment, zinc-based products (treatments): zinc oxide (inorganic zinc), zinc bis-glycinate (organic zinc), zinc nanoparticles (inorganic nanozinc) and organic zinc in nanoparticles (organic nanozinc) were supplemented in the tilapia diet at a concentration of 15 mg per kg of feed during 60 days. After this period, performance and hematoimmunological parameters, activity of antioxidant system enzymes, survival after infection with *Streptococcus agalactiae*, and zinc concentration in the muscle were evaluated. After bacterial challenge, MCHC increased significantly in tilapia treated with organic zinc, inorganic nanozinc, and organic nanozinc, while in the control group (inorganic zinc), MCHC remained unchanged. Regarding defense cells, inorganic nanozinc increased the number of basophils compared to organic zinc. The lymphocyte count increased after the challenge only in the treatments with organic zinc (bulk and nanoparticles). Neutrophils decreased in the control (inorganic zinc) and inorganic nanozinc treatments after the challenge. In the second experiment the following combinations of iron nanoparticles (IronNP) and copper nanoparticles (CopperNP) were evaluated for 9 weeks in the diet of the channel catfish: CopperNP only; IronNP only, and; CopperNP + IronNP, compared to a control diet supplemented with inorganic forms of iron and copper ($FeSO_4$ and $CuSO_4$). Growth and hematological parameters, proximal parameters of whole fish, intestinal microbiota, and experimental infection against *Edwardsiella ictaluri* were evaluated. No differences were detected for growth and survival parameters after experimental infection. The hematocrit and RBC of the fish fed the treatment containing copper nanoparticles was significantly lower than in control group. Higher relative abundance of lactic acid bacteria was observed in the digesta of fish fed diets containing copper nanoparticles. In conclusion, regardless of size, organic zinc increases the count of neutrophils and lymphocytes after bacterial infection, while in the inorganic form, it decreases the number of neutrophils after infection with *Streptococcus agalactiae*. Regardless of size, iron supplementation is necessary for the production of red blood cells. Copper nanoparticles increase the relative abundance of gram-positive bacteria in the gastrointestinal tract of the canal catfish. Overall, it can be concluded that zinc, copper, and iron nanoparticles do not affect fish's growth parameters and hematoimmunological parameters. Future studies should evaluate the implications of dietary copper nanoparticles on the gut microbiota.

Keywords: aquaculture; nanotechnology; fish nutrition; mineral supplementation; fish farming.

SUMÁRIO

1 INTRODUÇÃO

1.1 AQUICULTURA

A aquicultura é um importante setor contribuinte para o fornecimento global de proteína animal para consumo (COLGRAVE et al., 2021). Atualmente, aproximadamente 3,3 bilhões de pessoas possuem o peixe como sua fonte primária de proteína, sendo ela proveniente da captura ou aquicultura (FAO, 2022).

De acordo com a Food and Agriculture Organization (FAO), a produção mundial aquícola (animal) aumentou 20% de 2015 a 2020, atingindo 87,5 milhões de toneladas, sendo 54,4 milhões produzidos em águas continentais e 33,1 em águas marinhas (FAO, 2022).

Apesar da estimativa do consumo per capta de pescados ter diminuído de 20,5 para 20,3 kg de 2019 para 2020, há uma previsão para aumento da produção aquícola mundial devido ao aumento da demanda de pescados estimulada pela urbanização, aumento rendimentos da população e a tendencia de mudanças alimentares visando aspectos nutricionais dos alimentos de origem aquática (FAO, 2022; HENRIKSSON et al., 2018). A produção da aquicultura (excluindo algas) deve ultrapassar as 100 milhões de toneladas até 2030 (FAO, 2022).

Entretanto, o crescimento da aquicultura deve ser ordenado e apoiado nos pilares da sustentabilidade para garantir o abastecimento de proteína de qualidade para a população, a estabilidade da cadeia produtiva e a preservação do meio ambiente (BJØRNDAL; DEY; TUSVIK, 2024). A FAO destaca princípios que podem auxiliar no crescimento sustentável da aquicultura, como o desenvolvimento de programas de melhoramento genético, intensificação da produção, aumento da biosseguridade e controle de doenças, melhores políticas públicas voltadas para a aquicultura e a utilização de fontes de proteína alternativa nas rações (FAO, 2022; HENRIKSSON et al., 2018).

Entre os desafios enfrentados pelo setor aquícola para o aumento da sua produção, a proliferação de doenças está entre as principais preocupações (STENTIFORD et al., 2012). A necessidade da intensificação dos sistemas de criação para incrementar a produtividade da aquicultura aumenta os riscos de proliferação de doenças. Sistemas intensivos exigem mais conhecimento técnico em seu manejo, caso o sistema não seja manejado corretamente quanto ao tratamento da matéria orgânica e de efluentes, pode ocorrer aumento no risco de proliferação de doenças (IKEOGU; NSOFOR; IKPEZE, 2010). A nutrição tem um papel crucial no desenvolvimento sustentável da aquicultura. Os nutrientes, suplementos e aditivos fornecidos na dieta participam diretamente na resposta imunológica e no controle do estresse de peixes de cultivo, além disso, a qualidade da ração influencia diretamente a lixiviação de nutrientes para o meio ambiente (OLIVA-TELES, 2012; POHLENZ; GATLIN, 2014).

1.1.1 Indústria da tilápia do Nilo (*Oreochromis niloticus*)

A tilápia do Nilo é uma das espécies aquícolas com maior destaque no cenário mundial. Atualmente, é o terceiro peixe de água doce mais produzido no mundo, atrás apenas das carpas capim (*Ctenopharyngodon idellus*) e prateada (*Hypophthalmichthys molitrix*), com 5,8 e 4,9 milhões de toneladas produzidas em 2020, respectivamente, no mesmo ano foram produzidas 4,4 milhões de toneladas de tilápia do Nilo (FAO, 2022). Atualmente, o Brasil é o quarto produtor mundial de tilápia, e na piscicultura nacional, é a espécie mais produzida, representando aproximadamente 65% da produção nacional de peixes com 579 mil das 887 mil toneladas despescadas em 2023 (PEIXE BR, 2024). Nos últimos anos, a tilápia do Nilo esteve entre as espécies da aquicultura continental que mais aumentou de produção, foram 400 mil toneladas a mais despescadas entre 2015 e 2020 (FAO, 2022). No cenário nacional o aumento foi ainda mais expressivo em número relativos, entre 2014 e 2023 a produção passou de 285 mil toneladas para 587 mil toneladas (PEIXE BR, 2024).

O aumento da produção da tilápia do Nilo e seu destaque na produção mundial é justificado por diferentes fatores. Entre eles, estão as características que favorecem sua criação em cativeiro, como crescimento rápido, facilidade e tempo de reprodução, tolerância a variações ambientais, adaptação ao confinamento, resistência a doenças e boa aceitação de alimentos artificiais após fase larval (EL-SAYED, 2006). Adicionalmente, a adaptação da espécie às condições climáticas do país, qualidade do filé, programas de melhoramento genético e o aumento nas exportações favoreceram o aumento da produção nacional da tilápia do Nilo (FAO, 2022; NAKATA, 2021; PEIXE BR, 2024; YOSHIDA et al., 2022).

1.1.2 Indústria do catfish nos Estados Unidos

Apesar de não ter destaque no cenário mundial (568 mil toneladas despescdas em 2022), o catfish (bagre do canal) é a espécie mais produzida nos Estados Unidos com 149,2 mil toneladas (incluindo o bagre do canal, *Ictalurus punctatus*, e o híbrido, *Ictalurus punctatus* × *I. furcatus*). Mississippi, Arkansas e Alabama são os principais estados produtores. O maior produtor desta espécie é a China com 416,2 mil toneladas. Rússia, México e Itália, aparecem com uma produção menos expressiva comparada a China e EUA, somando menos de 300 toneladas produzidas em 2022 (FAO, 2024).

Os primeiros dados de produção do bagre do canal nos EUA são da década de 1950, a partir deste ano, a produção cresceu significativamente e atingiu seu auge no ano de 2003, com 300 mil toneladas. Após este ano, a produção anual diminuiu ano a ano, até chegar a 139 mil toneladas no ano de 2014 (FAO, 2024; USDA-NASS, 2024). Pesquisadores atribuem a queda

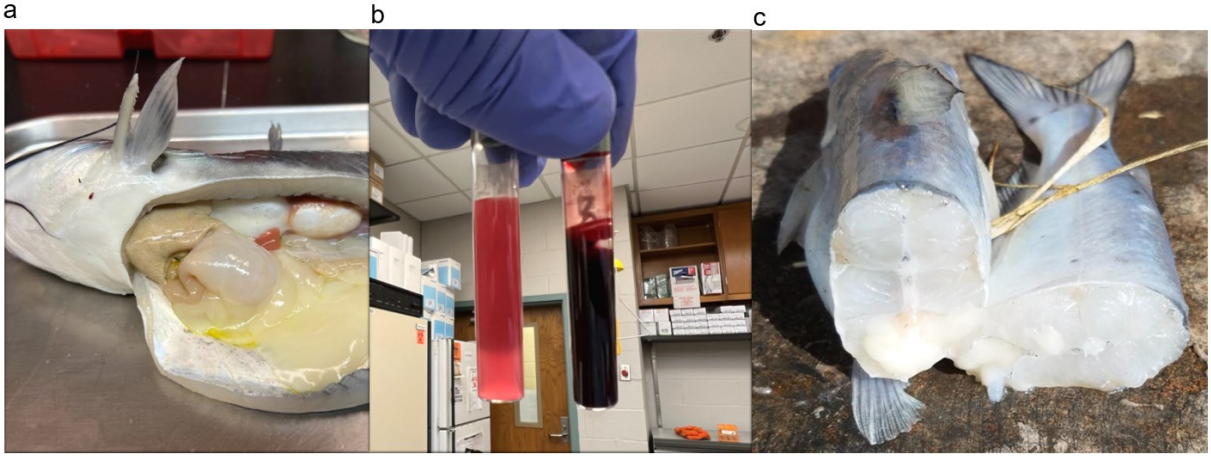
da produção neste período a fatores como a abertura das importações do peixe panga do Vietnã, aumento nos custos de produção e as recessões econômicas enfrentada em 2008 e 2012 (ENGLE; HANSON; KUMAR, 2022).

Apesar da diminuição da produção total, a indústria do bagre americano se tornou mais produtiva na última década. Em 2014, a área de lâmina d'água utilizada para produção dessa espécie foi de 31 mil hectares, em 2023, esse valor diminuiu para 22 mil hectares, enquanto a produção aumentou para 150 mil toneladas no mesmo ano (USDA-NASS, 2024). O emprego de novas tecnologias e medidas como aumento da aeração mecânica, sistemas de monitoramento de oxigênio com controle automático, adaptação de viveiros para o modelo de *split-ponds*, a produção do híbridos (*Ictalurus punctatus* × *I. furcatus*) e vacinação permitiu que a indústria do bagre do canal aumentasse sua produtividade (HEGDE et al., 2022).

1.1.2.1 Anemia idiopática do bagre do canal

Dentre os desafios enfrentados pela indústria de bagres nos EUA, produtores relatam anemia severa em alevinos de bagres do canal (*I. punctatus*) e bagres azuis (*I. furcatus*) desde a década de 1980 (BUTTERWORTH; PLUMB; GRIZZLE, 1986; PLUMB; LIU; BUTTERWORTH, 1994). Esta doença é caracterizada por peixes moribundos, hematócritos variando de 0 a 5%, brânquias pálidas ou brancas, rins e baço rosados e fígado cinza a bronzeado (Figura 1) (KLAR; HANSON; BROWN, 1986).

Figura 1 - (a) Bagre do canal acometido pela anemia com orgãos pálidos, (b) tubo da esquerda: sangue de bagre do canal com baixa presença de células vermelhas, tubo da direita: sangue de um bagre do canal saudável, (c) bagre do canal acometido pela anemia idiopática sem sinais de pigmentação no sangue. Fonte: (a) Dr. Lester Khoo, estudo de caso de animais vindos da Louisiana, EUA em 2023 (b) Dr. Fernando Y. Yamamoto, na estação experimental no estado Mississippi, EUA, em 2023, (c) relato documentado pelo produtor Darrell Bowers no estado do Texas, EUA, em 2022.



A anemia é uma doença que pode estar associada a fatores nutricionais, toxinas e bactérias ou vírus patogênicos (CLAUSS; DOVE; ARNOLD, 2008). Entretanto a maioria dos casos reportados atualmente são de anemia idiopática, ocorrendo no bagre do canal e no híbrido.

Produtores relatam maiores casos da doença na primavera e outono, com maior incidência no mês de outubro, em viveiros aleatórios da fazenda por até 5 meses seguidos, entre temperaturas de 17 a 27°C (CAMUS et al., 2014).

CAMUS et al., (2014) avaliaram o hematócrito de bagres do canal acometidos pela anemia idiopática após a injeção de ferro intramuscular (ferro dextrano), e após seis semanas o hematócrito aumentou gradativamente para os peixes anêmicos que receberam o tratamento suplementar de ferro. Além disso, o ferro no soro sanguíneo e no fígado também aumentaram após o mesmo período. Portanto, ficou constatado que as vias metabólicas envolvidas no transporte, armazenamento e síntese de hemoglobina funcionam normalmente nos peixes acometidos pela doença, e que possivelmente a causa da doença está relacionada com a biodisponibilidade do ferro ou na falha durante o processo de absorção. Pode-se dizer que a anemia idiopática do bagre do americano é microcítica (caracterizada por baixa produção de células vermelhas), normocrômica, (células vermelhas possuem coloração normal) e regenerativa (quando eritropoiese não está comprometida) (ARDELT; JOHNSON, 1994).

Atualmente, no estado do Mississippi, o maior produtor de bagre dos Estados Unidos, a indústria de ração tem suplementado as rações para esta espécie com concentrações de ferro acima do recomendado, entre 500 e 700 mg de ferro por quilo de ração, para evitar o aparecimento da doença (YAMAMOTO et al., 2023). Estudos recentes têm focado na avaliação de diferentes fontes e altas concentrações de ferro nas rações (BUYINZA et al., 2023; YAMAMOTO et al., 2023). Foi reportado que altas concentrações de ferro na ração não afetam parâmetros de crescimento, porém aumentam os níveis de hematócrito e o conteúdo de ferro no fígado.

1.2 NUTRIÇÃO E OS MINERAIS

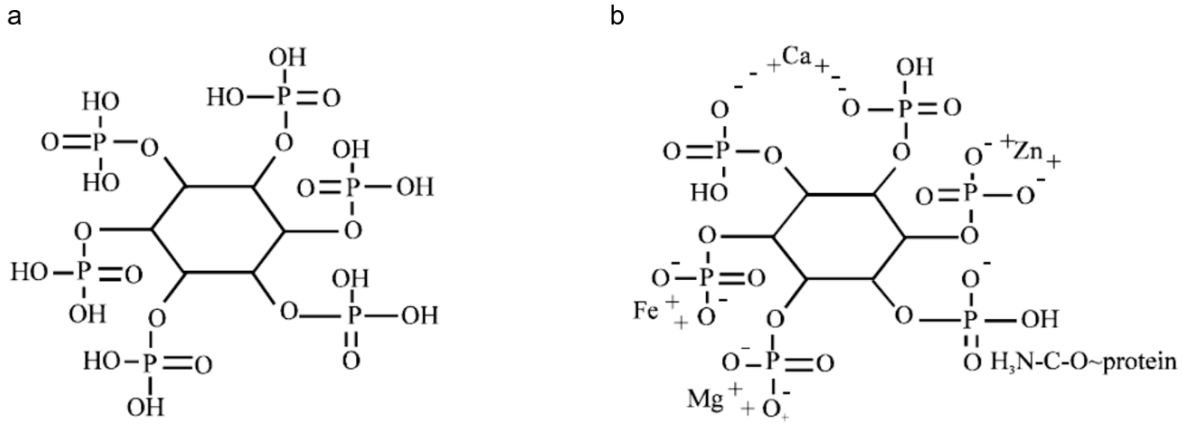
A nutrição é um dos principais componentes em produções aquícolas comerciais. Uma dieta equilibrada é formulada para incluir ingredientes que fornecerão os macronutrientes (proteínas, lipídios e carboidratos) e micronutrientes (vitaminas e minerais) necessários de acordo com as exigências nutricionais da espécie de interesse. É importante o fornecimento de uma dieta completa e balanceada para que os peixes possam expressar seu potencial de crescimento e resposta imunológica adequada frente a agentes infecciosos e variações ambientais (POHLENZ; GATLIN, 2014).

Até 2050, a previsão é que a aquicultura dobre sua produção (FAO, 2022). Grandes volumes de ração e consequentemente seus ingredientes, serão necessários para sustentar esse volume de produção. Tendo em vista o aumento populacional e a competição por recursos entre

setores de produção, a indústria de rações da aquicultura deverá aumentar a eficiência e versatilidade de seus ingredientes, como por exemplo empregando o uso de fontes alternativas de proteína, lipído, energia e mineral (FAO, 2022).

A substituição da farinha de peixe por ingredientes alternativos vêm sendo estudados pela aquicultura há décadas devido a estagnação da produção pesqueira e aumento da demanda por outros mercados consumidores, levando a um aumento do proibitivo do preço (FAO, 2022). Entre os ingredientes estudados estão os de origem vegetal, como o farelo de soja e o farelo caroço de algodão, que podem apresentar deficiência em minerais e fatores antinutricionais, como o fitato e o gossipol. (TACON; METIAN, 2008, NRC, 2011, SATOH, 2007). O fitato é relacionado com a redução da disponibilidade de microminerais na ração. Por ser carregado negativamente, o fitato pode se ligar a cátions, como zinco, magnésio, cobre e ferro (Figura 2). Da mesma forma, o gossipol é um antinutriente caracterizado pela sua alta afinidade por ferro e outros nutrientes como vitaminas. A formação destes complexos pode prejudicar a absorção destes microminerais e aumentar a necessidade de suplementação na ração (WEBSTER; LIM, 2015). Além disso, a baixa biodisponibilidade destes minerais e a presença de antinutrientes aumenta a lixiviação destes minerais não absorvidos no trato gastrointestinal para a água e o meio ambiente (CHENG; HARDY, 2004; SUGIUR, 1998).

Figura 2. (a) ácido fítico, (b) ácido fítico quelado a diferentes microminerais. Fonte: SINGH et al., (2018)



Os minerais podem ser classificados como macrominerais ou microminerais de acordo com a quantidade em que são exigidos na dieta e sua concentração no corpo dos organismos vivos. Cálcio, magnésio, fósforo, sódio, potássio, cloro e enxofre são exemplos de macrominerais. Zinco, ferro, cobre, manganês, iodo e selênio são exemplos de microminerais, mas também são conhecidos como elementos traço ou minerais traço (GATLIN III, 2010).

Os minerais desempenham funções vitais para os organismos vivos. No geral os macrominerais têm funções estruturais (e.g., ossos e escamas), atuam na osmorregulação e no

equilíbrio ácido-base no organismo, na homeostase e respiração celular (GATLIN III, 2010; LALL; KAUSHIK, 2021; WEBSTER; LIM, 2015). Microminerais têm função metabólica e atuam principalmente como componentes enzimáticos (GATLIN III, 2010), muitas dessas enzimas são necessárias para atividades metabólicas, como produção de energia, digestão de proteínas, replicação celular e atividade antioxidante (LALL; KAUSHIK, 2021).

A exigência dietética dos minerais varia de acordo com a fonte a qual o mineral é fornecido na dieta e sua biodisponibilidade, sua interação com outros nutrientes, características e particularidades da espécie alvo e o ambiente em que esse animal está inserido (AFSHARI et al., 2021; LALL; KAUSHIK, 2021; MOHAMMADY et al., 2023; NRC, 2011; WEBSTER; LIM, 2015).

1.2.1.1 Zinco

O zinco integra diversas enzimas do metabolismo, podendo ter função catalítica, ou seja, participa diretamente da atividade da enzima.; cocatalítica, atuando em conjunto com outro átomo de zinco; e estrutural, onde o zinco é necessário para a estabilidade estrutural da enzima (VALLEE; FALCHUK, 1993). Entre as enzimas para as quais o zinco é essencial estão superóxido dismutase (SOD), fosfatase alcalina, álcool desidrogenase e anidrase carbônica (WATANABE; KIRON; SATOH, 1997).

A superóxido dismutase (SOD) atua no sistema antioxidante, através redução da toxicidade de espécies reativas de oxigênio (íon superóxido) em uma forma menos danosa às células (peróxido de hidrogênio) (KOURY; DONANGELO, 2003). Além disso, o zinco é importante para a calcificação óssea, a transferência de dióxido de carbono nos glóbulos vermelhos e para a síntese e metabolismo de proteínas e ácidos nucleicos (NRC, 2011).

O zinco pode ser incorporado na dieta de animais através de sais inorgânicos como óxido de zinco (ZnO) e sulfato de zinco ($ZnSO_4$), como quelatos orgânicos (complexados a moléculas orgânicas) ou nanopartículas. Nos últimos anos, as pesquisas envolvendo a suplementação de zinco na dieta de peixes têm investido na avaliação das diferentes fontes e concentrações de zinco. Autores relatam que a utilização de nanopartículas, mesmo em menores concentrações, melhora dos parâmetros de crescimento, do sistema imune e sistema antioxidante em diferentes espécies.

KISHAWY et al., (2020) avaliaram a suplementação de zinco na formas inorgânica, orgânica e em nanopartículas nas concentrações de 20 e 40 mg/kg de ração na dieta de *Oreochromis niloticus*. Neste trabalho, os autores relataram ganho em peso semelhantes entre os tratamentos nanozinco (20 mg/kg), zinco orgânico (40 mg/kg) e zinco inorgânico (40

mg/kg). Além disso, os peixes que tiveram a dieta suplementada com nanozinco apresentaram melhora no sistema antioxidante e sistema imune.

Em experimento avaliando a inclusão de 10, 20 e 30 mg/kg de sulfato de zinco ($ZnSO_4$) e zinco em nanopartículas (Nano-ZnO) na ração de *Labeo rohita*, MONDAL et al., (2020) relataram melhora no sistema imune e aumento do crescimento no tratamento com 20 mg/kg de Nano-ZnO em comparação aos demais tratamentos. Segundo os autores, as nanopartículas de zinco possibilitaram maior absorção e biodisponibilidade no trato gastrointestinal devido ao seu tamanho. Da mesma forma IBRAHIM et al., (2022) relataram melhora nos resultados dos parâmetros de crescimento quando o Nano-ZnO (30 e 60 mg/kg de ração) foi suplementado na ração de tilápia do Nilo em comparação com ZnO.

1.2.1.2 Ferro

O ferro é um dos minerais traços essenciais para os vertebrados, desempenhando papel fundamental em processos bioquímicos como transporte de oxigênio, transferência de elétrons e eritropoiese (CLAUSS; DOVE; ARNOLD, 2008; LALL; KAUSHIK, 2021). A absorção de ferro ocorre tanto nas brânquias quanto no intestino, porém o principal sítio de absorção é no trato gastrointestinal (BURY; WALKER; GLOVER, 2003). As exigências nutricionais de ferro variam de acordo com a espécie, entre as espécies aquícolas esse valor varia entre 30,0 e 170,0 mg por quilo de ração (LALL; KAUSHIK, 2021).

Nos últimos anos, pesquisas envolvendo a suplementação de ferro na nutrição de espécies aquícolas têm focado na avaliação de diferentes concentrações e fontes de ferro. Os trabalhos mais recentes indicam que aparentemente a concentração de ferro no fígado de bagres aumenta de acordo com o aumento da concentração de ferro na ração, mas não é afetada pela fonte de ferro (inorgânica ou orgânica), além disso o desempenho zootécnico não é afetado pela concentração ou fonte de ferro, porém há um aumento linear do hematócrito de acordo com o aumento da concentração de ferro na dieta (BUYINZA et al., 2023; YAMAMOTO et al., 2023); Outros achados mostram que a suplementação de ferro em nanopartículas pode melhorar os parâmetros zootécnicos e aumentar os parâmetros hematológicos de tilápias do Nilo, *Labeo rohita* e *Acipenser stellatus* (BEHERA et al., 2014; EBRAHIMI et al., 2020; MOHAMMADY et al., 2023). Estudos utilizando a mesma espécie ou espécies diferentes podem apresentar resultados contraditórios. Autores relatam que 250 mg/kg de ferro pode causar danos ao fígado e intestino no catfish híbrido (*Ictalurus punctatus* × *I. furcatus*), porém até 1500 mg/kg de ferro não causaram danos histológicos aparentes no bagre do canal (BUYINZA et al., 2023; YAMAMOTO et al., 2023).

1.2.1.3 Cobre

O cobre é normalmente suplementado na forma inorgânica nas rações comerciais para aquicultura, através do sulfato de cobre. Outras fontes inorgânicas e orgânicas também podem ser encontradas. A exigência nutricional entre diversas espécies é entre 3,0 e 12,0 mg de cobre por quilo de ração (LALL; KAUSHIK, 2021). Além disso, a absorção do cobre ocorre nas brânquias e no intestino, através da alimentação. Porém o intestino é o principal local de absorção (BURY; WALKER; GLOVER, 2003).

Através das metaloenzimas, o cobre atua no metabolismo do ferro, na produção de energia celular (citocromo c oxidase) e no sistema antioxidante. Na aquicultura, a SOD e a ceruloplasmina são duas enzimas frequentemente estudadas quando a suplementação de cobre na dieta de peixes é avaliada, devido a sua capacidade de regular a atividade da enzima Zn-Cu-SOD (EYCKMANS et al., 2011), e sua função na ceruloplasmina que oxida o Fe^{2+} a Fe^{3+}, para que o ferro possa ser transportado pela transferrina até o local de formação dos eritrócitos (LALL; KAUSHIK, 2021).

Nos últimos anos, as pesquisas envolvendo suplementação de cobre em ração de peixes têm avaliado diferentes concentrações e fontes de cobre. Os autores relatam melhores resultados de crecimento e resposta imune em red sea bream (*Pagrus major*) quando o cobre é suplementado em nanopartículas (EL BASUINI et al., 2016). No esturjão *Acipenser gueldenstaedtii* como modelo de estudo, melhores resultados foram obtidos em relação a resistência a infecção com *Aeromonas hydrophila*, capacidade antioxidante e sistema imune através da suplementação de cobre em nanopartículas ou quelado a metionina, indicando que a suplementação de cobre poderia ser diminuída de 8 para 5 mg/kg de ração com a suplementação destes em substituição ao sulfato de cobre (WANG et al., 2018). Poucos trabalhos avaliaram a suplementação de cobre e ferro juntos na dieta de peixes, porém, estudos prévios relatam que em dietas práticas a suplementação de cobre e ferro afeta positivamente os parâmetros zootécnicos e parâmetros hematológicos da truta (*Schizothorax zarudnyi*), ou seja, quando suplementados separadamente, os resultados de crescimento são similares, porém quando suplementados juntos aumentam a performance zootécnica. Além disso, a suplementação em nanopartículas alteram os parâmetros de crescimento e hematológicos (hematócrito e RBC) comparada a suplementação na forma inorgânica (AFSHARI et al., 2021).

1.3 NUTRIÇÃO E SAÚDE

A saúde dos peixes refere-se ao estado geral de bem-estar físico e fisiológico. Isso inclui a ausência de doenças, a manutenção da homeostase e a capacidade de lidar com agentes

estressores (ASSEFA; ABUNNA, 2018; GATLIN III, 2003). O estudo da saúde dos peixes é crucial, pois a manutenção de um estoque de peixes saudáveis influencia diretamente na capacidade dos mesmos de expressar seu potencial de crescimento, na resistência a doenças e na qualidade do produto final (SEGNER et al., 2012). Medidas como vacinação, manejo adequado, controle de qualidade da água e uso de suplementos nutricionais são essenciais para garantir a saúde e o bem-estar dos peixes na aquicultura (ASSEFA; ABUNNA, 2018; SEGNER et al., 2012).

A nutrição adequada desempenha um papel crítico na manutenção do crescimento normal e da saúde dos organismos aquáticos. Todos os nutrientes essenciais devem ser fornecidos em quantidades adequadas na dieta para sustentar a saúde dos peixes (GATLIN III, 2010). Além disso, certos nutrientes, componentes dietéticos e práticas de alimentação podem influenciar a suscetibilidade dos peixes a doenças infecciosas e não infecciosas. A nutrição e a alimentação desempenham papéis essenciais na sustentação da saúde dos peixes produzidos na aquicultura (GATLIN III, 2003).

Na aquicultura, diferentes indicadores de saúde podem ser utilizados para avaliar a sanidade de peixes. Conhecer os parâmetros sanguíneos dos peixes, por exemplo, é crucial para avaliar a saúde e monitorar o estresse (DEKIĆ, 2005). Na hematologia, podem ser avaliados parâmetros como a contagem de eritrócitos, leucócitos, hemoglobina, hematócrito, índices hematológicos e contagem diferencial de leucócitos. A importância dessas avaliações reside na capacidade de fornecer informações sobre o estado fisiológico, identificar possíveis distúrbios de saúde, monitorar respostas a patógenos, estresse e qualidade do ambiente (DEKIĆ, 2005; FAZIO, 2019).

O estresse oxidativo é um desequilíbrio entre a produção de espécies reativas de oxigênio (ROS) e a capacidade do organismo de neutralizá-las(HOSEINIFAR et al., 2020). Isso pode levar a danos celulares, incluindo peroxidação lipídica, danos ao DNA, desnaturação de proteínas e até mesmo morte celular (SCHIEBER; CHANDEL, 2014). O sistema antioxidante é um conjunto de mecanismos de defesa do organismo contra o estresse oxidativo causado pelas espécies reativas de oxigênio (ROS). Ele inclui enzimas antioxidantes, como superóxido dismutase (SOD), glutationa peroxidase (GPx), glutationa redutase (GR) e catalase (CAT), além de antioxidantes não enzimáticos, como glutationa, tiorredoxina, vitamina C e vitamina E (HOSEINIFAR et al., 2020; MARTÍNEZ-ÁLVAREZ; MORALES; SANZ, 2005). Esses componentes trabalham em conjunto para equilibrar a produção e a remoção de ROS, protegendo as células contra danos oxidativos e mantendo a homeostase do organismo (HOSEINIFAR et al., 2020).

O microbioma intestinal influencia o metabolismo e a resposta a microrganismos com potencial patogênico. A composição do microbioma intestinal do peixe pode afetar diretamente sua saúde e bem-estar (TEPLITSKI; WRIGHT; LORCA, 2009; ZHANG et al., 2016). Fisiologicamente, as bactérias intestinais auxiliam na decomposição de certos componentes alimentares, como compostos xenobióticos e celulose, em fontes de energia digestíveis para o peixe. A microbiota intestinal interage com o sistema imunológico dos peixes, aprimorando a imunidade inata por meio de vários mecanismos, como competição por nutrientes, estimulação de respostas imunes não específicas e produção de moléculas antimicrobianas (YUKGEHNAISH et al., 2020).

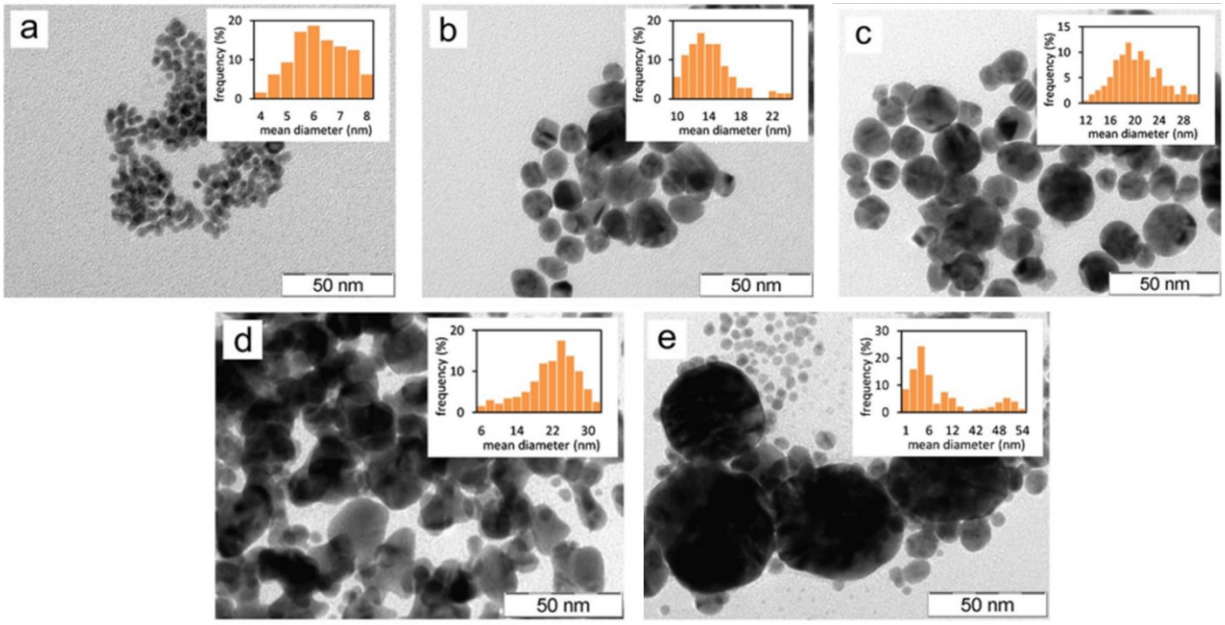
1.4 NANOTECNOLOGIA E AQUICULTURA

Nanotecnologia é a aplicação de conhecimentos científicos para manipular e controlar a matéria em nanoescala (1 até 100 nm) (ISO, 2023). A nanotecnologia é amplamente utilizada em diferentes segmentos da sociedade, como na medicina, no setor de alimentos, na agricultura e na aquicultura (MANJUNATHA; BIRADAR; ALADAKATTI, 2016; SARKAR et al., 2022; SOZER; KOKINI, 2009; SURENDIRAN et al., 2009).

Na aquicultura, a nanotecnologia pode ser aplicada de diversas formas. Nos últimos anos, pesquisas vêm sendo desenvolvidas com objetivo de avaliar o uso desta tecnologia na nutrição, saúde, desenvolvimento de vacinas, degradação de poluentes e sistemas de filtragem (SARKAR et al., 2022; SHAH; MRAZ, 2020).

Na nutrição, as pesquisas envolvendo nanotecnologia têm focado na absorção e transporte de nutrientes e aditivos. Substâncias como a quitosana e diferentes óleos, têm sido utilizados para nanoencapsular e aumentar a eficiência no transporte e utilização de ácido ascórbico, enzimas e vitaminas (SARKAR et al., 2022; SHAH; MRAZ, 2020). Além dessas aplicações, a suplementação mineral em nanopartículas vem sendo bastante estudada na nutrição aquícola. Em teoria, devido ao seu tamanho reduzido, as nanopartículas de minerais (Figura 3) possuem alta área superficial específica, o que pode aumentar sua biodisponibilidade, dessa forma, seria possível reduzir a concentração de minerais na formulação. Além de melhorar a absorção. Autores tem relatado melhoras nos parâmetros de crescimento, parâmetros sanguíneos e resposta imune (AFSHARI et al., 2021; BEHERA et al., 2014; DAWOOD et al., 2020; EBRAHIMI et al., 2020; MOHAMMADY et al., 2023).

Figura 3. Imagens de microscopia de transmissão e histogramas de distribuição granulométrica de nanopartículas de ouro em diferentes tamanhos.



Fonte: SUCHOMEL et al., (2018).

Dessa forma, a aplicação de nanopartículas de minerais como suplementos alimentares na dieta de peixes é uma das ferramentas que pode ajudar no desenvolvimento sustentável da aquicultura através do aumento da eficiência na absorção desses minerais, e possivelmente diminuindo a necessidade de suplementação e descarga destes nutrientes para o meio ambiente.

1.5 OBJETIVOS

1.5.1 Objetivos gerais

Avaliar a suplementação de minerais em nanopartículas na dieta de peixes quanto a parâmetros de crescimento e saúde.

1.5.2 Objetivos específicos

a) Analisar os efeitos da suplementação de nanopartículas de zinco, complexados a aminoácidos ou não, na dieta de juvenis de tilápia do Nilo, em relação aos parâmetros de crescimento, parâmetros hematoimunológicos, atividade de enzimas do sistema antioxidante e deposição de zinco no filé;

b) Avaliar os efeitos da suplementação de nanopartículas de zinco, complexadas a aminoácidos ou não, na dieta de juvenis de tilápia do Nilo, sobre a resistência a infecção experimental com *Streptococcus agalactiae*;

c) Analisar os efeitos de nanopartículas de cobre e ferro na dieta de juvenis de bagre do canal quanto a parâmetros de crescimento e hematológicos;

d) Avaliar os efeitos de nanopartículas de cobre e ferro na dieta de juvenis de bagre do canal sobre a resistência a infecção experimental com *Edwardsiella ictaluri*;
e) Analisar os efeitos de nanopartículas de cobre e ferro na dieta de juvenis de Bagre do canal sobre a microbiota intestinal.

2 ESTRUTURA DO TRABALHO

A estrutura desta tese está dividida em dois artigos científicos. O primeiro artigo da tese, intitulado "Effects of supplementation with different zinc-based products on the growth and health of Nile tilapia", está publicado na revista científica Fish and Shellfish Immunology e foi formatado segundo as normas da mesma, a qual é classificada com percentil 97% na base Scopus.

O segundo artigo científico da tese intitulado "Dietary supplementation of mineral nanoparticles for channel catfish (*Ictalurus punctatus*)" está formatado nas normas da revista científica Fish Physiology and Biochemistry, a qual o artigo foi aceito para publicação e é classificada com percentil 80% na base Scopus.

2.1 ARTIGO 1 - EFFECTS OF SUPPLEMENTATION WITH DIFFERENT ZINC-BASED PRODUCTS ON THE GROWTH AND HEALTH OF NILE TILAPIA

Vitor Fernandes Silva[1], Marília Tedesco[1], Silvia Terra Fontes[1], Marco Shizuo Owatari[1]*, Yuri Malaquias Gauglitz Gatto[1], Matheus Berlofa Ferreira[1], Paola Capistrano dos Santos[1], Gabriel Antonio Cuzma Costa[1], Adriano Faria Palmieri[1], Gracienhe Gomes dos Santos[1], Miguel Saldaña-Serrano[2], Afonso Celso Dias Bainy[2], Maurício Laterça Martins[1], José Luiz Pedreira Mouriño[1]

[1]AQUOS - Aquatic Organisms Health Laboratory, Department of Aquaculture, Federal University of Santa Catarina (UFSC), Florianópolis, Santa Catarina, Brazil.
[2]Laboratory of Biomarkers of Aquatic Contamination and Immunochemistry-LABCAI, Federal University of Santa Catarina, UFSC, Florianópolis, SC, 88037-000, Brazil.

*Corresponding author: M.S. Owatari
Email: owatarimarco@hotmail.com

Abstract

Zinc is one of the essential microelements for the metabolism of animals. Zinc nanoparticles may have higher bioavailability due to their low specific surface area, facilitating absorption by fish. The present study aimed to evaluate the effects of supplementation with different zinc-based products on the growth and health of Nile tilapia, *Oreochromis niloticus*. Zinc, in different sizes (nanoparticles or bulk) and forms (inorganic or organic), were used as a supplement in the tilapia diet at a dose of 15 mg kg feed^{-1} for 60 days. At the end of the feeding trial, production performance, hemato-immunological parameters, activity of antioxidant system enzymes, exposure to *Streptococcus agalactiae* and zinc concentration in the muscle were examined. After the bacterial challenge, the mean corpuscular hemoglobin concentration (MCHC) significantly increased in the fish treated with organic zinc, inorganic nano zinc, and organic nano zinc, while in the control group (inorganic zinc), MCHC remained unchanged. Regarding defense cells, dietary inorganic nano zinc increased the number of basophils (1.50 ± 1.10) compared to organic zinc (0.80 ± 0.90). Lymphocyte count increased after the challenge only in the organic zinc treatments (bulk and nanoparticles). Neutrophils decreased in the control (inorganic zinc) (2.20 ± 1.70) and inorganic nano zinc (2.60 ± 2.70) treatments after the challenge. When compared before and after the bacterial challenge, the plasma antimicrobial titer significantly increased after the bacterial challenge in all treatments. No significant differences were observed for total proteins, enzymes (SOD and CAT), cumulative survival and zinc deposition on fillet. In conclusion, organic zinc in nanoparticles or bulk size increased Nile tilapia innate defense during bacterial infection. However, the other parameters evaluated were not affected by zinc particle size or form (organic or inorganic), indicating that further evaluations should be conducted with organic zinc in nanoparticles or bulk size in the tilapia diet.

Keywords: aquaculture, immunity, minerals, nanoparticles, nutrition, oxidative stress.

2.1.1 Introduction

Nile tilapia *Oreochromis niloticus* is one of the most important cultured fish in the world. Marketability and rustic character promoted its cultivation in different regions of the world. However, to ensure sustainable tilapia farming, interactions between nutrition, disease resistance and immune function must be constantly investigated [1,2]. Minerals are essential for fish health and perform several metabolic and physiological functions, acting on the immune system and enzyme metabolism. In this respect, there are gaps related to Nile tilapia's dietary requirements for minerals such as zinc, whose dietary deficiency can cause significant negative health consequences [1]. The nutritional requirements of minerals vary depending on their structure, bioavailability and nutrient crosstalk, as well as rearing conditions and target species [2]. Zinc is one of the most important micronutrients for the enzymatic metabolism of fish and directly participates in enzymatic activity. Zinc is necessary for the structural stability of the enzyme. Among the zinc-containing enzymes, superox dismutase (SOD) is extremely important in the antioxidant activity of metabolism [3]. In addition, zinc is essential for bone calcification, the transfer of carbon dioxide in red blood cells, and the synthesis and metabolism of proteins and nucleic acids [4]. Zinc can be incorporated into aquafeed through inorganic salts such as zinc oxide (ZnO) and zinc sulfate ($ZnSO_4$), as organic chelates (complexed to organic molecules) or nanoparticles. In the intestine, zinc binds to mucus in the intestinal epithelium and is transported into epithelial cells as zinc ions or as ions bound to amino acids. Zinc nanoparticles pass through intestinal cells more quickly than other forms of zinc and can be an excellent alternative to the use of inorganic sources of zinc. Several studies have shown growth-promoting, antibacterial, and immunomodulatory effects [1,5,6]. Additionally, it is estimated that 17.3% of the global population is at risk of inadequate zinc intake [7]. Approximately 70% of dietary zinc in the United States comes from animal foods, such as fish and shellfish, which are good sources of zinc [8]. However, the tilapia industry is concerned with the lack of research on the effects of dietary zinc supplementation. Thus, zinc nanoparticles could simultaneously improve the health of tilapia and possibly enrich the zinc concentration in fish fillets. In this context, zinc supplementation in nanoparticles and organic forms in aquafeeds has potential to advance the nutrition of aquacultured species, aiming to optimize the absorption and use of this mineral, which acts as a health and growth promoter. Therefore, the present study aimed to evaluate the influence of particle size (nanoparticle or crude size) and zinc form (inorganic or organic) on production performance and health of *Oreochromis niloticus*.

2.1.2 Material and methods

The study was approved by the Animal Use Ethics Committee of the Federal University of Santa Catarina (UFSC), under protocol number 3015221121.

2.1.2.1 Zinc supplementation

Four products were used to evaluate zinc supplementation in fish diets. The products containing nanoparticles (particles size approximately 50 nm) of inorganic zinc (20% ZnO) and organic zinc (10% ZnO chelated with methionine) were supplied by TNS Nano (Florianopolis, SC, Brazil). The products containing inorganic zinc (ZnO 99.96%) and organic zinc (Zn 31.2% chelated with glycine) were purchased from SM Produtos Farmacêuticos (Florianópolis, SC, Brazil), and NPA (Jaboticabal, SP, Brazil), respectively. The zinc supplements were mixed with the vitamin and mineral premix, which were later added to the rest of the ingredients prior to extrusion.

2.1.2.2 Biological material

The Nile tilapia juveniles *O. niloticus* (GIFT lineage, reverted to male) with an average weight of approximately 10.0 ± 1.0 g were acquired from the company Aquacultura Nilótica (Ilhéus, SC, Brazil). After arrival at the laboratory, the fish were kept in the acclimatization process for 14 days and fed with the inorganic zinc diet formulated for this experiment.

2.1.2.3 Experimental design

The experiment was conducted at the Laboratory of Health of Aquatic Organisms (AQUOS), Department of Aquaculture, UFSC. The experimental units consisted of tanks with 70 L of useful volume, connected to an aquaculture recirculation system composed of mechanical and biological filters, ultraviolet reactor (Cubos-16 W, São Paulo, Brazil), individual submersible glass heater (AQUATOP GH-100 W) in each tank and central air blower (Asten P8474 0.3 C V) [9].

After acclimating period (14 days), 720 tilapia juveniles with an average initial weight of 11.12 ± 0.06 g were equally distributed in 24 experimental units (30 fish per tank). Four dietary treatments based on the size of zinc particles (bulk or nanoparticles) and the form of the zinc (inorganic or organic) were evaluated: diet supplemented with inorganic zinc (control); diet supplemented with organic zinc; diet supplemented with inorganic nano zinc, and diet supplemented with organic nano zinc. Each dietary treatment was assigned to a tank in a completely randomized design (n = 5). Zinc was supplemented at 15 mg kg^{-1} of diet in all diets.

The calculations of the daily feed supply were carried out based on the recommendations of the Agricultural Research and Rural Extension Company of Santa Catarina [10]. The physicochemical parameters of water quality for dissolved oxygen and temperature were monitored daily through a digital oximeter (YSI-550 A). A colorimetric kit (Alfakit®) measured pH, alkalinity, total ammonia, and nitrite twice weekly. The mean values of the water quality parameters throughout the experiment were dissolved oxygen 5.3 ± 1.1 mg L^{-1}, temperature 25.8 ± 2.1 °C, pH 7.6 ± 0.4, alkalinity 104.2 ± 21.3 mg L^{-1}, total ammonia 0.6 ± 0.4 mg L^{-1}, nitrite 1.8 ± 0.6 mg L^{-1} and salinity 1.15 ± 0.1 g L^{-1}.

2.1.2.4 Experimental diet

The basal diet (supplementary file 1) was formulated according to the nutritional requirements for Nile tilapia [4], except for the trace mineral zinc, which was evaluated in the treatments. The determinations of dry matter, crude protein, and mineral matter (supplementary file 2) of the diets were carried out at the Laboratory of Nutrition of Aquaculture Species of UFSC (LabNutri/UFSC) according to the methodologies described by the Official Methods of Analysis [11]: dry matter by method 950.01, mineral matter by method 942.05, protein by Dumas's method 990.03.

2.1.2.5 Zootechnical performance

After 60 days of feeding the experimental diets, the fish from each experimental unit were anesthetized with Eugenol (75 mg L^{-1}), counted, and weighed to evaluate weight gain (WG), feed efficiency (FE), and survival (S).

WG = [(final weight − initial weight) ÷ initial weight] × 100
FE = weight gain ÷ amount of feed offered
S = (final number of fish ÷ initial number of fish) × 100

2.1.2.6 Haematological and immunological analyses

Hematological and immunological analyses were performed before and after bacterial challenge, using six and four fish per experimental unit, respectively. The fish were anesthetized with eugenol (75 mg L^{-1}), and the blood was collected by puncturing the caudal vessel with heparinized syringes. Blood was used to prepare blood extensions, differential leukocyte count and total thrombocyte and leukocyte counts, hematocrit determination, quantification of the total number of erythrocytes, and hemoglobin. From the quantification of erythrocytes, hemoglobin, and hematocrit results, mean corpuscular hemoglobin, mean corpuscular volume, and mean corpuscular hemoglobin concentration were calculated. All the analyses mentioned

above were performed according to the methodologies described by Ranzani-Paiva et al. [12]. The remaining blood collected for hematological analysis was centrifuged at 3000 × g for 15 min to obtain blood plasma. The plasma of six fish per experimental unit was pooled and stored in a freezer at −20 °C for immunological assays. The plasma protein and immunoglobulin concentrations were measured with commercial kits (Labtest®, ref. 99 and 359 respectively) and were evaluated in a microplate reader. Plasma agglutinate and antimicrobial titers were performed in a 96-well microplate according to the method described by Silva et al. [13].

2.1.2.7 Bacterial Challenge

The experimental bacterial challenge was conducted with ten fish from each experimental unit. The initial water temperature was maintained at approximately 26 °C. Mortalities were assessed daily for 14 days to determine cumulative mortality. The strain *Streptococcus agalactiae* AQP/049 [14], was cultured in BHI broth at 30 °C for 24 h. The bacterial culture was centrifuged at 2100 × g for 30 min and resuspended in 10 mL of sterile saline solution (0.65% NaCl). The concentration of bacteria in the infection (1×10^7 CFU – colony-forming units – mL^{-1}) was previously defined by determining the lethal dose (LD50). The first infection was carried out via gavage with 1% of the body weight (mL g^{-1}). To ensure that all fish would receive the same number of bacteria, the temperature was gradually increased to simulate a natural field condition until it reached 28 °C. Subsequently, infections were performed on the days fourth and eighth by the infected feed method, according to Owatari et al. [15] to simulate prolonged exposure to *S. agalactiae*. The diets were inoculated with 100 µL of bacterial culture (1×10^7 CFU checked in feed) per gram of feed. To check the bacterial concentration present in the feed after inoculation, 1.0 g of feed was macerated in 1.0 mL of 0.65% sterile saline and subsequently diluted serially nine times in tubes at a factor of 1:10. Dilutions were seeded in Petri dishes containing BHI culture medium (HiMedia, Mumbai, India) to grow. The plates were stored in a bacteriological incubator at 32 °C for 24 h.

2.1.2.8 Antioxidant enzymes

The hepatopancreas tissue of three fish per experimental unit was homogenized in buffer solution (Tris HCl 20 mM, EDTA 1 mM, sucrose 0.5 M, KCl 0.15 M, DTT 1 mM, PMSF 1 mM, pH 7.6) using a tissue homogenizer in a ratio of 1:4 (g:L). The homogenate was centrifuged at 9,000 × *g* at 4 °C for 30 min to obtain the cytosolic fraction. The cytosolic fraction was divided into three aliquots, which were stored in a − 80 °C freezer for further analysis of the activity of SOD and catalase (CAT) enzymes. The concentration of total dissolved proteins in the cytosolic fraction (S9 fraction) was determined according to the methodology described

by Bradford [16] based on the absorption of the Comassie Brilliant Blue G-250 reagent, using bovine serum albumin as standard. The assay was performed on a microplate, and the data generated were used to calculate the specific activity of each enzyme. The SOD activity was measured by the method of inhibiting the reduction of cytochrome C by O_2^-, which is produced during the transformation of xanthine into uric acid by xanthine oxidase. During the reaction, O_2^- is transformed into H_2O_2 by SOD. The readings were performed on a microplate and at a wavelength of 550 nm for 90 s at 25 °C. This method was previously described by Mccord and Fridovich [17]. The activity of the CAT was measured according to the methodology proposed by Aebi [18], based on the decomposition of H_2O_2 ($\varepsilon = 0.0436$ mM − 1 cm^{-1}). The assay was performed on a Greiner microplate (UV-Star, North Carolina, USA) and measured at 240 nm for 90 s at 25 °C. The assay used a reaction medium of CAT buffer (Tris 1 M, EDTA 5 mM, pH = 7.8) and H_2O_2 10.4 mM. The enzyme activity was measured using the H_2O_2 consumption curve. Enzymatic activities are presented in U mg $protein^{-1}$.

2.1.2.9 Zinc deposition on fillet

The fillet samples of six fish per experimental unit were pooled, stored in a freezer at − 20 °C, and shipped to Aquavita Laboratório de Analises, Florianópolis, Brazil. Zinc was quantified in fish muscle using method AOAC 968.08–1969 of the Official Methods of Analysis, minerals in animal feed and pet food, which uses atomic absorption spectroscopy for the determination of zinc and other microminerals [11].

2.1.2.10 Statistical analysis

The data were subjected to the Shapiro-Wilk test to verify normality. A one-way analysis of variance (ANOVA) was performed for significant differences between treatments. Non-normal data were analyzed by Kruskal Wallis. If data presented normal distribution, a Tukey HSD test was performed for comparison of means. Cumulative survival during experimental infection was analyzed using the Kaplan-Meier survival test. All tests were analyzed at a significance level of 5% using the Statistica 14 software.

2.1.3 Results

2.1.3.1 Zootechnical performance

No differences between treatments were observed in the analyses of the growing performance of fish (Table 1).

Table 1. Growth parameters of Nile tilapia (means ± standard deviations) fed for eight weeks with diets containing different sources of zinc.

Variables	*Treatments*				*P-value*
	Inorganic zinc	*Organic zinc*	*Inorganic nano zinc*	*Organic nano zinc*	
Initial mean weight (g)	11.10 ± 0.10	11.10 ± 0.10	11.10 ± 0.10	11.10 ± 0.10	0.81
Final mean weight (g)	53.10 ± 2.50	51.30 ± 2.90	54.50 ± 3.20	51.40 ± 2.00	0.23
Weight gain (%)	42.00 ± 2.60	40.10 ± 2.90	43.30 ± 3.20	40.30 ± 2.10	0.41
Survival (%)	89.60 ± 12.50	90.40 ± 4.60	84.00 ± 10.20	82.40 ± 4.60	0.41
Feed efficiency	0.86 ± 0.10	0.85 ± 0.10	0.84 ± 0.20	0.77 ± 0.10	0.26

2.1.3.2 Hematological analyses

No differences between treatments were observed after or before the bacterial challenge (Table 2). However, after bacterial challenge, a significant increase ($p < 0.001$) was observed for erythrocytes (from 1.50 ± 0.50, 1.30 ± 0.20, 1.20 ± 0.30, 1.50 ± 0.40, to 1.90 ± 0.60, 2.10 ± 0.70, 2.30 ± 0.70, 2.20 ± 0.70 × 10^6 μL^{-1}) in all treatments. The mean corpuscular hemoglobin concentration (MCHC) increased significantly ($p < 0.001$) after the challenge (from 25.80 ± 2.20, 24.60 ± 2.70, 26.10 ± 0.80, to 30.60 ± 7.80, 27.20 ± 4.20, 30.30 ± 4.10 g dL^{-1}) in the organic zinc, inorganic nano zinc, and organic nano zinc treatments, respectively. However, in the control treatment (inorganic zinc), MCHC remained unchanged ($p = 0.240$) after challenge. On the other hand, after the bacterial challenge, a significant decrease ($p < 0.001$) was observed in all treatments for thrombocytes, hematocrit, hemoglobin, Mean Corpuscular Volume (MCV), and Mean Corpuscular Hemoglobin (MCH).

Table 2. Hematological parameters of Nile tilapia (means ± standard deviations) fed for eight weeks with diets containing different sources of zinc, before and after experimental bacterial challenge with *Streptococcus agalactiae*. Superscript uppercase letters mean differences between means of one treatment before and after infection with *S. agalactiae*. MCV = Mean Corpuscular Volume. MCH = Mean Corpuscular Hemoglobin. MCHC = Mean Corpuscular Hemoglobin Concentration.

Parameters	*Treatments*				*p-value*
	Inorganic zinc	*Organic zinc*	*Inorganic nano zinc*	*Organic nano zinc*	
Before experimental infection with S. agalactiae					
Erythrocytes (10^6 μL^{-1} cell)	1.50 ± 0.50^{B}	1.30 ± 0.20^{B}	1.20 ± 0.30^{B}	1.50 ± 0.40^{B}	0.05
Thrombocytes (10^3 μL^{-1} cell)	10.30 ± 7.80^{A}	7.70 ± 4.90^{A}	9.60 ± 8.60^{A}	11.80 ± 16.10^{A}	0.42
Hematocrit (%)	29.60 ± 1.40^{A}	29.60 ± 0.60^{A}	30.10 ± 1.10^{A}	29.90 ± 1.20^{A}	0.84
Hemoglobin (g dL^{-1})	7.60 ± 0.40^{A}	7.70 ± 0.60^{A}	7.40 ± 0.80^{A}	7.80 ± 0.40^{A}	0.51
MCV (fL)	2.20 ± 0.60^{A}	2.40 ± 0.30^{A}	2.70 ± 0.70^{A}	2.30 ± 0.80^{A}	0.05
MCH (pg)	5.60 ± 1.60^{A}	6.2 ± 0.7^{A}	6.4 ± 1^{A}	5.5 ± 1.0^{A}	0.05
MCHC (g dL^{-1})	25.80 ± 1.20	25.80 ± 2.20^{B}	24.60 ± 2.70^{B}	26.10 ± 0.80^{B}	0.65
After experimental infection with S. agalactiae					
Erythrocytes (10^6 μL^{-1})	1.90 ± 0.60^{A}	2.10 ± 0.70^{A}	2.30 ± 0.70^{A}	2.20 ± 0.70^{A}	0.55
Thrombocytes (10^4 cells μL^{-1})	7.70 ± 4.30^{B}	5.60 ± 2.70^{B}	6.10 ± 2.90^{B}	7.90 ± 3.60^{B}	0.09
Hematocrit (%)	22.00 ± 2.30^{B}	20.60 ± 3.20^{B}	22.20 ± 2.30^{B}	21.20 ± 2.40^{B}	0.62
Hemoglobin (g dL^{-1})	5.00 ± 1.00^{B}	6.30 ± 1.20^{B}	6.10 ± 1.20^{B}	6.20 ± 0.20^{B}	0.71
MCV (fL)	1.20 ± 0.40^{B}	1.00 ± 0.30^{B}	1.00 ± 0.20^{B}	1.10 ± 0.30^{B}	0.75
MCH (pg)	3.30 ± 0.10^{B}	3.40 ± 1.00^{B}	2.80 ± 0.80^{B}	3.30 ± 1.20^{B}	0.94
MCHC (g L^{-1})	27.90 ± 4.80	30.60 ± 7.80^{A}	27.20 ± 4.20^{A}	30.30 ± 4.10^{A}	0.41
p-value before vs. after experimental infection with S. agalactiae					
Erythrocytes	<0.001*	<0.001*	<0.001*	<0.001*	
Thrombocytes	<0.001*	<0.001*	<0.001*	<0.001*	
Hematocrit	<0.001*	<0.001*	<0.001*	<0.001*	
Hemoglobin	<0.001*	<0.001*	<0.001*	<0.001*	
MCV	<0.001*	<0.001*	<0.001*	<0.001*	
MCH	<0.001*	<0.001*	<0.001*	<0.001*	
MCHC	0.240	<0.001*	0.020*	<0.001*	

2.1.3.3 Defense cells and immunological analyses

The mean concentrations of the defense cells are shown in Table 3. Inorganic nano zinc showed a significantly increased ($p = 0.03$) number of basophils (1.50 ± 1.10) compared to organic zinc (0.80 ± 0.90). Lymphocytes were significantly increased ($p < 0.05$) after bacterial challenge only in organic zinc treatments (bulk and nanoparticles). Neutrophils were significantly decreased ($p < 0.05$) in the control (inorganic zinc) (2.20 ± 1.70) and inorganic nano zinc (2.60 ± 2.70) treatments after the challenge. On the contrary, neutrophils in organic treatments were significantly lower ($p < 0.05$) before the challenge.

Table 3. Leukocyte count of Nile tilapia means ± standard deviations) fed for eight weeks with diets supplemented with different sources of zinc before and after experimental infection with *Streptococcus agalactiae*. Superscript uppercase letters mean difference between means of one treatment before and after infection with *S. agalactiae*. Superscript lowercase letters mean differences between treatments after or before infection with *S. agalactiae*. No Basophils were found after the experimental infection.

White blood cells	*Treatments*				*p-value*
	Inorganic zinc	*Organic zinc*	*Inorganic nano zinc*	*Organic nano zinc*	
Before experimental infection with S. agalactiae					
Leukocytes (10^4 μL^{-1})	6.10 ± 2.00	6.00 ± 3.00	7.50 ± 2.70	6.00 ± 2.80	0.30
Lymphocytes (10^4 cell μL^{-1})	4.30 ± 2.00	4.10 ± 1.70^{B}	5.20 ± 2.30	4.30 ± 1.80^{B}	0.25
Monocytes (10^4 cel μL^{-1})	1.60 ± 0.80	2.10 ± 1.50	2.10 ± 1.50	1.60 ± 1.20	0.38
Neutrophils (10^3 μL^{-1} cells)	4.70 ± 7.80^{A}	1.10 ± 2.60^{B}	3.10 ± 5.30^{A}	1.60 ± 3.00^{B}	0.42
Basophils (10^3 cel μL^{-1})	1.00 ± 1.10^{ab}	0.80 ± 0.90^{b}	1.50 ± 1.10^{a}	1.10 ± 1.40^{ab}	0.03*
After experimental infection with S. agalactiae					
Leukocytes (10^4 μL^{-1})	9.30 ± 5.20	9.20 ± 3.80	12.50 ± 6.90	11.10 ± 4.80	0.28
Lymphocytes (10^4 cell μL^{-1})	8.40 ± 4.60	7.60 ± 3.50^{A}	10.80 ± 6.50	9.20 ± 4.40^{A}	0.05
Monocytes (10^4 cel μL^{-1})	1.20 ± 0.60	1.30 ± 0.70	1.40 ± 0.80	1.60 ± 0.70	0.14
Neutrophils (10^3 cel μL^{-1})	2.20 ± 1.70^{B}	2.20 ± 2.00^{A}	2.60 ± 2.70^{B}	3.30 ± 2.30^{A}	0.23
p-value after vs. before experimental infection with S. agalactiae					
Leukocytes	0.14	0.09	0.09	0.05	
Lymphocytes	0.08	0.04*	0.07	0.04*	
Monocytes	0.24	0.24	0.11	0.09	
Neutrophils	0.04*	0.04*	0.01*	<0.001*	

No significant differences ($p > 0.05$) were observed in immunological analyses of tilapia plasma after or before (Table 4). When compared before and after the bacterial challenge, the plasma antimicrobial titre significantly increased ($p < 0.05$) after the bacterial challenge in all treatments.

Table 4. Immunological analyses of Nile tilapia (means ± standard deviations) fed diets supplemented with different sources of zinc for eight weeks, before and after experimental infection with *Streptococcus agalactiae*. Superscript uppercase letters mean differences between means of one treatment before and after infection with *S. agalactiae*. Superscript lowercase letters mean differences between treatments after or before infection with *S. agalactiae*.

Immunological indexes	*Treatments*				*p-value*
	Inorganic zinc	*Organic zinc*	*Inorganic nano zinc*	*Organic nano zinc*	
Before experimental infection with S. agalactiae					
Total proteins (mg ml^{-1})	11.30 ± 1.10	12.80 ± 3.90	15.30 ± 2.90	13.90 ± 4.10	0.21
Immunoglobulin (mg ml^{-1})	5.50 ± 1.50	7.50 ± 3.80	6.30 ± 1.50	6.20 ± 1.80	0.61
Antimicrobial titre (log2, (x+1))	5.10 ± 2.20^{B}	4.20 ± 2.20^{B}	3.70 ± 1.60^{B}	3.74 ± 2.60^{B}	0.57
Agglutination titre (log2, (x+1))	4.60 ± 0.50	5.10 ± 2.40	6.10 ± 2.50	5.70 ± 1.90	0.24
After experimental infection with S. agalactiae					
Total proteins (mg ml^{-1})	13.10 ± 3.10	13.10 ± 1.50	13.10 ± 3.10	12.60 ± 6.80	0.26
Immunoglobulin (mg ml^{-1})	5.80 ± 2.90	6.40 ± 1.00	7.30 ± 1.20	6.40 ± 2.30	0.06
Antimicrobial titre (log2, (x+1))	7.60 ± 0.50^{A}	7.60 ± 0.50^{A}	7.80 ± 0.40^{A}	7.20 ± 0.40^{A}	0.30
Agglutination titre (log2, (x+1))	5.60 ± 2.30	5.60 ± 2.30	5.00 ± 1.00	6.30 ± 1.90	0.88
p-value after vs. before experimental infection with S. agalactiae					
Total proteins	0.69	0.88	0.26	0.25	
Immunoglobulin	0.91	0.57	0.26	0.83	
Antimicrobial titre	0.04*	0.01*	<0.001*	0.01*	
Agglutination titre	0.75	0.77	0.53	0.63	

2.1.3.4 Activity of antioxidant enzymes

The mean total liver proteins and antioxidant enzymes are shown in Table 5. No interactions between the factors or significant differences ($p > 0.05$) were observed for total proteins and enzymes SOD and CAT. Although no significant differences ($p > 0.05$) were observed between treatments, the control treatment (inorganic zinc) presented, in absolute values, total proteins levels above those of the other treatments.

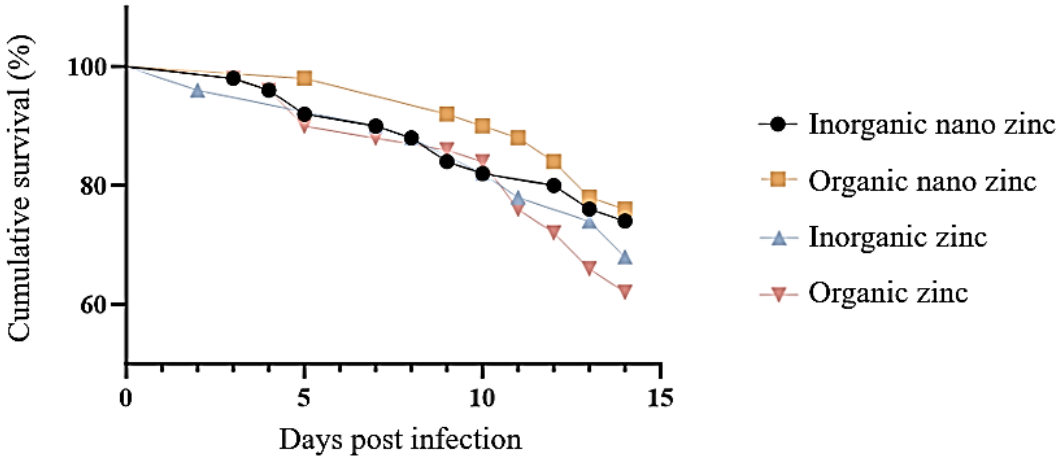
Table 5. Superoxide dismutase (SOD) and catalase (CAT) activity (means ± standard deviations) from liver tissue of Nile tilapia fed for eight weeks with diets containing different sources of zinc. Superscript uppercase letters mean differences between means of one treatment before and after infection with *Streptococcus agalactiae*. Superscript lowercase letters mean differences between treatments after or before infection with *S. agalactiae*.

Variables	*Treatments*				*p-value*
	Inorganic zinc	*Organic zinc*	*Inorganic nano zinc*	*Organic nano zinc*	
Total proteins (mg ml^{-1})	18.00 ± 3.20	17.10 ± 3.70	17.10 ± 5.70	17.40 ± 4.70	0.94
CAT activity (U mg protein^{-1})	237.60 ± 84.50	273.80 ± 91.20	192.60 ± 63.00	205.30 ± 111.40	0.07
SOD activity (U mg protein^{-1})	60.10 ± 18.90	64.70 ± 29.20	68.90 ± 19.30	59.10 ± 19.70	0.63

2.1.3.5 Bacterial Challenge

After 14 days of observation of cumulative survival after the bacterial challenge (Figure 1), no significant differences (p = 0.42) were detected between treatments.

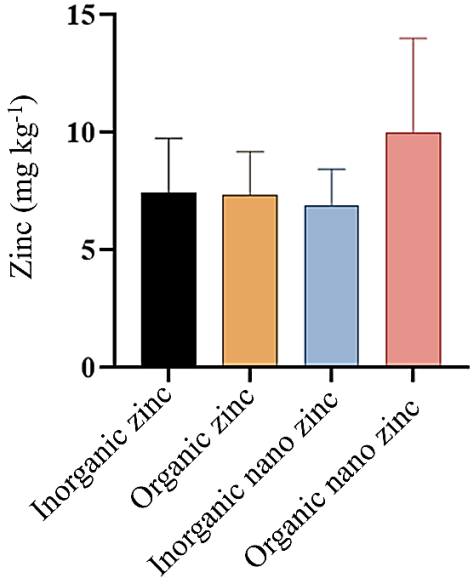
Figure 1. Cumulative survival of Nile tilapia fed for eight weeks with diets containing different sources of zinc after infection of *S. agalactiae*. No significant differences (p = 0.42) were detected between treatments.



2.1.3.6 Zinc deposition on fillet

The average zinc concentrations in the fish fillet of all treatments are shown in Fig. 2. No significant differences (p = 0.28) between treatments were observed.

Figure 2. Zinc concentration in the fillet of Nile tilapia fed for eight weeks with diets containing different sources of zinc. No significant differences (p = 0.28) between treatments were observed.



2.1.4 Discussion

Optimal zinc diet supplementation for tilapia was established based on growth and ranges between 37.2 and 52.1 mg kg^{-1} [19,20]. This study examined the possibility of reducing zinc concentration using zinc nanoparticles in the tilapia diet. However, unlike the present

study, studies that evaluated different sources of zinc showed better growth performance when zinc in nanoparticles or organic form was supplemented between 20 and 40 mg kg^{-1} compared to the inorganic form [23,24].

Indeed, zinc nanoparticles in fish feed improve the fish health, reflecting on the growth performance of fish [25]. Faiz et al. [26] and Sallam et al. [27] reported superior growth performance for *Ctenopharyngodon idella* and *Siganus rivulatus*, respectively, when nano zinc was supplemented at 30 and 60 mg kg^{-1}. Despite this, in the present study, zootechnical equity between treatments means the different forms of zinc did not cause impairments in tilapia growth.

According to Kumar et al. [25], zinc plays an important role in fish immunity, as this nutrient is a key co-factor for several metabolic and enzymatic systems. Unlike the present study, several authors have reported differences in the blood parameters of fish fed diets supplemented with inorganic, organic, and nanoparticle zinc. Faiz et al. [26] and Yaqub et al. [28] reported increased hematocrit, MCV, MCH, and MCHC in treatments where zinc was supplemented in nanoparticles. However, Ibrahim et al. [24] reported lower MCV at the highest concentration of zinc in nanoparticles at 60 mg kg^{-1} and an increase in HCM at 30 mg kg^{-1}.

On the other hand, El Badawy et al. [29] reported higher values of hemoglobin (Hb), hematocrit (Hct), and red blood cell count (RBCs) for fish fed a diet containing 35% protein level supplemented with zinc oxide nanoparticles than those fed with bulk zinc oxide. Therefore, no standard effect of dietary zinc nanoparticles was observed regarding the blood parameters in studies found in the literature. Regarding studies of the effects of different forms of zinc on the immune system, an increase in immunoglobulin concentration, white blood cells, and antimicrobial title were commonly reported in dietary treatments supplemented with zinc nanoparticle compared to inorganic zinc [24,25,28–30].

These benefits observed after ingestion of enriched diets with zinc nanoparticles can explain the higher cumulative survival rates of fish experimentally infected with different bacteria. Some authors have reported higher cumulative survival in experimental infections in treatments when the fish diet was supplemented with zinc in nanoparticles and infected with *Aeromonas hydrophila*, *A. sobria*, and *Staphylococcus aureus* [28,30,31]. In the present study, an increase in th number of basophils observed in the inorganic nano zinc group before infection means an improvement in the resistance of tilapia during the experimental challenge. Although no statistical differences in survival were observed, numerically, the organic zinc treatment had lower survival rates than inorganic nano zinc.

Zinc nanoparticles play a pivotal and complex role as catalysts for several enzymes and reducing oxidative stress in fish [25]. SOD and CAT are enzymes of the antioxidant system, reducing reactive oxygen species into molecules that are less harmful to other cells from the host [27]. Several authors have reported higher activity and gene expression of SOD and CAT in Nile tilapia and other fish species, such as common carp (*Cyprinus carpio*), Marbled spinefoot (*Siganus rivulatus*), and Grey mullet (*Liza ramada*) fed zinc in nanoparticles or organic forms when compared to inorganic zinc [23,27,28,30,32,33]. However, in the present study, no differences were found in SOD and CAT activity between the treatments evaluated. Despite this, these findings can be considered relevant, considering that apparently, no oxidative damage was caused by the different forms of zinc.

Studies in the literature report a significant accumulation of zinc in tilapia muscle when zinc supplementation in the diet is performed with nanoparticles or in organic form. Sherif et al. [30] reported higher zinc concentration in muscle when zinc in nanoparticles was supplemented with 60 mg kg^{-1}. However, supplementation with nano zinc at 30 mg kg^{-1} and inorganic zinc at 60 mg kg^{-1} did not cause differences in zinc concentration in muscle. Kishawy et al. [23] zinc in nanoparticles or organic form, especially at 40 mg kg^{-1}, showed a higher zinc concentration in the fillet than inorganic form. Contradicting the results found in the literature, in the present study, no differences were observed for the analyses performed. Other studies evaluated higher concentrations than those tested in the present work in semi-purified or practical diets. In addition, the zinc concentration in the inorganic nano zinc treatment was approximately 10 mg kg^{-1} higher than in the other treatments. Therefore, the conditions evaluated between the studies were similar, and low zinc concentration was not a limiting factor for the lack of significant differences between treatments.

Nanoparticles are classified by their small size (between 1 and 100 nm) [34]; this is the main characteristic that increases the bioavailability of minerals in nanoparticles by intestinal cells [5]. Other factors can classify mineral nanoparticles in addition to their size, such as the zeta potential and the agglomeration rate of the particles. The environment in which the nanoparticles are embedded, the feed manufacturing processes, and the fish's gastrointestinal tract can alter the nanoparticles' characteristics. Stability, in terms of particle aggregation, sedimentation, and dissolution of zinc, is critical to determine the fate and transport of nanoparticles in an aqueous environment. Increasing the pH, for example, may decrease the zeta potential and increase the particle size and aggregation rate of zinc nanoparticles; in addition, at pH between 4 and 6, zinc has a high dissolution rate [35,36].

A possible explanation for the lack of significant differences in the present study may be changes in the characteristics of the products throughout the feed production process or in the gastrointestinal tract of the animals. In this context, it is suggested that further experiments evaluate the stability of nanoparticles during the extrusion and digestion processes.

2.1.5 Conclusion

In the present study, organic zinc in nanoparticles or bulk size increased the number of blood defense cells of lymphocytes and neutrophils during bacterial infection. However, the other parameters evaluated in Nile tilapia are not affected by zinc particle size or form (organic or inorganic), indicating that further evaluations should be conducted with organic zinc in nanoparticles or bulk size in the tilapia diet.

2.1.6 Supplementary material

2.1.6.1 Supplementary file 1 - basal diet formulation

Table 6. Formulation of the basal diet and zinc concentration in each diet.

Ingredients	Inclusion (g kg^{-1})
Soybean meal	394.0
Corn meal	300.0
Poultry by-product meal	187.0
Wheat meal	80.0
Dicalcium phosphate	13,6
Soybean oil	13.0
Zinc-devoided vitamin and mineral premix[1]	8.0
Tryptophan	3.0
L-Threonine	0.8
DL-Methionine	0.5
Treatment	**Zinc (mg kg^{-1})**
Inorganic zinc	66.84
Organic zinc	67.71
Inorganic nano zinc	77.83
Organic nano zinc	70.72

[1]Premix composition (kg $feed^{-1}$): calcium carbonate (3.36 g kg^{-1}), sodium chloride (2.04 g kg^{-1}), chelated iodine (1.2 g kg^{-1}), iron chelate (0.24 g kg^{-1}), chelated selenium (0.07 g kg^{-1}), vit. A (2.2 mg kg^{-1}), vit. D3 (0.1 mg kg^{-1}), vit. E (89.8 mg kg^{-1}), vit. K2 (5.3 mg kg^{-1}), vit. B2 (7.0 mg kg^{-1}), folic acid (0.93 mg kg^{-1}), vit. B1 (3.44 mg kg^{-1}), vit. B6 (11.8 mg kg^{-1}), vit. C (24 mg kg^{-1}), inositol (480 mg kg^{-1}).

2.1.6.2 Supplementary file 2 - diets dry matter, protein and mineral matter

Table 7. Dry matter, crude protein, and mineral matter of the diets were evaluated in each treatment.

Treatment	Content		
	Dry matter (%)	Crude protein (%)	Mineral matter (%)
Inorganic zinc	90.84	33.51	9.26
Organic zinc	89.9	33.01	9.19
Inorganic nano zinc	89.56	32.33	9.15
Organic nano zinc	89.61	30.36	8.34

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Credit authorship contribution statement

Vitor Fernandes Silva: Data curation, Formal analysis, Investigation, Writing – original draft. Marília Tedesco: Formal analysis, Investigation, Methodology. Silvia Terra Fontes: Formal analysis, Investigation, Methodology. Marco Shizuo Owatari: Formal analysis, Writing – original draft, Writing – review & editing. Yuri Malaquias Gauglitz Gatto: Investigation, Formal analysis. Matheus Berlofa Ferreira: Formal analysis, Investigation, Methodology. Paola Capistrano dos Santos: Investigation, Methodology. Gabriel Antonio Cuzma Costa: Formal analysis, Investigation, Methodology. Adriano Faria Palmieri: Formal analysis, Investigation, Methodology. Gracienhe Gomes dos Santos: Conceptualization, Formal analysis, Investigation, Methodology. Miguel Saldana-Serrano: ˜ Data curation, Formal analysis, Investigation, Methodology. Afonso Celso Dias Bainy: Data curation, Formal analysis, Investigation, Methodology. Maurício Laterça Martins: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Writing – review & editing. Jose ´ Luiz Pedreira Mourino: ˜ Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing. Data availability Data will be made available on request. Acknowledgements The authors are grateful for the financial support from

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2.2 ARTIGO 2 – DIETARY SUPPLEMENTATION OF MINERAL NANOPARTICLES FOR CHANNEL CATFISH (*ICTALURUS PUNCTATUS*)

Vitor F. Silva [1,2], José Luiz P. Mouriño [1], Maurício L. Martins[1], Pedro L. P. F. Carvalho [2], Edgar D. Rodrigues [2,3], Delbert M. Gatlin III [2],*, Matt J. Griffin [4,5], Caitlin E. Older [6], and Fernando Y. Yamamoto [4,7],*.

[1] AQUOS - Aquatic Organisms Health Laboratory, Department of Aquaculture, Federal University of Santa Catarina (UFSC), Florianópolis, SC, 88034-257, Brazil.

[2] Department of Ecology and Conservation Biology, Texas A&M University, College Station, TX, 77845, USA.

[3] Department of Breeding and Animal Nutrition, Sao Paulo State University, Botucatu, SP, 18610-034, Brazil

[4] Thad Cochran National Warmwater Aquaculture Center, Stoneville, Mississippi Agriculture and Forestry Experiment Station, Mississippi State University, Stoneville, MS, 38776, USA.

[5] Department of Pathobiology and Population Medicine, College of Veterinary Medicine, MS, 38776, USA.

[6] Warmwater Aquaculture Research Unit, Agricultural Research Service, U.S. Department of Agriculture, Stoneville, MS, 38776, USA.

[7] Department of Wildlife, Fisheries and Aquaculture, Mississippi State University, Starkville, MS, 39762, USA

*Correspondence: fyy5@msstate.edu (F.Y.Y.)

Abstract

In recent years, the application of mineral nanoparticles as dietary supplements has increased in aquaculture research because nanoparticles have a higher surface area which should facilitate enhanced mineral bioavailability. This study evaluated the supplementation of iron and copper nanoparticles in channel catfish diets and their influences on growth and health. A comparative feeding trial was carried out for 9 weeks to evaluate different combinations of iron nanoparticles (IronNP) and/or copper nanoparticles (CopperNP): only CopperNP, only IronNP, and CopperNP + IronNP, relative to a control diet supplemented with iron and copper inorganic forms ($FeSO_4$ and $CuSO_4$). Growth performance, hematological parameters, whole-body proximate composition, and intestinal microbiota. After the 9-week feeding trial, fish were subjected to a bacterial challenge against *Edwardsiella ictaluri* to evaluate the contribution of the experimental treatments to fish health status. No statistical differences were detected for catfish fed the various diets in terms of production performance or survival after bacterial challenge. The hematocrit and red blood cell counts from fish fed the diet containing copper nanoparticles were significantly lower than the control group. Higher relative abundance of the bacterial taxa *Weissela*, Enterococcus, *Leuconostocaceae*, Lactobacillales, *Streptococcaceae*, *Lactococcus*, Actinobacteriota, and Actinobacteria were found in the digesta of catfish fed diets containing copper nanoparticles. Furthermore, in the context of hematology, iron nanoparticles did not impact the blood parameters of channel catfish, however, reduced hematocrits were observed in fish fed the copper nanoparticle diet lacking additional dietary iron, thus reinforcing the importance of dietary iron to catfish hematobolic regulation. Nonetheless, additional studies are needed to investigate the effects of dietary copper nanoparticle supplementation in catfish diets to better illuminate its effects on the intestinal microbiota.

Keywords: *Ictalurus punctatus*, fish nutrition, mineral supplementation, trace element, aquafeed.

2.2.1 Introduction

Catfish (*Ictalurus* spp.) farming is the largest aquaculture industry in the United States, with the majority of production located in the southeastern states of Mississippi, Alabama, Arkansas and Texas (Hegde et al. 2022). Among the several challenges faced by the catfish industry, idiopathic catfish anemia, colloquially known as “no-blood” or “white lip” in channel (*Ictalurus punctatus*) and hybrid (*I. punctatus* × *I. furcatus*) catfish (Camus et al. 2014) is characterized by low hematocrit values ranging from 0 to 5%, pale or white gills, pink kidneys and spleen, and gray to tan liver, resulting in moribund fish and weekly mortalities (Klar, Hanson, et al. 1986). Isolated cases of catfish anemia have been associated with nutritional disorders, feed contaminants, environmental toxins, and pathogenic bacteria or viruses (Clauss et al. 2008). However, the majority of severe anemia outbreaks in the industry are attributable to feed-related issues (Butterworth et al. 1986; Plumb, et al. 1994) or idiopathic iron deficiency (Camus et al. 2014)

Optimal nutrition is a principal component of commercial animal production, providing essential nutrients for optimal growth and metabolism. Additionally, it equips fish with the necessary resources to combat both infectious and noninfectious diseases, adapt to various environmental conditions, and withstand stress. A balanced diet is formulated to provide the required macronutrients (proteins, lipids, and carbohydrates) and micronutrients (vitamins and minerals). Iron is one of the essential trace minerals required by various fish species, playing a pivotal role in oxygen transport and erythropoiesis (Clauss et al. 2008; Lall and Kaushik 2021). Copper is another essential trace mineral, acting as a cofactor for important proteins, such as superoxide dismutase (Bury et al. 2003) and other metalloenzymes involved in iron metabolism (Linder 2002). Copper-containing enzymes (e.g., ceruloplasmin) oxidize Fe^{2+} to Fe^{3+}, to be transported by transferrin to hematopoietic tissues. Dietary copper deficiency can cause alterations in enzyme activities, deformations, and decreased growth (Damasceno et al. 2016; Lall and Kaushik 2021).

The supplementation of dietary minerals varies according to their physical and chemical characteristics, as well as their potential interaction with other dietary components, species-specific requirements, and the environment (Tawfik et al. 2017). In the last few decades, forage fish-derived protein feedstuffs has begun to be replaced with renderer’s by-products and plant-based ingredients in the aquafeed industry (Hodar et al. 2020). Generally, these ingredients are more economically viable and sustainable (Sales 2009; Macusi et al. 2023). Among the ingredients used in catfish feeds, soybean meal and cottonseed meal are the main protein ingredients (Robinson and Li 2020). However, soybean meal and cottonseed meal

contain high levels of certain antinutrients, such as phytic acid and gossypol, respectively (Krogdahl and Bakke 2015). These antinutritional factors have a high affinity for minerals and amino acid groups, thus potentially hindering the uptake of certain nutrients during digestion and ultimately requiring increased supplementation in feed formulations (Webster and Lim 2015).

Recently, the application of mineral nanoparticles as dietary supplements has gained increased attention in aquaculture nutrition (Vijayaram et al. 2023). Nanoparticles have greater surface area compared to conventional inorganic minerals, which may enhance their bioavailability, improving micronutrient uptake and metabolism (Patra and Lalhriatpuii 2011). Over the past decade, the catfish feed industry has been supplementing diets with high concentrations (500 to 700 mg kg^{-1}) of inorganic iron to prevent onset of idiopathic catfish anemia (Yamamoto et al. 2023). Mineral. Mineral nanoparticle supplementation may be an innovative strategy to enhance catfish iron uptake and assist the catfish industry in mitigating catastrophic outbreaks of idiopathic anemia. Therefore, this study aimed to evaluate the effects of dietary supplementation of iron nanoparticles and copper nanoparticles on channel catfish production performance, hematology, intestinal microbiota and susceptibility to *Edwarsiella ictaluri* challenge.

2.2.2 Materials and Methods

2.2.2.1 Experimental diets

A plant-based diet (Table 1) was formulated to contain 32% crude protein and 16.67 MJ/kg crude energy to meet the nutritional requirements of channel catfish, as outlined by the National Research Council (NRC 2011). Ingredients were individually weighed and mixed for 25 minutes in a V-mixer machine (Blend Master, Buflovak Inc., Tonawanda, NY). The resulting mixture was transferred to an orbital mixer (Mixer-A200, Hobart Inc., Offenburg, BW), and the oils were gradually included while mixing for 10 min, followed by the addition of water (30%) for 10 minutes. The resultant moistened mixture was cold pelleted (Hobart Inc., Offenburg, BW) using a 3-mm die, and the experimental diets dried at room temperature with forced ventilation for 24 h. Each experimental diet was analyzed for proximate composition (Table 1), following the recommended procedures outlined by the Association of Official Analytical Chemists (AOAC 2006).

Table 1. Feed formulation, proximate composition of the basal diet, iron, and copper concentrations.

Basal diet	
Ingredients	Inclusion %
Soybean meal[1]	45.8
Cottonseed meal[1]	15.0
Corn meal[1]	24.6
Fish oil[2]	2.0
Soybean oil[3]	3.0
Vitamin premix[4]	3.0
Mineral premix[5]	4.0
Carboxymethyl cellulose[2]	2.0
L-Lysine[6]	0.3
DL-Methionine[6]	0.3
Proximate analysis (%)	
Dry matter	89.14
Crude protein (% dry matter)	32.14
Moisture (% dry matter)	10.86
Lipid (% dry matter)	4.97

[1]Producers Cooperative Association, Bryan, TX, USA.
[2]MP Biomedicals, LLC, Solon, OH, USA.
[3]Hill Country Fare, TX, USA.
[4]Basal vitamin premix composition (g kg^{-1}): ascorbic acid 50.0, dl-calcium pantothenate 5.0, choline chloride 36.2, inositol 5.0, menadione sodium bisulfite 2.0, niacin 5.0, pyridoxine HCl 1.0, riboflavin 3.0, thiamine mononitrate 0.5, dl-alpha-tocopherol acetate (250 IU g^{-1}) 8.0, vit. A palmitate (500,000 IU g^{-1}) 0.2, vitamin micro-mix 10.0, cellulose 874.0. Vitamin micro-mix (g × 100 g^{-1}): biotin 0.5, folic acid 1.8, vitamin B12 0.02, cholecalciferol (40 IU ug^{-1}) 0.02, cellulose 97.66.
[5]Basal mineral premix concentration: Ca: 2.6 g kg^{-1}, P: 3.7 g kg^{-1}, K: 4.3 g kg^{-1}, Na: 1.8 g kg^{-1}, Cl: 1.1 g kg^{-1}, Mg: 0.5 g kg^{-1}, Al: 1.2 mg kg^{-1}, I: 4.6 mg kg^{-1}, Cu: 5.1 mg kg^{-1}, Mn: 9.1 mg kg^{-1}, Co: 9.9 mg kg^{-1}, Zn: 27 mg kg^{-1}, Fe: 40.2 mg kg^{-1}, Se: 0.2 mg kg^{-1}.
[6]Ajinomoto Co., Inc., USA.

Initially, a basal mineral premix was formulated devoid of iron and copper. Iron sulfate ($FeSO_4.7H_2O$), copper sulfate ($CuSO_4.5H_2O$), copper nanoparticles (Cu 60 to 80 nanometers #0821XH, Skyspring Nanomaterials, Inc.), and iron nanoparticles (Fe 60 to 80 nanometers #0923XH, Skyspring Nanomaterials, Inc.) were added to the mineral premix to generate a total of four experimental diets (Table 2). The analyzed Fe and Cu in all treatments are shown in Table 2.

Table 2. Iron and copper sources for the control and each treatments.

(continua)

Control or Treatment	**Iron source**	**Copper source**
Control	Ferrous sulfate ($FeSO_4 \cdot 7H_2O$)	Copper sulfate ($CuSO_4.5H_2O$)
CuNP	-	Copper nanoparticles[1]
FeNP	Iron nanoparticles[2]	-
FeNP + CuNP	Iron nanoparticles[2]	Copper nanoparticles[1]
Treatment	**Analyzed iron (mg kg^{-1})**	**Analyzed copper (mg kg^{-1})**
Control	637.0	25.9

Table 2. Iron and copper sources for the control and each treatments.

(conclusão)

Treatment	Iron source	Copper source
CuNP	104.0	99.3
FeNP	595.0	17.7
FeNP + CuNP	482.0	94.7

[1]Cu 60 to 80 nanometers, 99.9% Purity – Skyspring Nanomaterials, Inc

[2]Fe 60 to 80 nanometers, 99.7% Purity – Skyspring Nano-materials, Inc

2.2.2.2 Experimental design

Channel catfish juveniles were acquired from the A. E. Wood State Fish Hatchery (San Marcos, TX, USA), and transferred to the Aquacultural Research and Teaching Facility at Texas A&M University (ARTF; College Station, TX, USA). A total of 425 fingerlings (8-13 cm; ~25 g) were equally distributed to their respective experimental units (25 aquaria with 110 L volume operating as a recirculating aquaculture system) and acclimated for 10 days. After acclimatization period, during which fish were fed the basal control diet, the dietary treatments were distributed in a completely randomized design, with five replicates per treatment. Fish were fed twice daily for 9 weeks at a fixed rate ranging from 3 to 3.5% of the biomass. Fish were weighed weekly, and the daily feed ratio adjusted accordingly. Growth performance parameters were evaluated as presented below.

Weight gain (%) = [(final weight – initial weight) ÷ (initial weight)] × 100
Feed efficiency = (weight gain) ÷ (dry feed intake)
Survival (%) = [(final number of fish) ÷ (initial number of fish)] × 100

2.2.2.3 Whole-body proximate composition

At the beginning of the experiment, a composite pool of 12 individual juvenile catfish, with a collective wet-weight of 300 g wet-weight was sampled for initial whole-body proximate composition. After 9 weeks of feeding, two fish per aquarium were euthanized in a 300 mg L^{-1} MS-222 solution, and stored at -20°C. Frozen fish were ground using a meat grinder (Hobart Inc., Offenburg, Germany) then dried in a forced ventilation oven at 60°C for 24 h. After drying, fish were ground using a blender for whole-body proximate composition analysis. Moisture, crude protein, fat, and ash content of whole-body samples were analyzed following AOAC (AOAC 2006) procedures. Protein conversion efficiency (PCE) was calculated based on a dry-matter basis according to the following equation.

PCE (%) ={[(final weight (g) × final protein (%)) – (initial weight (g) × initial protein (%))] ÷ protein intake (g)} × 100

2.2.2.4 Blood parameters

Two fish per tank were anesthetized with MS-222 (100 mg L^{-1}), and blood collected from the caudal vein with heparinized syringes. Hematocrit and red blood cell (RBC) counts were determined following previously published protocols (Witeska et al. 2022). Mean Corpuscular Volume was calculated according to the following formula:

MCV (fL) = (hematocrit × 10) ÷ (n° of erythrocytes)

2.2.2.5 Intestinal microbiota

At the end of the feeding trial, remaining fish were fed their experimental diets for an additional 4 days. One day before collecting digesta, each tank was fed to apparent satiation, staggered in 5-minute intervals to ensure roughly equivalent transit times across all experimental units. Digesta were sampled from respective tanks 18 hours after the staggered feeding, following the same order as the 5-minute feeding intervals. Transient digesta samples was collected aseptically from the whole intestine of two catfish per tank and pooled into 15-mL tubes (1 tube per tank). The total digesta was homogenized in PBS at a 1:1 ratio (w/v), then flash-frozen in liquid nitrogen. Samples were subsequently stored at -80° C until processing.

For DNA extraction, samples were thawed and vortexed, and 500 µL of each sample was pelleted by centrifugation (15,000 × *g* for 1 minute) in 1.5 microcentrifuge tubes. The supernatant was removed and genomic DNA isolated from the pellet using the DNeasy Power Soil Pro Kit (QIAGEN, Hilden, Germany). Amplification and sequencing of the V3-4 region (341F/805R) of the 16S rRNA gene was performed using an Illumina MiSeq plataform (Gohl et al. 2016) at the University of Minnesota Genomics Center. Sequencing data were processed in QIIME2 (v. 2023.7) (Bolyen et al. 2019), where primers were removed using cutadapt (Martin 2011) and, reads quality filtered using DADA2 (Callahan et al. 2016). Reverse and forward reads were trimmed at 280 and 180 respectivel and merged with 30 nt overlap. Taxonomic classification was performed using a Naïve Bayes classifier (Bokulich et al. 2018) trained on the SILVA SSU NR99 database (v138; Quast et al. 2013). Sequences were inserted into the SILVA 128 SEPP (Stefan et al. 2018) to generate a phylogenetic tree for phylogenetic-based diversity metrics. Prior to alpha and beta diversity calculations, samples were rarefied to 37,502 sequences/samples.

2.2.2.6 Bacterial challenge

Bacterial challenges were performed at the Thad Cochran National Warmwater Aquaculture Center (NWAC), in Stoneville, MS, USA. Fish of each dietary treatment were transferred from the ARTF to the NWAC in adapted 56.7-L volume plastic tanks. Oxygen was kept close to saturation by continuous diffusion using pure oxygen through air stones. Salinity was maintained at 5 g L^{-1}, and the survival rate after transport was 100%. At the NWAC facility, ten fish were stocked and acclimated in aquaria containing 22 liters of well water (~24-26 C) in a flow-through system with supplemental aeration provided by a regenerative blower. Control and treatments were kept with five replicates per group.

Edwardsiella ictaluri isolate S97-773 (GenBank Acc. No.: CP084524) was revived from cryogenic storage by isolation streaking on Tryptic Soy Agar plates supplemented with 5% defibrinated sheep blood and incubated for 72 hr at 28 °C. An individual colony was expanded in 9 ml of porcine Brain Heart Infusion broth (pBHIb) for 16 h static at 27° C. Subsequently, 3 mL of culture was inoculated in 3 L of pBHIb and cultured for 16 h (170 rpm at 27° C). The Colony Forming Unity (CFU) was measured by serial dilution in BHI agar plates, and the final concentration was approximately 9.6×10^{10} CFU mL^{-1}. Prior to challenge, water flow was suspended, and 75-mL of the straight culture added to each tank, yielding an approximate challenge dose of 3.2×10^{8} CFU mL^{-1}. The flow of water was resumed after 1 hour. Fish were monitored for morbidity and mortality for 14 days and cumulative percent survival determined.

2.2.2.7 Statistical analysis

Data was validated for normal distribution using the Shapiro-Wilk test, followed by one-way ANOVA or Kruskal-Wallis in case of non-normal distribution. If significances for the dietary treatments ($p<0.05$) were found, post-hoc testing was performed using the Tukey HSD test. Kaplan–Meier cumulative survival analysis was conducted in GraphPad Prism 10.1.0. Intestinal microbiota alpha diversity results were subjected to the nonparametric Kruskal–Wallis rank sum test using the R package stats. Beta diversity distance matrices were analyzed with analysis of similarities (ANOSIM) tests using the R package vegan. Testing for bacteria with differences in relative abundance was performed with the linear discriminant analysis (LDA) effect size (LEfSE) (Segata et al. 2011).

2.2.3 Results

2.2.3.1 Production performance and whole-body proximate composition

No significant differences were observed for the production performance parameters, survival and whole-body proximate composition (Table 3).

Table 3. Production performance (mean ± standard deviation) and whole-body proximate composition of juvenile channel catfish (*Ictalurus punctatus*) after 9 weeks of feeding experimental diets supplemented with Fe and Cu nanoparticles.

Variables	Treatments				p-value
	Control	CuNP	FeNP + CuNP	FeNP	
Initial weight (g)	26.6 ± 0.9	26.6 ± 0.9	26.3 ± 0.6	26.4 ± 0.9	0.89
Final weight (g)	55.2 ± 7.7	58.7 ± 4.6	58.8 ± 4.4	57.6 ± 5.0	0.84
Weight gain (%)	107.7 ± 29.4	120.4 ± 17.0	124.1 ± 21.1	118.6 ± 21.2	0.69
Survival (%)	87.1 ± 19.7	82.3 ± 11	95.3 ± 2.6	90.6 ± 12.2	0.22
Feed efficiency	0.4 ± 0.1	0.4 ± 0.04	0.4 ± 0.04	0.4 ± 0.1	0.82
PCE (%)	18.1 ± 7.6	16.5 ± 2.0	18.3 ± 2.5	20.4 ± 2.6	0.58
Whole-body proximate analysis					
Protein (%)	58.3 ± 1.8	57.2 ± 2.5	56.4 ± 1.9	57.4 ± 2.0	0.84
Lipid (%)	26.8 ± 3.1	27.5 ± 2.5	29.9 ± 3.4	27.8 ± 1.3	0.51
Dry Matter (%)	26.2 ± 2.2	26.7 ± 0.7	27.2 ± 1.6	26.9 ± 1.5	0.92
Ash (%)	13.4 ± 2.1	12.7 ± 2.4	13.6 ± 0.9	13.5 ± 0.4	0.84

Abbreviations: PCE, Protein conversion efficiency

2.2.3.2 Blood parameters

Hematocrit and RBC counts were significantly ($p<0.05$) affected by dietary treatments (Table 4). Hematocrit and RBC counts in the control group were significantly higher ($p<0.05$) than those in the CuNP treatment, but not the FeNP and FeNP + CuNP treatments groups. Similarly, no significant treatment effects were observed for mean corpuscular volume (MCV).

Table 4. Channel catfish red blood cell (RBC) counts, hematocrit, and mean corpuscular volume (MCV) after 9 weeks of feeding experimental diets supplemented with Fe and Cu nanoparticles.

Treatment	RBC (x 10^6 μL^{-1})	Hematocrit (%)	MCV (fL)
Control	2.8 ± 0.4^{a}	29.3 ± 5.3^{a}	11 ± 0
CuNP	2.1 ± 0.3^{b}	18.6 ± 4.8^{b}	8.9 ± 0
FeNP	2.4 ± 0.3^{ab}	24.1 ± 2.7^{ab}	10.3 ± 0
FeNP + CuNP	2.4 ± 0.3^{ab}	24.5 ± 2.3^{ab}	10.4 ± 0
p-value	0.02*	0.02*	0.06

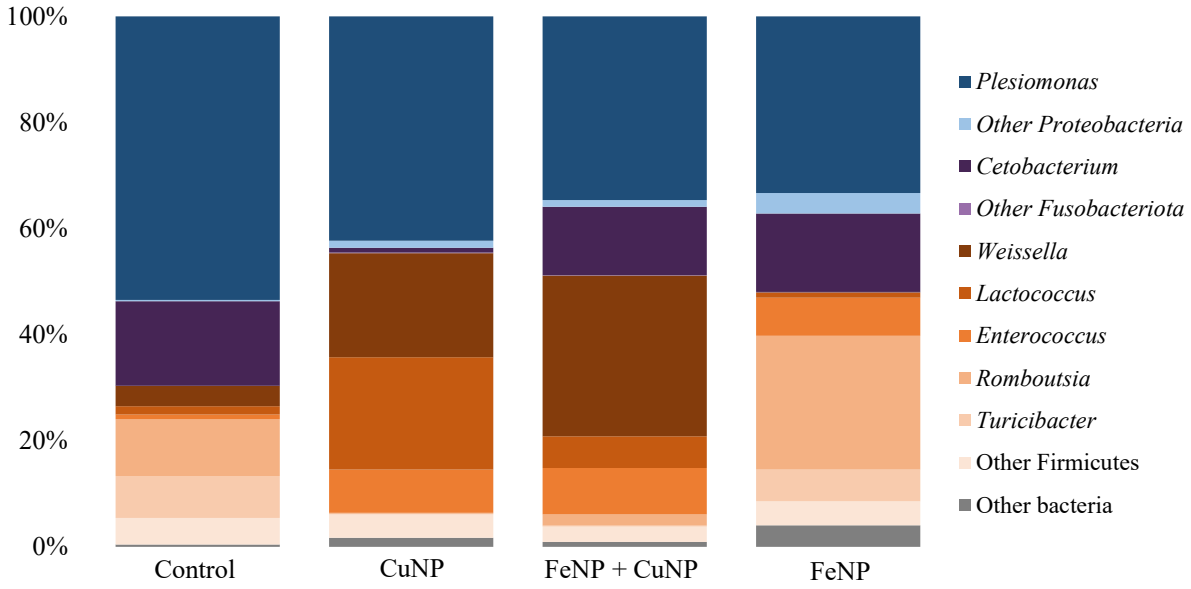
Superscript lowercase and "*" mean significant differences between groups

Abbreviations: MCV, Mean Corpuscular Volume.

2.2.3.3 Intestinal microbiota

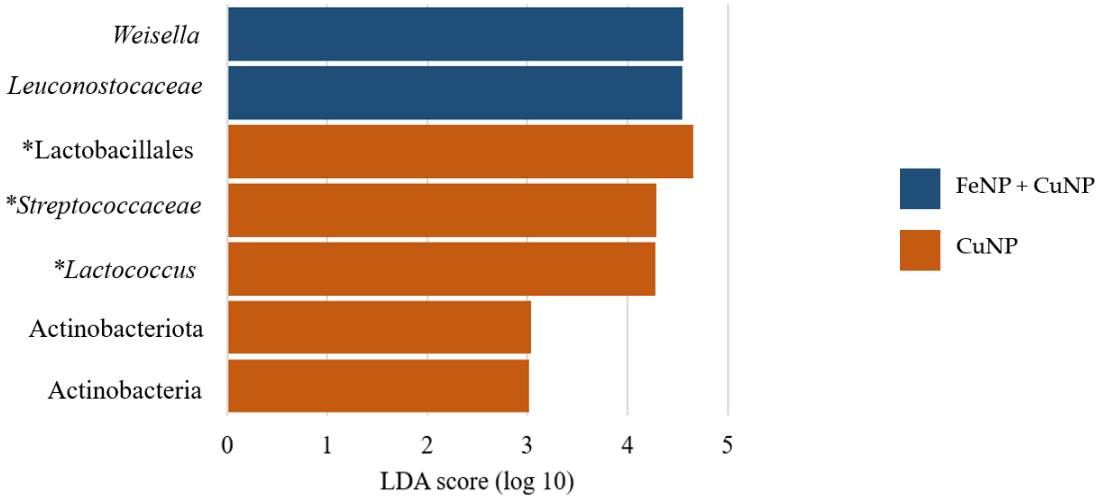
Overall, a predominance of *Plesiomonas*, *Cetobacterium*, and *Romboutsia* were the predominant taxa observed in the intestinal microbiota of fish fed the Control and FeNP diets (Fig 1). For treatments containing copper nanoparticles, higher relative abundances of the phylum Firmicutes were observed. *Weissella*, *Lactococcus*, and *Enterococcus* were the genera within Firmicutes with the highest relative abundances (Fig 2).

Fig 2 Relative abundance of bacteria across treatments and the control after 9 weeks of feeding experimental diets



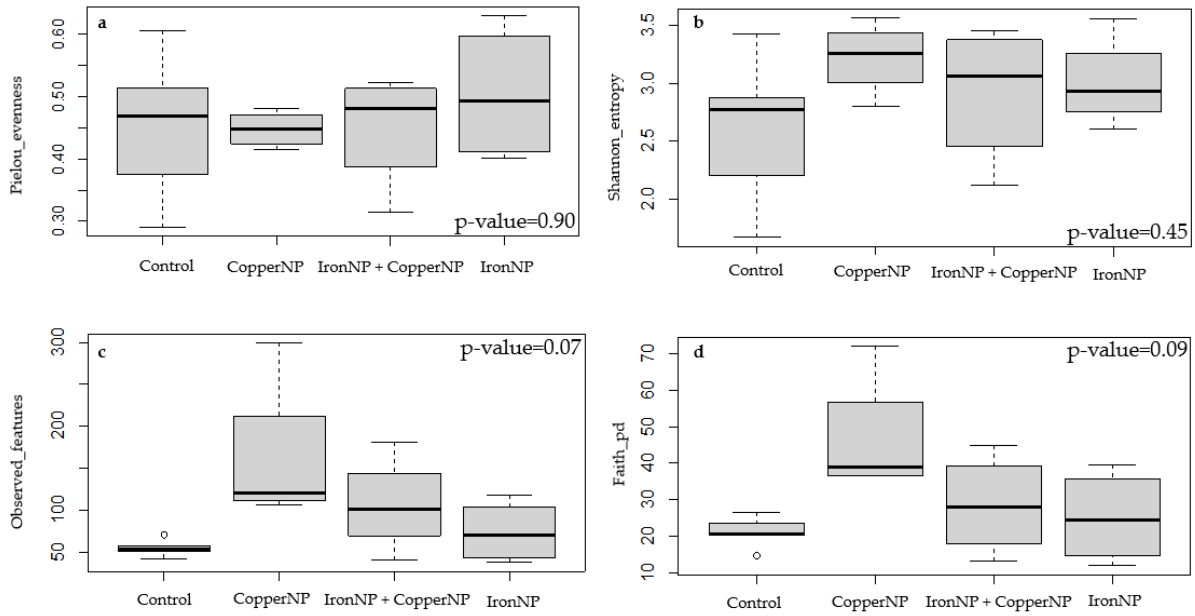
The taxa *Weissella* and *Leuconostocaceae* presented a higher relative abundance in the FeNP + CuNP treatment when compared to the other treatments. Lactobacillales, *Streptococcaceae*, *Lactococcus*, Actinobacteriota, and Actinobacteria had higher relative abundance in the CuNP treatment compared to the other treatments (Fig 3).

Fig 3 Significant results (LDA score [log 10] >3, p< 0.05) from the linear discriminant analysis effect size (LEfSe) analysis to identify differentially abundant taxa. An asterisk "*" indicates p<0.01



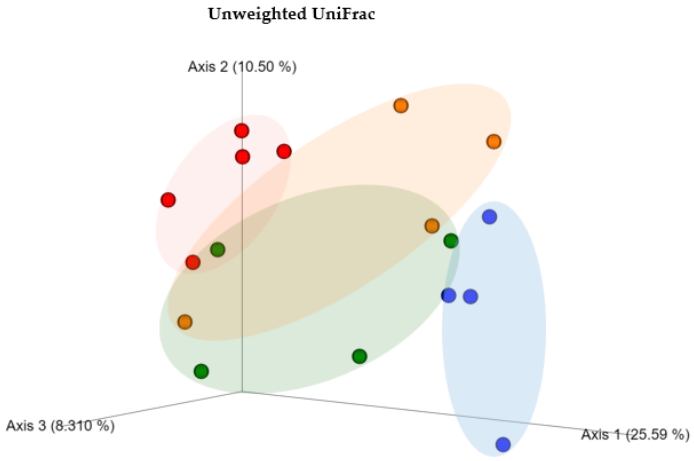
No significant differences were observed for alpha diversity metrics (Fig 4). In particular, the intestinal microbiota in the CuNP group appeared to have higher richness than other groups.

Fig 4 Alpha diversity metrics for (a) Pielou's evenness (p 0.90); (b) Shannon entropy (p 0.45), (c) observed features (p 0.07), (d) Faith's phylogenetic diversity (p 0.09)



Significant differences were observed between treatments for beta diversity metrics Jaccard (p=0.03, R=0.24) and Unweighted UniFrac (p=0.007, R=0.35) analysis (Fig 5). Comparing beta diversity calculated with abundance-based metrics Bray-Curtis (p=0.057, R=0.18) and weighted UniFrac (p=0.093, R=0.11) did not indicate significant differences between treatments.

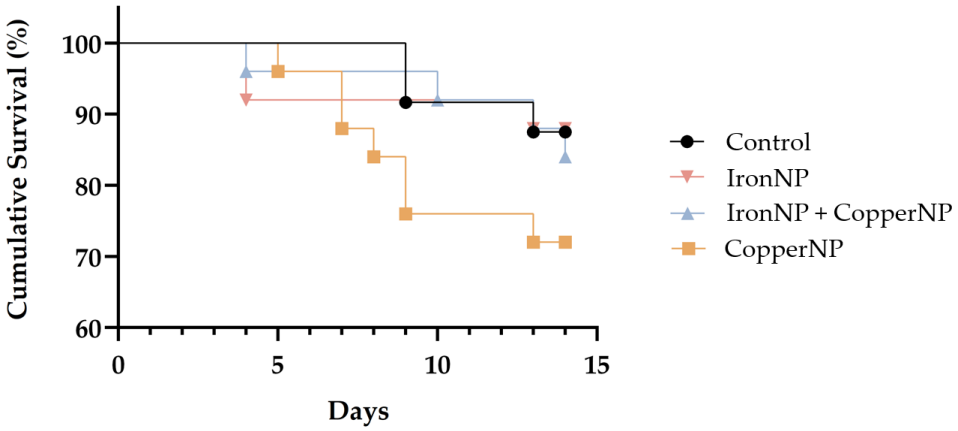
Fig 5 Principal coordinate analysis (PCoA) plot unweighted UniFrac metric for beta diversity



2.2.3.4 Bacterial challenge

No significant differences were observed (p=0.36) in survival among the experimental treatments and the control group following bacterial challenge with *Edwardsiella ictaluri* (Fig 6).

Fig 6 Cumulative survival estimations for channel catfish (~50 g) following immersion challenge with *Edwardsiella ictaluri* isolate S97-773 (estimated concentration: 3.2 × 10^8 CFU mL-1) administered after receiving the experimental diets for nine weeks. Differences in survival between treatments were insignificant (p>0.05).



2.2.4 Discussion

Given its crucial role in red blood cell formation and it's the role of iron deficiency in idiopathic catfish (Camus et al. 2014), dietary iron supplementation has been thoroughly investigated in the channel catfish diet. The earliest study established the minimum dietary iron requirement of channel catfish fed semi-purified diets supplemented with iron sulfate to be 30 mg Fe per kg diet (Gatlin and Wilson 1986). In this context, researchers have conducted investigations to establish optimal concentrations of iron sources in practical diet formulations, which have yielded conflicting results (Yamamoto et al. 2023; Buyinza et al. 2023). Research

has shown a linear relationship between hematocrit and incremental levels of dietary iron sulfate was recently reported in hybrid catfish (*I. punctatus* × *I. furcatus*), with no discernible clinical or histological signs of toxicity or tissue damage and no detrimental effects on production performance (Yamamoto et al. 2023). This is in contrast to similar studies in channel catfish fed inorganic iron and organic iron, which reported increased hepatitis and gastroenteritis at the highest doses (Buyinza et al. 2023).

Research investment in dietary mineral nanoparticle supplementation in aquaculture has increased in recent years (Vijayaram et al., 2023). However, at present, supplementation with iron and copper nanoparticles has not been investigated for channel catfish or channel x blue hybrid catfish. In the present study, decreased red blood cell (RBC) counts and hematocrit levels were observed in channel catfish fed the CuNP diet without additional iron supplementation. This finding is consistent with previous research (Afshari et al., 2021), where lower RBC counts and hematocrit levels were reported for snow trout (*Schizothorax zarudnyi*) receiving diets supplemented with copper in the absence of additional iron supplementation. These results underscore the importance of iron for erythropoiesis, regardless of the iron source.

In other species, dietary iron nanoparticle supplementation improved growth performance and blood parameters. In one study, higher growth performance, hematocrit, and hemoglobin in Nile tilapia (*Oreochromis niloticus*) fed practical diets supplemented with iron nanoparticles when compared to feed formulated with bulk iron (Fe_2O_3) (Mohammady et al. 2023). Similarly, rohu (*Labeo rohita*) fed a semi-purified diet, and iron nanoparticles had higher RBC and hemoglobin levels than fish fed a diet supplemented with standard iron sulfate (Behera et al. 2014). Lastly, in contrast with the findings of the present study, snow trout fed a combination of iron and copper nanoparticles yielded improved growth performance, higher RBC counts, and increased hematocrit compared to diets containing the nanoparticles alone or combined in their respective inorganic forms (Afshari et al. 2021).

Increased lysozyme activity, serum bactericidal activity, and oxidation resistance have been reported for red sea bream (*Pagrus major*) and common carp (*Cyprinus carpio*) fed diets supplemented with copper nanoparticles, which should in turn reduce the susceptibility to disease (El Basuini et al. 2016; Dawood et al. 2020).

Comparably, previous work reported varied levels of dietary iron did not influence susceptibility of channel catfish to *Edwardsiella ictaluri* infection (Yamamoto et al. 2023), however diets deficient or overloaded with iron may impact the susceptibility of fish to bacterial infection. Given the critical role of iron in essential functions such as cellular and metabolic processes, host defense, and immune activation, controlling iron availability is crucial during

infections. It is hypothesized that macrophage iron content can influence the activity of pro-inflammatory transcription factors, thereby affecting the initiation and amplification of the immune response (Haschka et al. 2021). The onset of mortality in channel catfish challenged with *E. ictaluri* was accelerated in fish receiving iron-deficient feed compared to those fed diets containing 300 or 3000 mg of iron per kg of feed. Conversely, there are reports of increased susceptibility to bacterial infection in vivo or using cell lines when iron is highly available in Atlantic salmon (Valenzuela-Muñoz et al. 2020; Díaz et al. 2021). In vertebrate tissues, bacterial pathogens encounter an iron-restricted environment, as intracellular iron is incorporated into metalloproteins or stored in association with ferritin. Most extracellular iron is complexed to hemoglobin in circulating erythrocytes, which limits access for invading bacterial pathogens, thus inhibiting bacterial replication and growth (Haschka et al. 2021; Murdoch and Skaar 2022).

With the increasing affordability of next-generation sequencing technologies and increased recognition of the importance of the role of gut microbiota on fish health (Xiong et al. 2019) the impacts of dietary micronutrients on the intestinal microbiome has become an important area of study (Barra et al. 2021). The differences in beta diversity between the CuNP-containing treatments, the standard copper sulfate supplement, and controls in the present study could have been driven by higher concentration of available copper in the CuNP-containing treatments, which can have antibacterial properties. In contrast to the present study, graded dietary copper sulfate concentrations did not change in the intestinal microbiota of white-leg shrimp *Litopenaeus vannamei* (Zhou et al. 2017). Overall, research has shown the antibacterial activity of copper nanoparticles is more effective against gram-negative bacteria than gram-positive (Valodkar et al. 2011; Nowak et al. 2014; Peszke et al. 2016), attributed to the depolarization of the cell membrane and the overproduction of reactive oxygen species (ROS), leading to lipid peroxidation, protein oxidation, and DNA degradation (Chatterjee et al. 2014). Furthermore, copper can be used to accelerate lactic acid production during the co-fermentation of food waste (Ye et al. 2018), where a higher hydrolysis of carbohydrate and glucose was observed along with a higher relative abundance of *Lactobacillus*. In the present study, the relative abundance of the lactic acid taxa *Weissella*, *Leuconostocaceae*, Lactobacillales, *Streptococcaceae*, *Lactococcus*, Actinobacteriota, and Actinobacteria was higher in fish fed diets containing copper nanoparticles suggesting copper nanoparticles may stimulate growth of lactic acid bacteria and/or inhibit the gram-negative bacteria production.

It is important to highlight that some lactic acid bacteria can be potentially pathogenic for fish. For example, *Enterococcus* spp. are known fish pathogens from the order

Lactobacillales, and members of the *Streptococcaceae* (*Lactococcus garvieae*, *L. petauri* and *Streptococcus* spp.) have been implicated in significant losses in cultured fish (Abdelsalam et al. 2021; Swaminathan et al. 2021; Egger et al. 2023; Saticioglu et al. 2023; Heckman et al. 2024). Unfortunely, the methods used to characterize the bacterial microbiota in the present study do not provide the proper resolution to discriminate among species (Mignard and Flandrois 2006; Winand et al. 2020). Further study is warranted to better understand the mechanisms by which dietary copper nanoparticles influence the intestinal microbiota of farmed fish.

2.2.5 Conclusions

In conclusion, iron nanoparticle supplementation is an innovative strategy to enhance catfish iron uptake. However, the growth performance and hematological parameters of channel catfish were not affected by nanoparticles supplementation. Furthermore, the differences in the hematological parameters of catfish fed control or CuNP dietary treatment reinforce the importance of dietary iron supplementation to maintain fish health regardless of iron source. Future studies should evaluate the implications of dietary copper nanoparticles on the intestinal microbiota.

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3 CONCLUSÕES GERAIS

a) O zinco em nanopartículas na forma inorgânica e orgânica não afetou os parâmetros de crescimento e hematoimunológicos, a atividade das enzimas do sistema antioxidante superóxido dismutase e catalase, deposição de zinco no filé e a resistência a infecção bacteriana;
b) Independentemente do tamanho, o zinco orgânico, aumenta a contagem de neutrófilos e linfócitos após a infecção com *Streptococcus agalactiae*;
c) Na forma inorgânica, o zinco diminui o número de neutrófilos após infecção com *Streptococcus agalactiae*;
d) Independentemente do tamanho, a suplementação com ferro é necessária para a produção de células vermelhas;
e) Cobre em nanopartículas aumenta a abundância relativa de bactérias gram-positivas no trato gastrointestinal do bagre do canal;
f) Cobre e ferro em nanopartículas não afetam os parâmetros de crescimento, parâmetros hematológicos e a resistência a infecção bacteriana.

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