

UNIVERSIDADE FEDERAL DE VIÇOSA

**Development and evaluation of chimeric recombinant antigen, nano vaccine
and probiotics for fish to *Aeromonas hydrophila* and *Pseudomonas
fluorescens* control**

Jojo Rodrigues
Doctor Scientiae

**VIÇOSA - MINAS GERAIS
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JOJO RODRIGUES

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and probiotics for fish to *Aeromonas hydrophila* and *Pseudomonas
fluorescens* control**

Thesis submitted to the Agricultural
Microbiology Graduate Program of the
Universidade Federal de Viçosa in partial
fulfillment of the requirements for the
degree of *Doctor Scientiae*.

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I dedicate this thesis to all trans people and also to those who came before me and were prevented by the stigma of an intolerant and prejudiced society from enjoying a more dignified and loving life. I dedicate this thesis to these people in the certainty that one day we will be occupying spaces that are rightfully ours and that were denied to us. As many trans people as researchers, teachers, doctors, lawyers, engineers, biologists, influencers, politicians... so be it.

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ABSTRACT

RODRIGUES, Jojo, D.Sc., Universidade Federal de Viçosa, February, 2025. **Development and evaluation of chimeric recombinant antigen, nano vaccine and probiotics for fish to *Aeromonas hydrophila* and *Pseudomonas fluorescens* control.** Adviser: Tiago Antonio de Oliveira Mendes. Co-advisers: Jener Alexandre Sampaio Zuanon and Sheiliza Carmali.

Aquaculture is an aquatic food production system whose main products are the cultivation of fish, bivalves and algae. It contributes approximately US\$230 billion to the world economy annually. Growth estimates project trends until 2050 and, therefore, aquaculture is considered one of the most promising global markets. The high consumption of fish in the human diet has intensified in recent years due to the benefits it brings to human health and nutrition, since fish are a source of proteins, vitamins, minerals and “good” fats, which help prevent cardiovascular, bone, ophthalmological and degenerative diseases. However, fish production has been threatened by the establishment of pathogenic bacteria that cause a wide range of diseases and comorbidities in fish and are associated with high mortality rates and production losses in aquaculture production. The species *Aeromonas hydrophila* and *Pseudomonas fluorescens* are representatives of a group of Gram-negative bacteria that threaten fish health and have an alarming zoonotic potential. Vaccination of fish, as well as alternative methods such as the use of probiotics in supplementation, are considered the gold standard in the fight against these infectious agents. Therefore, this study aimed to develop and evaluate a chimeric, nanoencapsulated, probiotic antigen for fish against pathogenic strains of *Aeromonas hydrophila* and *Pseudomonas fluorescens*. The antigen was constructed using immunoinformatics tools using the sequence of the protein UDP-N-acetylmuramate-L-alanine ligase, which is important for the biogenesis of the precursor of the cell wall of bacteria, as a skeleton. A prediction of B and T cell epitopes was performed on the BepiPred 2.0 and NetCLT web servers, and the most immunogenic protein sequences of both bacteria were used to construct the chimeric antigen. The evaluation of the immunogenic property was evaluated in the animal model, zebrafish (*Danio rerio*), and the gene expression of genes related to the fish immune system was evaluated. Protection after immunization was evaluated by challenging the LD50 or lethal doses of *A. hydrophila* in vivo and survival was recorded over a period of seven days after challenge. The nano antigen was also nanoencapsulated by nanostructured lipid nanocarriers and the physicochemical characteristics of the formulations as well as the immunogenic and protective properties in vivo were evaluated. In

addition, the antimicrobial and probiotic potential of two strains, *Lactococcus lactis* and *Lactobacillus acidophilus*, was performed and characterized in vivo. The results were promising and demonstrated an ease of production of the antigen on a large scale through expression in bacterial systems, as well as an immunogenic and protective potential culminating in the upregulation of genes of the fish immune system and in the high survival percentage of immunized fish that were challenged with the LD50 and lethal doses of *A. hydrophila*. The results were even more promising when the antigen was nanoencapsulated, demonstrating that after nanoencapsulation, the protection and survival of the fish were significant at low concentrations of the antigen and challenge of the fish with a highly lethal dose of *A. hydrophila*. However, the probiotic and antimicrobial evaluation of the strains of *L. lactis* and *L. acidophilus* was positive only in vitro, culminating in a direct antimicrobial activity against pathogenic strains of *A. hydrophila* and *P. fluorescens*, but the same was not observed in vivo, and the protection and survival of the fish that received the probiotic treatments were not significant in relation to the fish that were not treated. The data presented in this work demonstrate the power of using immunoinformatics and nanotechnology for the construction and production of products with antimicrobial and vaccine potential against pathogenic agents

Keywords: Aquaculture; Fish; Pathogenic bacteria; *Aeromonas hydrophila*; Vaccination; Immunoinformatics.

RESUMO

RODRIGUES, Jojo, D.Sc., Universidade Federal de Viçosa, fevereiro de 2025. **Desenvolvimento e avaliação de um antígeno quimera recombinante, nanovacina e probióticos para peixes como métodos de controle contra infecção por *Aeromonas hydrophila* e *Pseudomonas fluorescens*.** Orientador: Tiago Antonio de Oliveira Mendes. Coorientadores: Jener Alexandre Sampaio Zuanon e Sheiliza Carmali.

A aquicultura é um sistema de produção de alimentos aquáticos que tem como principais produtos o cultivo de peixes, bivalves e algas. Contribui anualmente com cerca de 230 bilhões de dólares para a economia mundial. A estimativa de crescimento projeta tendências até 2050 e, portanto, a aquicultura é considerada um dos mercados mundiais mais promissores. O elevado consumo de peixes na dieta humana tem sido intensificado nos últimos anos devido os benefícios que trazem para a saúde e nutrição humana uma vez que os peixes, são uma fonte de proteínas, vitaminas, minerais e gorduras “boas”, que auxiliam na prevenção de doenças cardiovasculares, ósseas, oftalmológicas e degenerativas. Entretanto, a produção de peixes tem sido ameaçada pelo estabelecimento de bactérias patogênicas que causam as mais diversas doenças e comorbidades nos peixes e que estão associados a altas taxas de mortalidade e perdas de produção na produção da aquicultura. As espécies, *Aeromonas hydrophila* e *Pseudomonas fluorescens*, são representantes de um grupo de bactérias Gram negativas que ameaçam a saúde dos peixes e possuem um potencial zoonótico alarmante. A vacinação dos peixes bem como métodos alternativos aliados como é o caso dos uso de probióticos na suplementação são considerados modelo ouro no combate contra estes agentes infecciosos. Este trabalho, portanto, teve como objetivo o desenvolvimento e avaliação de um antígeno quimérico, nano encapsulado e de probióticos para peixes contra cepas patogênicas de *Aeromonas hydrophila* e *Pseudomonas fluorescens*. O antígeno foi construído por meio de ferramentas de imunoinformática usando como esqueleto a sequência da proteína, UDP-N-acetylmuramate-L-alanine ligase, importante para a biogênese do precursor da parede celular das bactérias. Uma predição de epítomos de células B e T foi realizada no servidor de web BepiPred 2.0 e NetCLT, e as sequências mais imunogênicas da proteína de ambas as bactérias foram usadas para construção do antígeno quimérico. A avaliação da propriedade imunogênica foi avaliada no modelo animal, zebrafish (*Danio rerio*), e a expressão gênica de genes relacionados ao sistema imunológico de peixes foi avaliada. A proteção após a imunização foi avaliada com desafio das

doses de LD50 ou letal de *A. hydrophila* in vivo e a sobrevida registrada em um período de sete dias após o desafio. O antígeno nano foi também nano encapsulado por nano carreadores lipídicos nano estruturados e as características físico-químicas da formulações bem como a propriedade imunogênica e protetiva in vivo foram avaliados. Além disso, o potencial antimicrobiano e probiótico de duas cepas, *Lactococcus lactis* e *Lactobacillus acidophilus*, foi performado e caracterizado in vivo. Os resultados foram promissores e demonstraram uma facilidade de produção do antígeno em larga escala através da expressão em sistemas bacterianos, bem como um potencial imunogênico e protetor culminando na regulação positiva de genes do sistema imunológico de peixes e no alto percentual de sobrevida dos peixes imunizados e que foram desafiados com as doses de LD50 e letal de *A. hydrophila*. Os resultados foram ainda mais promissores quando o antígeno foi nano encapsulado, demonstrando que após o nano encapsulamento a proteção e sobrevida dos peixes foi significativa em concentrações baixas do antígeno e desafio dos peixes com uma dose altamente letal de *A. hydrophila*. Entretanto, a avaliação probiótica e antimicrobiana das cepas de *L. lactis* e *L. acidophilus* foi positiva apenas in vitro, culminando em uma atividade antimicrobiana direta contra cepas patogênicas de *A. hydrophila* e *P. fluorescens*, porém o mesmo não foi observado in vivo, e a proteção e sobrevida dos peixes que receberam os tratamentos probióticos não foram significativas em relação aos peixes que não foram tratados. Os dados apresentados neste trabalho demonstram o poder do uso da imunoinformática e

Palavras-chave: Aquicultura; Peixes; Bactéria patogênica; *Aeromonas hydrophila*; Vacinação; Imunoinformática.

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WORK OVERVIEW

Dear reader, the focus of this work was to explore new methods to control aquatic pathogens that compromise fish health and aquaculture production. The content of this work is based on a literature review to introduce a background in aquaculture reports and importance, the field of fish vaccine, including new vaccines approach based on nanotechnology, fish bacteria pathogens, the current status of fish vaccine and probiotic control methods used in aquaculture and some considerations for the potential application of smart lipid nano vaccine in the aquaculture.

In the chapter 1, the reader will be emerged in the exploration of the immunoinformatic tools to enhance the antigenic response of no conventional antigen strategies. The content of the chapter is based on the design and fish protection and immune response evaluation of a chimeric recombinant protein built from a cytoplasmatic cell wall biosynthesis enzyme as a backbone.

In the chapter 2, the content pervades the nanotechnology tools, more specifically the antigen nanoencapsulation technique. In this chapter the reader will have accesses to chimeric recombinant antigen nanostructured lipid formulation as well as the standardization of the process, nanoparticles characterization, in vitro evaluation, antigen release profile and storage conditions analysis.

The chapter 3 brings crucial information about the nano vaccine produced in this work, focusing in the fish protection assay, nano vaccine adjuvant evaluation, nano formulation cytotoxicity analysis as well as the oxidative stress profile from the nano formulations compared with traditional adjuvants available on market. In this chapter the first reported application of nano vaccine based on vaccine with antigen encapsulated in nanostructured lipid will provide for the reader the initial analysis for aquaculture application.

Finishing the experience, the reader will have access in the chapter 4 the evaluation of probiotic profile of two lactic acid bacteria isolated from human commercial supplements and their antimicrobial activity in vitro and in vivo.

LITERATURE REVIEW

THE AQUACULTURE OVERVIEWS

The aquaculture is a food production system based on aquatic animals, plants and seaweeds culture. The production can be located in a range of niches such as freshwater, brackish, marine and hypersaline water, and the main products are crustaceans, bivalves, algae and fish (Lucas, 2015a). Current studies are reporting an increasing of 46% in aquatic animals' production which 52% of fish consumed by human were provided by aquaculture in 2018. The freshwater system represents 62% of current production and Asia continent is considered the main area of production, however, non-Asiatic countries production are growing (Fiorella et al., 2021).

The global aquaculture was responsible by 80 million tons of food fish and in 2010 occupied 18 million ha of land space represented by 12 million in inland and 6 million in coastal areas. Countries such China, Indonesia, India, Vietnam, Thailand, Egypt, Norway, Chile and Brazil are the major producers, and carp, catfish, tilapia, shrimp and salmon considered the most high-value species (FAO, 2018). The aquaculture market contributes with 232\$ billion dollars annually for world economy and the Latin America is one the most promising non-Asiatic region in growth. The coastal aquaculture dominates the sector with Latin America exporting tones of aquatic animals, although, the exportation of tilapia farmed in freshwater, is also contributing to aquaculture production with Brazil, Chile, Peru, Mexico and Colombia responsible by the major production (Gyalog et al., 2022).

Brazil has a privilege geographic distribution with the largest hydrographic basis in the world representing approximately 12% of all the surface freshwater and wide coastal area. The aquaculture production in Brazil is recent and dated from 1970 but was in the 2000s that this system gained strength. The statistics data of aquaculture activity in Brazil are poor, by the other hand, with the huge increasing activities the Brazilian Institute of Geographic and Statistics (IBGE) since 2016 has updated the official data and estimate that in 2019 the fish production was over than 800,000 tones with a national production around US\$ 1 billion with focus on southern and southeastern regions (Lima Junior et al., 2018; Valenti et al., 2021).

Aquaculture foods provide a range of benefits with the finality to reduce malnutrition, diseases, social and economic inequities. The combination of proteins, amino acids, vitamins, minerals and great fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) present in the aquatic food play an important role on brain and eyes development in

addition to contribute with the automatic nervous, metabolic and immune systems. For aquaculture to continue growing and meet the demand for high-quality animal protein, production systems must be intensified to meet market demand without expanding farming areas. However, in intensive systems, fish are subjected to high stocking densities and frequent classification procedures that cause stress and, consequently, lower efficiency in using nutrients for growth and lower immune system activity. Therefore, actions are needed to minimize such impacts, maintaining productivity without losing the quality of the final product. In addition, the use of bioactive immunostimulants present in all living microorganisms (plants, animals and microbes) as feed additive can contribute with the fish health, production and nutrients variability (Vijayaram et al., 2022). The aquaculture comprises a significant market with growth projections until 2050 but high demands of production brings some challenges such as infection diseases by pathogen microorganisms (Norman et al., 2019).

The production has been threatened by some virus, bacteria, parasites and fungi infections and 10% of aquaculture production is wasted due to high mortality rate of aquatic animals, mainly fish (Adams, 2019; Pérez-Sánchez et al., 2018). The heterogeneous environmental is propitious to bacteria establishment and in case of a stressful exposure due to high production levels, the fish immune system can be committed favoring bacterial infection (Nayak, 2020a). The most common genus of bacteria founded in infected fish are *Aeromonas*, *Edwardsiella*, *Vibrio* e *Pseudomonas*, consider responsible by severe damages to fish health like skin ulcers, hemorrhages and internal organs necrosis (Buján et al., 2018; de Guzman and Shotts, 1988; Xu et al., 2022).

BACTERIAL PATHOGENS THAT THREAT THE AQUACULTURE PRODUCTION AND THE FOOD SECURITY

A food system production must guarantee the robust control quality to avoid damage products available in market that compromise the environment, animal and human security, in the same is expected for aquaculture system production of animal and plantae. Fish meat is often included in diseases outbreaks with a significant number of people getting infections from contaminated fish, data collected from Foodborne Diseases Outbreak Surveillance System (FDOSS) shown at least 857 outbreaks associates with contaminated fish in the USA. The zoonic potential, infection diseases that is transmitted from animals species to humans, of some bacteria, virus, fungi and parasites need to be investigated to avoid loss for aquaculture production and human diseases (Norman et al., 2019; Ziarati et al., 2022).

Zoonotic transmission between contaminated fish and humans occurs mainly by bacteria pathogens such as the *Vibrio* and *Mycobacterium* genus, however, *Aeromonas* and *Pseudomonas* genus are also included, presenting several clinical manifestations in humans (Ziarati et al., 2022).

The *Aeromonas* genus include gram negative bacteria, anaerobic facultative, positive oxidase, huge spread in aquatic environmental, responsible by aeromonosis outbreaks in fish, resulting in high mortality and economic loss. In humans the *Aeromonas* infection occurs through of contact of ulcers or ingestion, symptoms such as bacteremia respiratory infections, gastroenteritis, sepsis, urinary tract infection and diarrhea are reported. The *Aeromonas hydrophila* species can be found in freshwater, polluted water and saline water with reports of fish infected, meat and vegetables threatening the food safety. The virulence factor associated with *A. hydrophila* include enterotoxin, cytotoxin, hemolysins, surface polysaccharides, proteases, lipases, extracellular enzymes, iron system binding, fimbriae and flagella contributing with their pathogenicity. Some fish species such as carps, *Cyprinus carpio*, yellow catfish, *Aspistor luniscutis*, channel catfish, *Ictalurus punctatus*, rainbow trout, *Oncorhynchus mykiss* and Nile tilapia, *Oreochromis niloticus* were often diagnosed with ulcers, hemorrhage, necrosis and organs damage due *A. hydrophila* infection (Bandeira Junior et al., 2019; Bandeira Junior and Baldisserotto, 2021; Cao et al., 2022; Çiftci et al., 2016; Kumar et al., 2022).

The *Pseudomonas* genus include gram-negative bacillus highly spread in all environmental, presenting virulence factors such as enzymes, pili, flagella, LPS and quorum sensing. *Pseudomonas* species can act as opportunistic pathogen. However, some non-pathogenic strains can be found in plants and soils. Virulent strains of *Pseudomonas fluorescens* were found infecting carps, Nile tilapia and turbot (*Scophthalmus rhombus*) culminating in high mortality rates above 60 and 80 %, in addition, *P. fluorescens* were founded infecting birds and humans. Studies have reported peculiar characteristics of *P. fluorescens* co-infection with parasites in infection sites (Attia et al., 2022; Hardi et al., 2019; Sun and Sun, 2015).

Alternative control methods have been reported to handle with fish infection diseases avoiding traditional strategies such as antibiotics, since they can promote genetic resistance mechanisms resulting in Multi-resistant bacteria. The vaccination combined with probiotic strategies are been considered a high potential control method to explore and apply in aquaculture (Soliman et al., 2019).

CONTROL METHODS AGAINST BACTERIAL PATHOGENS IN AQUACULTURE

The use of antibiotics in aquaculture as control method against pathogenic bacteria is not new and have been applied by decades contributing with exchange of resistant genes among bacteria often found in human environmental. The main antibiotics used by aquaculture are oxytetracycline, florfenicol, erythromycin, amoxicillin and enrofloxacin which belongs the class list used in human medicine as reported by World Health Organization (WHO). The not sustainable use of antibiotics in aquaculture and others food systems influenced organizations such as WHO, Food and Agriculture Organization (FAO) and The World Organization for Animal Health (WOAH) to deploy safe strategies to minimize the use of antibiotics, and nowadays those rules are being followed by nations like United States, Japan, Colombia and Sweden. However, efficiently alternative control methods must be encouraged to explore as the case of vaccines, probiotics and prebiotics that if combined, could be a gold standard to handle with fish infection (Du et al., 2022a; Hegde et al., 2023; Soliman et al., 2019).

FISH VACCINATION

Vaccination is the best sustainable control method option to deal with aquatic pathogens, currently, many aquatic vaccine developments have been in progress in the last years. Since 1940, the vaccine development for aquaculture has been successfully established with the first inactivated oral fish vaccine against *Aeromonas salmonicida* implemented. Currently, there are about 50 vaccines available on the market with a major focused on inactivated vaccine and injection delivery, although, some challenges in protection, immunogenicity, delivery and cost of production difficult the implementation of new vaccines for the market (Adams, 2019; M. Zhang et al., 2023).

The technologies applied for vaccines include some strategies and can classify them by antipathogen properties as bacterial, viral or parasitic, by composition as monovalent, polyvalent or multivalent and by the method of preparation as live, inactivated, subunit, recombinant or nucleic acid. Advantages and disadvantages are present in all preparation methods, briefly, the inactivated vaccine is easy to prepare and can include multivalent composition with high immunogenicity, although, requires multiple vaccinations due the short period of immunity and induce majority humoral immunity, with less induction of cellular and mucosal immunity. The live attenuated vaccine induce strong and long protection and can be delivery by oral and injection routs, but, has questionable integrity and stability and a main

concern about the risk of reverse mutation, the subunit and recombinant vaccine can bring some advantages such as bioinformatic enhancement, high safety, strong immunity, high purity and stability and large production scale, however, some disadvantages such as need of adjuvant, boost doses and sometimes less ability to induce cellular and mucosal immunity. The nucleic acid vaccine can be constructed to enhance immune protection with durable response, safety storage and safe application, although, DNA can induce autoimmune response, expression of foreign antigens, antigen uptake is only after intramuscular injection and immune response influenced by many factors (Du et al., 2022b; Flores-Kossack et al., 2020; Jose Priya and Kappalli, 2022; T. Zhang et al., 2023).

The vaccines available on market are focus in protection of important fish species around the world such as Atlantic salmon, rainbow trout, catfish, common carp, koi carp, Indian major carps and tilapia, and this protection is against *Aeromonas salmonicida*, *Vibrio salmonicida*, *Yersinia ruckeri*, *Vibrio anguillarum*, *Edwardsiella ictalurid* and *Aemonas hydrophila* (Jose Priya and Kappalli, 2022). The protective antigens, rout of administration, environmental safety and the immunogenicity are the most important topics when aquaculture vaccines is in discussion and challenges must be overcome (Adams, 2019). So, the fish vaccine challenges include factors based on the type of pathogen, fish species, rout of administration, antigen prediction and production and models of experimentation (Bedekar and Kole, 2022).

The aquaculture environmental is heterogeneous and the presence of diverse strains of pathogenic bacteria, with different range of antigen epitopes, can difficult the vaccine efficacy, in addition, the diverse range of fish species, as well as the lack of comprehension about bacteria and fish interactions, brings a challenge to produce universal vaccines. An oral vaccinology is frequently required for aquaculture due the laborious injection method and weak immunostimulation of immersion strategy, however, oral vaccine must be carefully designed to avoid mucosal tolerance, antigen denaturation and lack of immunogenicity. Therefore, the rout of administration is so far the main challenge to aquaculture vaccine production. The production of a highly protective antigen able to induce a strong immune response with low cost of production is crucial for vaccine progress difficulted by the lack of an universal animal model to test the antigen protection and standardization of the bacteria diseases (Adams, 2019; Bedekar and Kole, 2022).

Currently, progress in bioinformatics, reverse vaccinology, new delivery strategies and adjuvants approaches are been active in fish vaccinology to overcome the challenges (Bedekar and Kole, 2022). The advanced of bioinformatics tools made it possible the low-cost of sequencing methods and a diverse range of fish genomes and pathogens genomes are available

for use by reverse vaccinology to identify antigens with high immune response prediction. Nanotechnology is also a great approach for fish vaccinology, making possible the target delivery of antigens for immune cells resulting in a robust immune response, in addition, enhances the mucosal immune due to small size crossing the mucosal barrier and protecting the antigen against host cleaning mechanism and harsh conditions from gastrointestinal tract. Depending on the nature of the nanoparticles, their adjuvant role can be accessed and culminate in vaccine efficacy (Adams, 2019; Bedekar and Kole, 2022; Du et al., 2022a; Nayak, 2020b; Thompson et al., 2023a).

PROBIOTICS IN FISH FEEDING

Probiotics can be defined as live microorganisms that promote beneficial host roles improving their metabolism, immune response, antimicrobial resistance and growth performance. Probiotic microorganisms are been identified in aquatic animals and other vertebrates with significant quantities of 10^8 CFU/g than pathogenic microorganisms 10^3 CFU/g and this fact is related to the probiotic mechanism of site competition with pathogenic bacteria establishment (El-Saadony et al., 2021).

The probiotic introduction in aquaculture system is not recent and comes since 90s, and since these microorganisms are present in water ecosystem, they were classified as “water additive”. Some criteria are required to consider a microorganism as probiotic and include: nontoxic effect or pathogenic profile for all live hosts, free from plasmids that containing antibiotic resistance genes, ability to live in low pH and high bile salts content, and need to demonstrate mucus adherence, immunostimulant role, property of stimulating growth and range spectrum of antagonist effect against pathogenic bacteria (El-Saadony et al., 2021; Simón et al., 2021a; Wanka et al., 2018). Currently is known that the probiotic mechanisms against fish pathogenic bacteria include: competition for nutrients and energy, exclusion by antimicrobial production, competition for site of adhesion, reduction of virulence by quorum sense manipulation, enhancement of immune response and host digestion (El-Saadony et al., 2021; Simón et al., 2021a).

Extensive studies have reported the role of lactic acid bacteria (LAB) as probiotic microorganisms due the production of compounds with antimicrobial activity such as lactic acid, acetic acid, hydrogen peroxide and bacteriocin (Cano-Lozano et al., 2022). The genus *Lactococcus* and *Lactobacillus* are some examples of LAB bacteria widely investigated as fish probiotic since *Lactococcus lactis* species, an gram-positive, catalase-negative, facultatively

anaerobic, non spore-forming and nonmotile bacteria, have been recognize as safe (GRAS) and *Lactobacillus acidophilus*, an gram-positive non-forming spore bacteria, was founded in animal intestinal tract promoting a beneficial microbe balance (Aly et al., 2008; Cogan, 1999; Dawood et al., 2016; Litopoulou-Tzanetaki and Tzanetakis, 1999; Wu et al., 2023).

NANOTECHNOLOGIES TO IMPROVE THE FISH VACCINE IMMUNE STIMULATION AND DELIVERY

The long immune protection of recombinant vaccines is often putted in question and nanotechnologies are emerging as approaches to deal with recombinant fish vaccine challenges that include low and short immune stimulation and vaccine delivery. In the field of vaccine technologies, the nanoparticles is gaining attention due to their proprieties of antigen encapsulation, antigen delivery in specific sites, slow release, possibility to improve the oral delivery and effective fish immune system stimulation (Harshitha et al., 2023; Thompson et al., 2023b).

Different nanomaterials are been used in medicine and veterinary applications and are divided into groups based on their proprieties: ultrafine nano-size particles presenting in nature and the engineered nanoparticles produced in controlled space (Shalan et al., 2016). Engineered nanomaterials based on nanoparticles, micelles, liposomes, emulsions, biopolymers, protein-carbohydrate complexes, dendrimers, solid nano-lipid particles and nanostructured lipid carriers are recognized as systems already applied on agriculture field due their proprieties of bioavailability, stability improvement and controlled release, reducing the process cost and economic waste (Fajardo et al., 2022).

Bio nanomaterials are the components to construct bio-nanoparticles and their properties of immune-stimulation, environmental safety and facility to design have gain attention in fish vaccinology mainly those based on nano-lipid materials (Vinay et al., 2018). The use of lipid-based nanoparticles makes it possible the encapsulation of hydrophobic and hydrophilic antigens protecting them from enzymatic degradation (Thompson et al., 2023c).

Oral delivery vaccine is by far the route of administration preferred for fish vaccinology field, however, peptides administrated orally have to deal with the inactivation by low pH of gastrointestinal tract, enzymatic degradation and poor membrane permeability, although, this challenges can be overcome by introduction of lipid-based nanocarriers such as oil-in-water nanoemulsions, solid lipid nanoparticles (SLN), nanostructure lipid carriers (NLC), liposomes and micelles, all prepared by surfactants and lipids approved by FDA (Haddadzadegan et al.,

2022).

Solid lipid nanoparticles and nanostructure lipid carriers can be promises nanocarriers to be delivered and applied in aquatic environmental, keeping the peptide integrity. SNL consist in combination of solid lipids and surfactants and differ from NLC by the presence of liquid lipids in the NLC composition overcoming the challenges with protein loading and storage observed in SNL. Differently from the other lipid nanocarriers, SLN and NLC, remain in the solid state after oral delivery, protecting the peptide from gastrointestinal and epithelium barriers (Haddadzadegan et al., 2022; Plaza-Oliver et al., 2021; Subramaniam et al., 2020).

MAIN GOAL

Based on the facts presented above, the main goal of this work was to develop and evaluate a chimeric recombinant antigen, nano vaccine and probiotics for fish to *Aeromonas hydrophila* and *Pseudomonas fluorescens* control.

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CHAPTER 1

RECOMBINANT CHIMERIC ANTIGEN ZEBRAFISH PROTECTION AGAINST *A. hydrophila* AND ALUMINUM HYDROXIDE ADJUVANT AND TOXIC PROFILE EVALUATION

PAPER 1

PROTECTION AND SAFETY OF ALUMINUM HYDROXIDE ADJUVANT AND CHIMERIC ANTIGEN CONTAINING PREDICTED EPITOPES FROM BACTERIA MURC GENE IN AEROMONAS HYDROPHILA FISH INFECTION

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ABSTRACT

The aquaculture is a food production system based on fish, plant and algae from fresh water, marine waters, brackish and hypersaline environmental. The aquaculture production is increasing and have a significant impact in world economy and human nutrition. Infection diseases are threatening the fish production culminating in high mortality rates. *Aeromonas hydrophila* is a gram-negative zoonotic bacterium associated with outbreaks on aquaculture and fish disease and mortality. Vaccine is an efficient control method to avoid the pathogen spread and establishment. The aim of this work was to evaluate a zebrafish protection against *A. hydrophila* infection using a recombinant antigen based on cytoplasmatic enzyme involved on cell wall biosynthesis built by immunoinformatic prediction as well as the toxic impacts of aluminum hydroxide adjuvant. The chimeric recombinant antigen was constructed based on murC gene product and submitted to cells B and T prediction, cloned on Pet28a plasmid and expressed on bacteria system. The toxic profile of *A. hydrophila* was accessed in vivo and fish mortality recorded. Immunization assay was performed in adult zebrafish intraperitoneally and two booster of chimeric protein (PTN), aluminum hydroxide (ADJ), chimeric protein combined with aluminum hydroxide (ADJ+PTN) and saline buffer (PBS) was applied every 7 days. The zebrafish protection assay was evaluated after 7 days of the last immunization with administration of a LD₅₀ dose of *A. hydrophila*. The liver, spleen, intestine and gills were harvested before and after bacterium challenge and submitted to histopathological analysis and qRT-PCR to access the immune-related genes profile. The results showed acute toxic profile of *A. hydrophila* in vivo with high mortality in 24 hours, however, a antigenic profile from the chimeric antigen was observed and was able to protect zebrafish from mortality after *A. hydrophila* challenge. The chimeric antigen had no toxic activity for fish and achieved a targeted response in the spleen based on up-regulation of IL10, IFN1, MHCII and IL6 without acute inflammatory response, although, aluminum hydroxide 0,2% showed a toxic profile resulting in fish mortality, fish tissues damage and acute inflammation. In conclusion, new targets from mur family, murA to murF, all involved in the bacteria cell wall biosynthesis as well as other cytoplasmatic enzymes important for bacteria virulence must be explored to achieve their immunogenic profile and construct an efficient multiepitope vaccine against aquaculture pathogenic bacteria, in addition, novel adjuvants for aquatic vaccines as sustainable alternative with no toxicity profile.

1. INTRODUCTION

The consumption of fish has significantly increase by the last decades and 52% of all fish market was provided by Aquaculture industry, a food production system based on aquatic animals species, plants and seaweeds culture(Fiorella et al., 2021; Lucas, 2015a). Fish is considered one of the most important food for human diet helping to prevent cardiovascular diseases, neuro and hepatic disorders as well as malnutrition; rich in vitamins, amino acids, unsaturated fatty acids and bioactive molecules with anti-oxidation, anti-inflammation and antimicrobial properties(Chen et al., 2022; Thong and Solgaard, 2017).

Brazil is one of the largest fish producers on the world and Nile Tilapia, *Oreochromis niloticus*, the focus of production, however, Brazilian fish consumption is lower (8 kg per capita) of recommended by World Health Organization (WHO). The consumption in the five macro-regions differ and the population of the north region has a higher preference of this protein source then other meat options. Despite, those findings, Brazilian aquaculture market is considered a competitive world market in growth by Food and Agriculture Organization of the United Nations (FAO)(“© FAO. 2025. Brazil. Text by Suplicy, F.M.. In: Fisheries and Aquaculture.” n.d.; GUZMÁN-PINCHEIRA et al., 2024; Lopes and de Freitas, 2023).

The aquaculture projections estimate a growth until 2050 placing the aquaculture market as a promiser world economy tendency, contributing with 232 billion dollar per year already, although, bacteria infections have emerged as a challenge for fish production resulting in high mortality rates, economic loss and zoonotic proliferation (Ahmed and Thompson, 2019; Fiorella et al., 2021; Lucas, 2015a; Ziarati et al., 2022). Bacteria genera such as *Aeromonas*, *Edwardsville*, *Vibrio* and *Pseudomonas* are considered responsible by severe damages to fish health like skin ulcers, hemorrhages and internal organs necrosis (Buján et al., 2018; de Guzman and Shotts, 1988; Xu et al., 2022). The intensification of production systems makes it possible to meet the increased demand for fish but exposes fish to stressors such as high stocking densities and classification managements that cause compromised immune systems and greater susceptibility to harmful bacteria such as *Aeromonas hydrophila*, a zoonotic gram-negative bacterium founded in fresh and marine water, responsible for invaluable loss on fish production due to aeromonosis outbreaks as well as human enteritis due to contaminated fish consumption (Bandeira Junior et al., 2019; Bandeira Junior and Baldisserotto, 2021; Cao et al., 2022).

Vaccination is by far the best control method against infection diseases and since 1940 when the first vaccine for fish was reported (Duff, 1942), the aquatic vaccines have gained

attention, although, the lack of information about fish immune system, response depending of fish species, route of administration for aquatic vaccines, vaccine evaluation and effectivity, contributed for few vaccines available on market for fish(Adams, 2019; Bedekar et al., 2022; Bedekar and Kole, 2022). The inactivated and injection route is currently the main strategy present on these vaccines; however, other strategies must be explored such as the subunit antigen vaccine and oral route of administration, overcoming the laborious administration and non-specific response present in the traditional strategies (Du et al., 2022b; Flores-Kossack et al., 2020; Jose Priya and Kappalli, 2022; T. Zhang et al., 2023).

Subunit and recombinant vaccines have the advantage of the use of bioinformatic enhancement producing antigens with high safety, strong immunity stimulation, high purity, stability and scalable production(Adams, 2019; Du et al., 2022c; Flores-Kossack et al., 2020; Nayak, 2020b). Currently, the strategy for subunit vaccines for fish is based on the immunogenic and the antigen exposition on the cell outer membrane, characteristics of the outer membrane proteins (OMPs) (Mzula et al., 2019; Thirumalaikumar et al., 2022; M. Zhang et al., 2023), however, new potential antigens strategies must be explored to produce effective vaccines and join forces against bacteria fish infections. The enzymes and products involved on the bacteria cell wall biosynthesis have gained attention as antimicrobial targets and murC gene code important proteins related in these pathway(Berckx et al., 2020; Kumar et al., 2023).

Evaluate the immunogenic profile of murC gene product to achieve a target with antimicrobial and antigenic properties could be a start to produce effective subunit vaccines for fish, therefore, the aim of this work was to construct by immunoinformatic prediction a vaccine able to protect zebrafish against *A. hydrophila* mortality. In addition, to investigate the adjuvant and toxicity profile of Aluminum hydroxide, an important approved adjuvant already used in human vaccines (He et al., 2015; Zou et al., 2024).

2. MATERIAL AND METHODS

2.1 BACTERIA STRAIN AND CULTURE CONDITIONS

The *A. hydrophila* strains (ATCC 7966) and (IOC23116) were genteelly donated by Enterobactérias Laboratoty from Fundação Oswaldo Cruz (FIOCRUZ) and were aerobic cultured in Trypticase Soy Broth (TSB) medium at 30 °C under agitation of 180 rpm when necessary.

2.2 ANIMAL ETHICS APPROVAL AND MAINTENANCE

The experiments with zebrafish, *Danio rerio*, were conducted after approval of Animal Production Ethic Committee (CEUAP) from Federal University of Viçosa, Brazil (Protocol 39/2023). The zebrafish were obtained from Pisciculture market and manipulated in Laboratory of Physiology Applied to Pisciculture and Laboratory of Proteomic and Bioinformatic from Instituto de Biotecnologia Aplicada a Agropecuária (BIOAGRO-UFV). The fish manipulation and maintenance respected the Zebrafish Breeding Manual (Dammski et al., n.d.). Briefly, fish were kept in tank of 20 L (30 N) or 10 L (10 N) with aerated water quality guaranteed, water temperature of 25-30 °C, light and dark cycle by 12h/12 h and fed twice by day *ad libitum*. Fish health was evaluated by behavior (movement/ appetite) and clinical diseases signs were also evaluated during the experiments.

2.3 INFECTION AND TOXICITY EVALUATION

The zebrafish toxicity of *A. hydrophila* IOC23116 and ATCC 7966 strains were evaluated by injection of 10µl of each strain in peritoneum. Male and Female zebrafish adults with 0,50 g body weight were randomly distributed in tanks of 10 L (10N) and injected with bacteria suspension at 0,5, 1 and 2 Mcfarland standard ($1,5 \times 10^8$, 3×10^8 and 6×10^8 respectively). The control group received 10 µl of saline solution (NaCl 0,9%) and the toxicity was evaluated by clinical signals (skin discoloration, ulcers, hemorrhage, lack of appetite) and mortality during 7 days (Pacheco et al., 2021a; Yi et al., 2019).

2.4 ANTIGEN DESIGN

The antigen construction was performed by immunoinformatic tools as following described. The target, murC gene product named UDP-N-acetylmuramate-L-alanine ligase, involved on cell wall biosynthesis was chosen after literature review. Posteriorly the protein antigen sequence was submitted to UNIPROT data bank to access the protein sequence from *Aeromonas hydrophila* and *Pseudomonas fluorescens* strains. The FASTA sequences were globally aligned in MEGA software 11.0 and a phylogenetic tree was constructed by Clustal Omega and the most conserved sequence chosen from each bacteria specie and submitted to epitopes prediction. The web server BepiPred 2.0 and NetCLT were used to predict the Cells B and T epitopes based on “mouse” because “fish” or related organism is not available. The

peptides score above 0,5 that presented 5 amino acids were selected and only epitopes that can stimulate Cells B and TCD4 were included because the pathogens are not intracell, epitopes that can stimulate Cells TCD8 were excluded. After, chimeric protein was constructed by epitopes sequence linking from each strain. The flexible link (GSGSGS) was used in the middle of the epitopes to improve the solubility and avoid interactions between epitopes neighbors. The synthetic gene sequence was submitted to codon enhancement to expression in *Escherichia coli* (<https://www.idtdna.com/pages/tools/codon-optimizationtool?returnurl=%2FCodonOpt>) (Menezes-Souza et al., 2014; Pereira et al., 2020).

2.5 CHIMERIC ANTIGEN CONSTRUCTION AND EXPRESSION

The DNA sequence from chimeric antigen was synthesized in PET28a plasmid vector and used to *E. coli* BL21 and Artic Express (DE3) cells transformation by thermal shock. The transformants were cultured in Agar Luria-Bertani (LB) medium culture containing 50µg/mL of Kanamycin and 20µg/mL of Gentamycin when necessary. The Polymerase chain reaction (PCR) was performed to confirm the success of transformation with specific primers and afterwards the cells were cultured in LB Broth and after Optical density of 0.6 the protein expression was induced with 0,5 µmol/mL of IPTG under agitation at 37°C or 12°C. Posteriorly the induction time the cells were centrifuged and submitted to sonication and the insoluble fraction was harvested and submitted to purification process using His-trap column. The insoluble fraction was analyzed by SDS-PAGE and Western blotting as following steps, the samples were denatured at 95 °C for 10 minutes after mixed with 2-mercaptoethanol and submitted to electrophoresis gel and posteriorly stained with Coomassie blue. After the transfer process to a nitrocellulose membrane the blocking procedure was conducted with skimmed milk by 1 hour and the membrane was washed three times with PBS/tween buffer and submitted to antibodies reaction (anti His and anti Igg conjugate with peroxidase). Posteriorly antigen-antibodies reaction the membrane was submitted to staining with 3,3-diaminobenzidine tetrahydrochloride (DAB) and hydrogen peroxide under dark conditions (Pereira et al., 2020).

2.6 PROTECTIVE EVALUATION OF CHIMERIC ANTIGEN AGAINST AEROMONAS HYDROPHILA

The total of 90 adult zebrafish were vaccinated intraperitoneally with 1 microgram of chimeric antigen (PTN group) or with 10 µL of aluminum hydroxide 0,2 % mixed with 1

microgram of chimeric antigen (PTN +ADJ) or with 10 µL of saline solution (PBS) or only aluminum hydroxide 0,2 % (ADJ) as control groups. After 10 days of first immunization a boost dose was applied as described. Posteriorly 7 days after the boost immunization 30 adult zebrafish distributed in three tanks of 20 L were challenge intraperitoneally with 10 µL of *Aeromonas hydrophila* ATCC 7966 strain at 2 McF concentration and the mortality evaluated during 7 days (Kole et al., 2019a, 2019b).

2.7 RELATIVE EXPRESSION OF IMMUNE-RELATED GENES (QRT-PCR)

The Rna was extracted from spleen, gill and intestine by TRI Reagent® (Sigma-Aldrich) method following manufacture instructions. The RNA samples were measured by NanoDrop™ spectrophotometer (Thermo Scientific™) and diluted to achieve a concentration of 500 µg, treated with Dnase kit (Promega) and submitted to cDNA reverse transcription kit (Applied Biosystems™) to achieve a cDNA concentration of 1µg. Posteriorly, 1 µl of cDNA samples was used to perform qRT – PCR assay using Go Taq® qPCR SYBR kit (Promega). The primers sequence for immune-related genes (IL10, IFN1, MHCII and IL6) were obtained after literature review (Kavaliauskis et al., 2016; Pacheco et al., 2021b). The 18 s from zebrafish was used as an internal control. The list with all primer sequences can be seen in table 1. A standard curve assay was submitted for all primers to evaluate the primers percentage of efficiency and to achieve the equation of line that was used to calculate the relative expression. The results were submitted to test-t and were considered statistically significant with p value <0,05 (Xiu et al., 2024).

Table 1:Immune-related gene prime sequences

Genes	Primer sequences	Reference
18 s	F: 5´ ACCACCCACAGAATCGAGAAA3´ R: 5´ GCCTGCGGCTTAATTTGACT3´	(Kavaliauskis et al., 2016)
IL10	F:5´CCCTATGGATGTACGTCATG3´ R:5´GCATTTACCATATCCCGC3´	
Ifn1	F: 5´ GAGCACATGAACTCGGTGAA3´ R: 5´TGCGTATCTTGCCACACATT3´	
MHCII	F: 5´ TGACTCAACTGTCCGTGATA3´	

	R: 5' CCATTAGCCATCTCCATAGTG3'	
IL6	F:5'TCAACTTCTCCAGCGTGATG-3'	(Pacheco et al., 2021b)
	R: 5'-TCTTTCCTCTTTTCCTCCTG-3'	

2.8 HISTOPATHOLOGIC EVALUATION

The liver, intestine and gill of six zebrafish were collected to histopathologic evaluation of aluminum hydroxide toxicity profile. The samples were fixed with 10 % of formaldehyde and dehydrated in a graded series of ethanol and xylene and posteriorly embedding in paraffin wax. The samples were cut into sections of 5 μ m thickness and stained with hematoxylin and eosin (H&E) for liver and intestine and toluidine blue for gills and posteriorly observed under light microscope. The results were submitted to test-t and were considered statistically significant with p value <0,05 (He et al., 2017).

2.9 STATISTICAL ANALYSIS

Statistical significance was performed using the GraphPad Prism (<https://www.graphpad.com/scientific-software/prism/>). Comparison between columns in different assays was conducted using analysis of variance (ANOVA) and between two groups was used test-t non-parametric. The was considered significant with p value <0,05.

3. RESULTS AND DISCUSSION

3.1 IN VIVO TOXIC PROFILE OF AEROMONAS HYDROPHILA

The *in vivo* toxicity and clinical signs of the strains *A. hydrophila* ATCC 7966 and IOC23116 were accessed by injection of 10 μ L of bacteria solution in different concentrations (Mcfarland 0.5, 1 and 2 scale) and evaluated during 7 days. The survival curve (Fig 1A) and clinical signs (Fig 1 B) showed high toxicity in McF concentration 2 for *A. hydrophila* ATCC 7966, with 40 % of survival and 60% of mortality registered in 1 day after challenge. The clinical signs revealed apparent tegument depigmentation, hemorrhage and necrosis. The McF concentration 1 also registered toxicity profile but only 20% of mortality and no apparent toxicity was accessed in McF concentration 0.5. The IOC23116 strain isolated from infected

fish didn't show toxic and clinical signs during 7 days, with no mortality registered in zebrafish. In addition, histopathological photographs of control group (PBS) from immunization assay before and after challenge with *A. hydrophila* ATCC 7966 McF 2 revealed characteristic pathologies of a tissue damaged by *A. hydrophila* infection, on liver, presenting steatosis (Fig 1 C, D and E) and vascular congestion (Fig 1 F,G and H), on intestine, presenting inflammatory infiltrate (Fig 1 I, J and K) and on gill, presenting destruction of lamellae, dilatation of the central venous sinus and aneurysm (Fig 1 L, M, N and O, P), which was not observed before challenge.

Toxicity profile of *A. hydrophila* ATCC 7966 in zebrafish

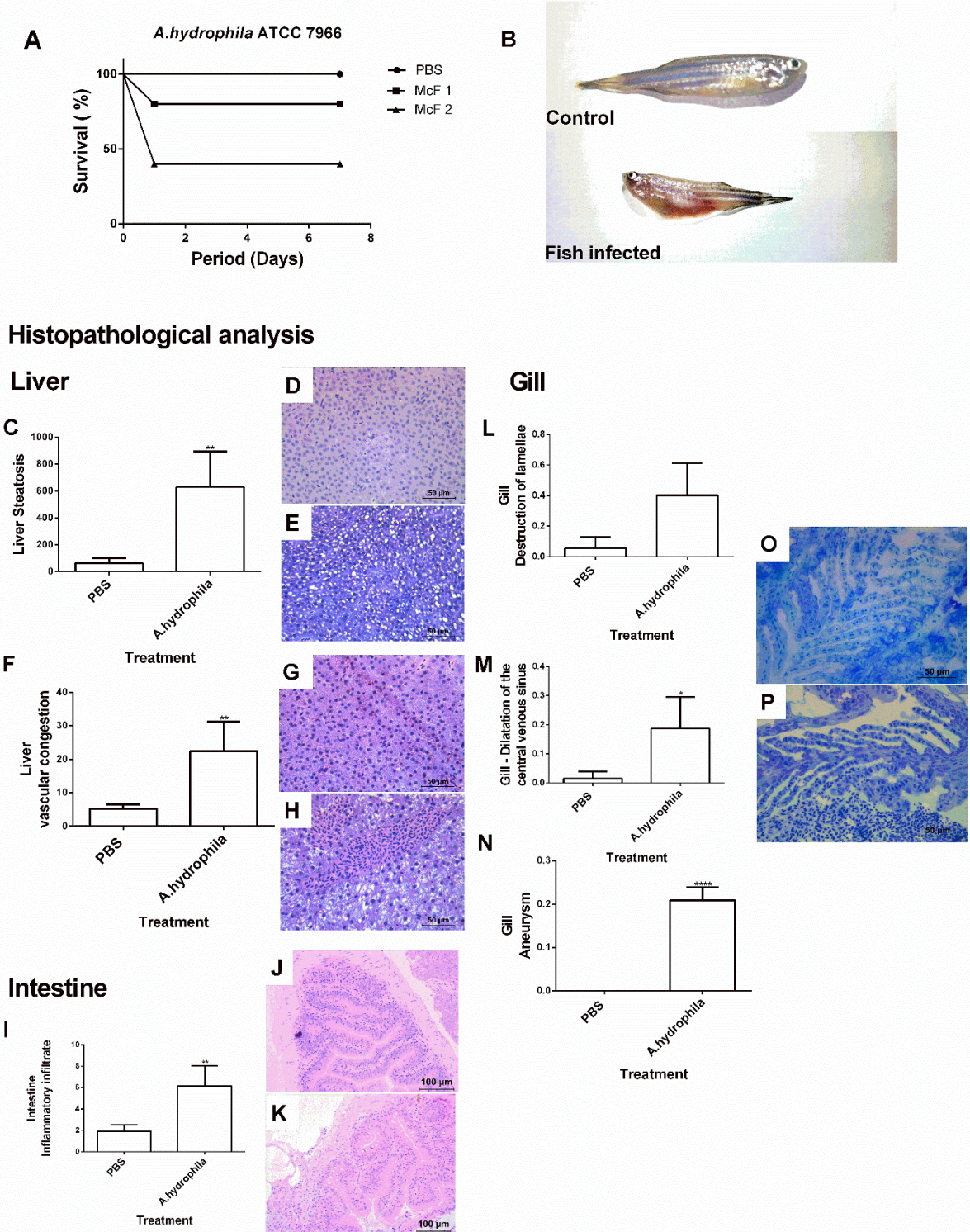


Figure 1: Toxicity of *A. hydrophila* in infected Zebrafish. (A) zebrafish survival curve after injection with 10 μL of saline (PBS) and McF concentrations 0.5, 1 and 2 *A. hydrophila* ATCC 7966 strain. (B) Clinical signs (hemorrhage in body and skin, skin discoloration and necrosis) of zebrafish infected and non-infected (control). Tissues of the control group (PBS) from immunization assay before and after challenge with *A. hydrophila* ATCC 7966 strain were submitted to histopathological evaluation and showed, liver steatosis damage: (C) significance, photo graphics before (D) and after challenge (E), liver vascular congestion: significance (F), photo graphics before

(G) and after challenge (H). Intestine inflammation: significance (I), photo graphics before (J) and after challenge (K). Gill destruction of lamellae, dilatation of the central venous sinus and aneurysm (L, M and N respectively), photo graphics before (O) and after challenge (P). The statistic analysis were made in GraphPrism 7.0 and the survival percent was considered significant with p value <0,05. The test-t unpaired was chosen to achieve the significance into two groups in the histopathological analysis and p value <0,05 was considered statistically significant and represented by asterisks (*).

The *Aeromonas hydrophila* fish toxicity profile was also accessed by (Dias et al., 2016) and the results were similar those founded in this work, the *A. hydrophila* toxicity was evaluated in *Arapaima gigas* fish and doses such as 10^9 CFU/mL and 10^{10} CFU/mL injected intraperitoneally were responsible for fish mortality in 24 hours, although, the intradermal injection resulted in more prominent aggressive profile with a concentration of 10^8 CFU/mL responsible for 91% of mortality. The apparent clinical signs like depigmentation in tegument, hemorrhages and internal organ necrosis were observed and the histopathological analysis emphasize a tissue damage caused by *A. hydrophila* infection. The liver, gills and intestine photo graphics after infection demonstrate the aggressive profile of *A. hydrophila* based on the following pathologies: steatosis and vascular congestion on liver, compromising of gill lamellae and sites of inflammation on intestine, suggesting the reason of high mortality rate. Histopathological results presented by (AlYahya et al., 2018) of liver, gills and intestine from infected Blue tilapia, *Oreochromis aureus*, with *A. hydrophila* support the findings of this work revealing hemocytes aggregation and cell necrosis in all organs.

3.2 IMMUNOINFORMATIC PREDICTION

The peptide epitopes were carefully selected to immunostimulate the Cells B and TCD4 response and avoid TCD8 because *Aeromonas hydrophila* and *Pseudomonas fluorescens* are not un intracell pathogen (Fig 2). The protein selected was based on bacteria cell wall biosynthesis enzyme which name is UDP-N-acetylmuramate-L-alanine ligase. The UDP-N-acetylmuramate-L-alanine ligase sequences related to *A. hydrophila* present in UNIPROT were approximately 10 and all of them showed 99% of similarity and the sequence identified as **A0A347A1E0** was selected to prediction and epitopes from 150 to 380 aa sequence that achieved a score above 0.5 were chosen to construct the chimeric protein antigen as can be seen in figure 2A. The UDP-N-acetylmuramate-L-alanine ligase sequences related to *P. fluorescens* present in UNIPROT were approximately 84 and a phylogenetic tree was constructed and 6 clusters were selected based on sequences similarity, the **A0A7Z6MYC5**, **A0A1B3CMK7**,

A0A5E7TXI2, A0A5E7KJZ6, A0A5E7TQB6 and A0A160A4J6 sequences. After the alignment of 6 sequences and epitopes prediction the epitopes from 49 until 210 aa sequence were chosen, as can be seen in figure 2B.

Immunoinformatic prediction of UDP-N-acetylmuramate-L-alanine ligase from:
Aeromonas hydrophila *Pseudomonas fluorescens*

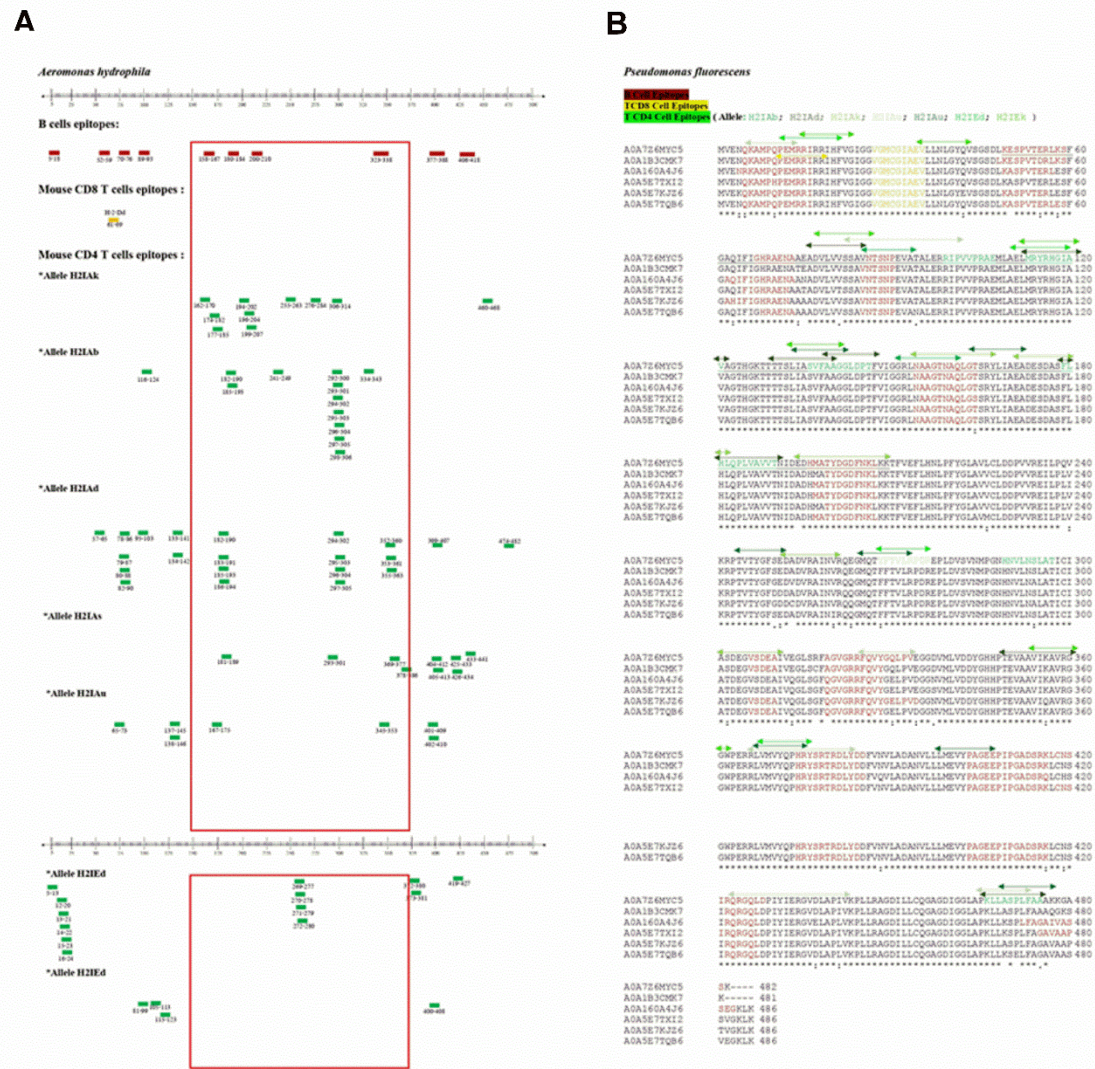


Figure 2: *Aeromonas hydrophila* and *Pseudomonas fluorescens* antigen epitopes prediction. The UDP-N-acetylmuramate-L-alanine ligase protein has 484 aminoacids and after Cells B (epitopes portion in red) and TCD4 (epitopes portion in green) and TCD8 (Epitopes portion in yellow) prediction the most abundant part from 150 to 380 amino acids sequence were selected avoiding TCD8 epitopes (epitopes portion presenting in red box) for A. hydrophila sequence **A0A347A1E0**. *Pseudomonas fluorescens* UDP-N-acetylmuramate-L-alanine ligase protein has 484 aminoacids and the most conserved site in all 6 sequences analyzed (**A0A7Z6MYC5**, **A0A1B3CMK7**, **A0A5E7TXI2**, **A0A5E7KJZ6**, **A0A5E7TQB6** and **A0A160A4J6**) was selected after Cells B (epitopes portion in red) and TCD4 (epitopes portion in green and alleles epitopes portion in arrows) prediction resulting in selection

of epitopes from 49 to 210 amino acids sequence avoiding TCD8 epitopes (epitopes portion in yellow).

The computational vaccinology has emerging as an important tool to produce effective vaccines with the capacity of to map epitopes, select and design antigens, overcoming the vaccine challenges. The immunoinformatic tools are currently been used in the cancer therapy in intention to produce vaccines that can induce only cancer cells epitopes avoiding the receptors from non-cancer cells. The magnification of these tools for animal vaccinology can improve methods already applied and support those that are emerging (De Groot et al., 2020). Vaccines based on recombinant protein are interesting for immunoinformatic application playing like a platform able to select multiple proteins related to disease or etiological agents and link the epitopes after cells B and T prediction, resulting in multiepitope vaccines desirable for aquatic vaccine field to control a wide range of pathogens (Unajak et al., 2022). Recently (Jungi et al., 2023) showed a strong protection role of a recombinant peptide after epitopes prediction from Nervous necrosis virus coat protein, demonstrating a immunogenic response in Asian seabass based on antibodies able to neutralize the Nervous necrosis virus.

The UDP-N-acetylmuramate-L-alanine ligase is a product of the gene *murC*, and catalyzes the ATP-dependent transfer of L-alanine to UDP-N-acetyl-muramic acid (UNAM), the precursor of the peptide chain to form UNAM pentapeptide, crucial for mature peptidoglycan production(Humnabadkar et al., 2014a). Antimicrobial drugs for inhibition of the mur family, *murA* to *murF*, are been explored (Ehmann et al., 2004; Gaur and Bera, 2022), however, this work had a propose to investigate the *murC* gene product, UDP-N-acetylmuramate-L-alanine ligase, antigenic profile after immunoinformatic analysis.

3.3 CONSTRUCTION AND EXPRESSION OF CHIMERIC RECOMBINANT PROTEIN RCAPA

The chimeric gene was successfully constructed and cloned in the PET28a plasmid vector (Fig 3A) which is an expression vector used in *E. coli* strains to recombinant expression. The vector containing the chimeric protein gene has the length of 6499 base pairs and was identified as rCAPA (recombinant chimeric *Aeromonas* and *Pseudomonas* antigen). The total length comprises the antigen gene combined with His-tag sequence (1209bp), *lacIq* promoter, Kanamycin selection marker and origin of replication.

C4, C5 and C6) and BL21 colony (line C7) and Artic express non-induced colonies (lines C8, C9 and C10), (M-molecular marker). (F) Analysis of chimeric protein expression, present most of it in insoluble fraction (line 2) compared to soluble fraction (line 1), (M-molecular marker). (G) Chimeric protein purification fractions (lines 1, 2, 3, 4 and 5) eluted by 20% of buffer containing imidazole and yield of 3,5 mg/mL.

The electrocompetent cells BL21 and Artic express were submitted to transformation and the PCR products (amplicon of 125bp) obtained using the specific primers present in table 2 confirmed the success of the process. All transformants colonies were tested for BL21 (Fig 3 B) and Artic express (Fig 3 C) and presented the PCR products as expected.

Table 2: Chimeric gene primer sequence

Chimeric antigen gene primer sequence	
Forward	GCAAGGACGCAGGCGAATTG
Reverse	TGCGCGAATGATAGCGTCGT

Posteriorly transformation the positive transformants cells were cultured in LB medium broth in Erlenmeyer's of 250 mL and the expression was induced with 0.5 μ mol of IPTG after cells achieve optical density of ≤ 0.6 . The expression was confirmed by SDS-PAGE (Fig 3 D) and Western blotting (Fig 3 E), as can be seen the SDS-PAGE of Artic Express showed an intensity band of 45 kda in all colonies induced (C1, C2, C3, C4, C5 and C6) when compared to non-induced colonies (C7 and C8) confirming the expression of the protein with the same molecular weight as expected for the recombinant antigen constructed. The Western blotting with specific antibodies that can recognize the His-tag presenting in the final portion of the recombinant antigen showed an intense marking in all colonies that were submitted to protein expression presenting an significant band of 45 kda. The Artic express colonies induced (C1, C2, C3, C4, C5, and C6) presented an stronger band than observed in BL21 cell induced (C7) and for this reason the Artic express (DE3) was selected as expression model for this antigen. This results also showed an expression of this antigen even in non-induced colonies (C8, C9 and C10), indicating a strong relation between the replication, transcription and translation cellular machinery with the rCAPA plasmid constructed.

Posteriorly antigen expression confirmation, samples were selected and analyzed in which fraction (soluble or insoluble) the protein was concentrated (Fig 3 F). The results showed that the most of it is present in insoluble fraction (Fig 3 F line 2) and easily purified in a

preparative His-tag column (Fig 3 G). After purification the protein concentration was accessed by Bradford assay showing a yield of 3,5 mg/mL considerate un desire feature for recombinant protein production with vaccinology interest.

Some desirable vaccine characteristics are important to construct an effective fish vaccine which include strong immune antigens with capacity to stimulate the fish immune system and to produce memory cells. In general, the safety for the host and for other organisms present in the ecosystem must be guarantee and peptide vaccines can overcome the safety challenge from attenuated and inactivated vaccines widely produced for aquaculture. The huge range spectrum of protection against multiple stirps conferring multiprotection and low cost production also are factors present in peptide vaccines (Bedekar et al., 2022). The vaccine manufactory method proposed in this work is ally with characteristics that need to be in a vaccine, the use of immunoinformatic tools and the use of bacterial expression model proved to be strategic and low-cost methods to produce a polyvalent fish vaccine with high yield and stable product. The vaccine cost of production impacts negatively or positively the manufacturing and low-cost methods are required to produce safe, effective and consistent long-life vaccines (Plotkin et al., 2017). Recombinant vaccines can be produced by some expression models such as: bacteria, yeast, insect cells, mammalian cells and plants and all these models have advantages and disadvantages and depends on the antigen proprieties and necessities. Recombinant peptides were established on market by decades working as drugs or vaccines components and with the molecular biology, bioinformatic and engineer advance new approaches such as peptide immune prediction, peptide nanoencapsulation and advanced bacterial expression model have been developed (de Pinho Favaro et al., 2022).

3.4 ZEBRAFISH PROTECTION ASSAY AND IMMUNE-RELATED GENES EXPRESSION

The protection assay (Fig 4 A) was accessed after two immunization doses with 1 microgram of the antigen combined or not with aluminum hydroxide adjuvant. The results showed that zebrafish vaccinated intraperitoneally with chimeric antigen (PTN) was able to protect fish against *Aeromonas hydrophila* ATCC 7966 infection when compared to zebrafish control group (PBS). As can be seen after a challenge with 10 μ L of *A. hydrophila* the majority of group control did not resist the infection in 24 hours resulting in survival percent of 40 % in

the end of 7 days while the group vaccinated with chimeric protein (PTN) was capable to resist the infection resulting in a significant survival percent of 90%. Interestingly, the groups with aluminum hydroxide (ADJ or ADJ+PTN) presented high toxicity profile during the immunization and a strong mortality was accessed and discussed in the next sections.

Protection assay

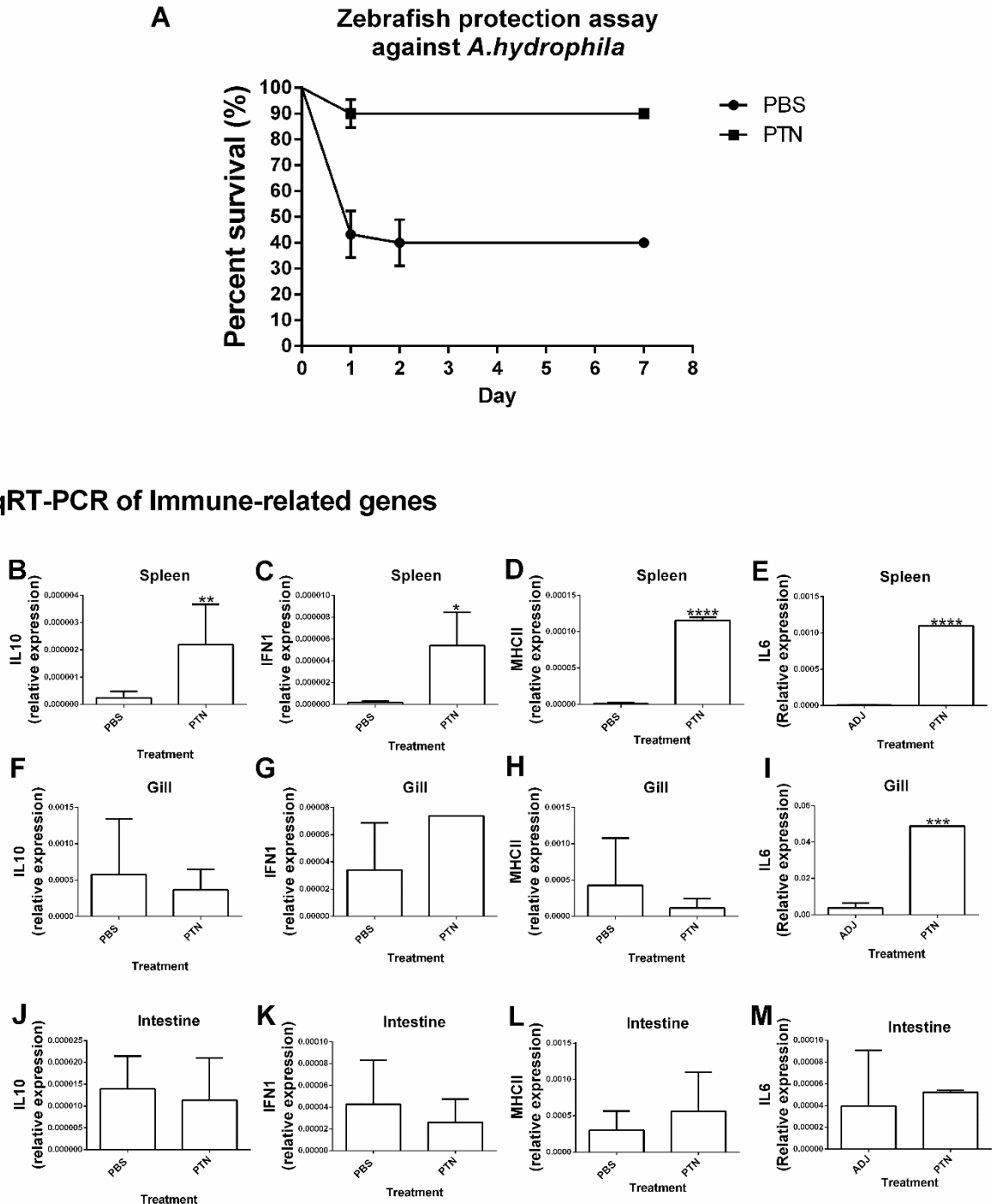


Figure 4: Zebrafish percent survival against *Aeromonas hydrophila* challenge. (A) The adult zebrafish (N: 30 in

each group) were vaccinated with chimeric antigen (PTN), or with saline solution (PBS) two times and after the immunization period were challenge with 10 μ L of *Aeromonas hydrophila* ATCC 7966 strain. Zebrafish vaccinated with PTN presented a percent survival of 90% and the most of group control (PBS) did not resist the infection presenting just 40% of percent survival. The statistical analysis was made in GraphPrism 7.0 and the survival percent was considered significant with p value $<0,05$. The Lon-rank test was chosen to evaluate the significance and the p value was **** ($<0,0001$). The immune-related genes (IL10, IFN1, MHCII and IL6) of zebrafish were accessed after immunization period on spleen (B, C, D and E), Gills (F, G, H and I) and Intestine (J, K, L and M). The test-t unpaired was performed and p value $<0,5$ consider statistically significative represented by (*).

The search for protein targets used as antigen against bacterial infection is based on characteristics virulence factors founded in pathogenic bacteria strains. Common targets such as outer membrane proteins (OMPs), lipopolysaccharides (LPS), aerolysin, glycoprotein and S-layer protein are strategies used currently for recombinant antigen vaccine against bacterial infection (Mzula et al., 2019), however, other strategies must be explored and the main goal of this work was to evaluate the role of cell wall biosynthesis genes products in to confer protection against *A. hydrophila* infection. The murC gene was chosen among others mur family, although could be interesting to explore all due to their important role in catalyze the first steps of peptidoglycan biosynthesis. The focus of antimicrobial studies against mur genes family is based on their properties to be present only in bacteria as well as strongly expressed on pathogenic stirps, indispensable characteristics to recombinant vaccines development (Humnabadkar et al., 2014b; Mol et al., 2003). The cytoplasmatic proteins immunogenic profile have been investigated, (Kaur et al., 2021) showed by reverse vaccinology the strong immunogenic role of Alanine-tRNA ligase, also involved in peptidoglycan biosynthesis, from fish pathogenic *A. hydrophila* strain as well as (S. et al., n.d.) showing after in silico studies glycan biosynthesis enzymes non-host homology, Alanine-tRNA ligase and UDP-N-acetylmuramate-L-alanine ligase, as essential for *Flavobacterium columnare* virulence in zebrafish suggesting a antimicrobial and vaccine target role.

The zebrafish immune-related genes expression was accessed after the immunization period to understand the mechanisms involved on chimeric recombinant antigen protection (Fig 4 B to M), however, the immunological system response for teleost fish remains unclear in many aspects despite been related to mammalian. Zebrafish is considering the animal model to comprehend the fish immune-related genes and response mechanisms and is already know that thymus, kidney, spleen, intestine, gills and liver are consider secondary lymphoid organs for fish because different from mammals there's no lymph nodes and germinal centers in the spleen of zebrafish(Lieschke and Trede, 2009; Somamoto and Nakanishi, 2022). The relative

expression of IL10, IFN1, MHCII and IL6 was accessed from Spleen, gills and intestine of adult zebrafish vaccinated with two doses of 1 µg of chimeric recombinant antigen and compared with the control group the received saline solution (PBS). As can be seen, a targeted response was observed in the spleen (Fig 4 B, C, D and E) with all immune factors significantly expressed on the PTN group suggesting the importance of this organ in protect zebrafish mortality after *A. hydrophila* challenge. Regarding the immune profile of gills and intestine organs, a statistically significant response was observed in the gills only for IL6, however, IFN1 can be involved in regulate this response (Fig 4 I and G respectively) as well as MHCII and IL6 slightly up-regulated in the intestine of vaccinated group with chimeric protein then group control (PBS) (Fig 4 L to M respectively).

The targeted response on spleen showed clearly results suggesting an strong immune response of the chimeric antigen, the IL10 and IFN1, cytokines involved on anti-inflammatory response, mainly IL10 associated with adaptative fish response and IFN1 with a regulatory cytokine, as well as IL6 with pro and anti-inflammatory role could be involved on the up regulation of MHCII response, immune stimulating the macrophages and cells T, similar mammalians, known as antigen presenting cells in fish(Sakai et al., 2021; Zou and Secombes, 2016). The spleen is the second crucial lymphoid organ where adaptative response can generate and because the lack of lymph nodes in fish the filtration of blood seems more important then mammalian. The presence of a fish splenic white pulp, formed for basal lamina, reticular cells and macrophages, suggest an effective filtration and antigen recognition supported by studies showing a strong presence of Mhc-II class positive cells, majority the macrophages and T cells, distributed in this compartment(Buchmann and Secombes, 2022). Similar the results found in this work, (Guo et al., 2018), showed a protective efficacy as well as MHCII and IL10 Up-regulation of four iron related recombinant proteins against *A. hydrophila* infection in zebrafish.

The protection and immune-related gene regulation of the chimeric recombinant antigen were carefully discussed, however, the adjuvant role of aluminum hydroxide was a crucial point of this work, our hypothesis was that the protein combined with the aluminum hydroxide could amplify the directed immune response and efficiently protect fish against *A. hydrophila* infection, although, this protection was associated with high toxicity profile responsible for zebrafish mortality.

3.5 ALUMINUM HYDROXIDE ADJUVANT AND TOXICITY PROFILE IN ZEBRAFISH

Aluminum hydroxide is currently used as adjuvant to human's vaccine and has a great role in potentialize the immunological vaccine response. The toxic effect wasn't showed in humans, therefore, this work aimed to use the aluminum hydroxide to improve the recombinant fish vaccine response in zebrafish animal model. Curiously after zebrafish immunization with aluminum adjuvant mixed with PBS or with recombinant protein the zebrafish mortality was observed but no mortality was registered in the group vaccinated only with chimeric recombinant antigen. In the end of the immunization period (21 days) the mortality percentual was accessed (table 2). The zebrafish immunized only with aluminum hydroxide presented 22,3% of mortality. This result showed an aluminum toxic role in zebrafish animal model and the histopathological profile and immune-related genes were investigated as can be seen in figure 5.

Table 2: Zebrafish mortality after aluminum hydroxide injection

Groups		N° zebrafish before injection	N° zebrafish after injection	% Mortality	
Zebrafish injected with PBS	1	30	30		
	Tank	2	30	30	0 %
	3	30	30		
Zebrafish injected with aluminum hydroxide	1	30	25		
	Tank	2	30	25	22,3 %
	3	30	20		

*Zebrafish injected only with Recombinant protein did not show mortality

*Zebrafish injected recombinant chimeric protein combined with aluminum hydroxide also showed a mortality percent of 22%

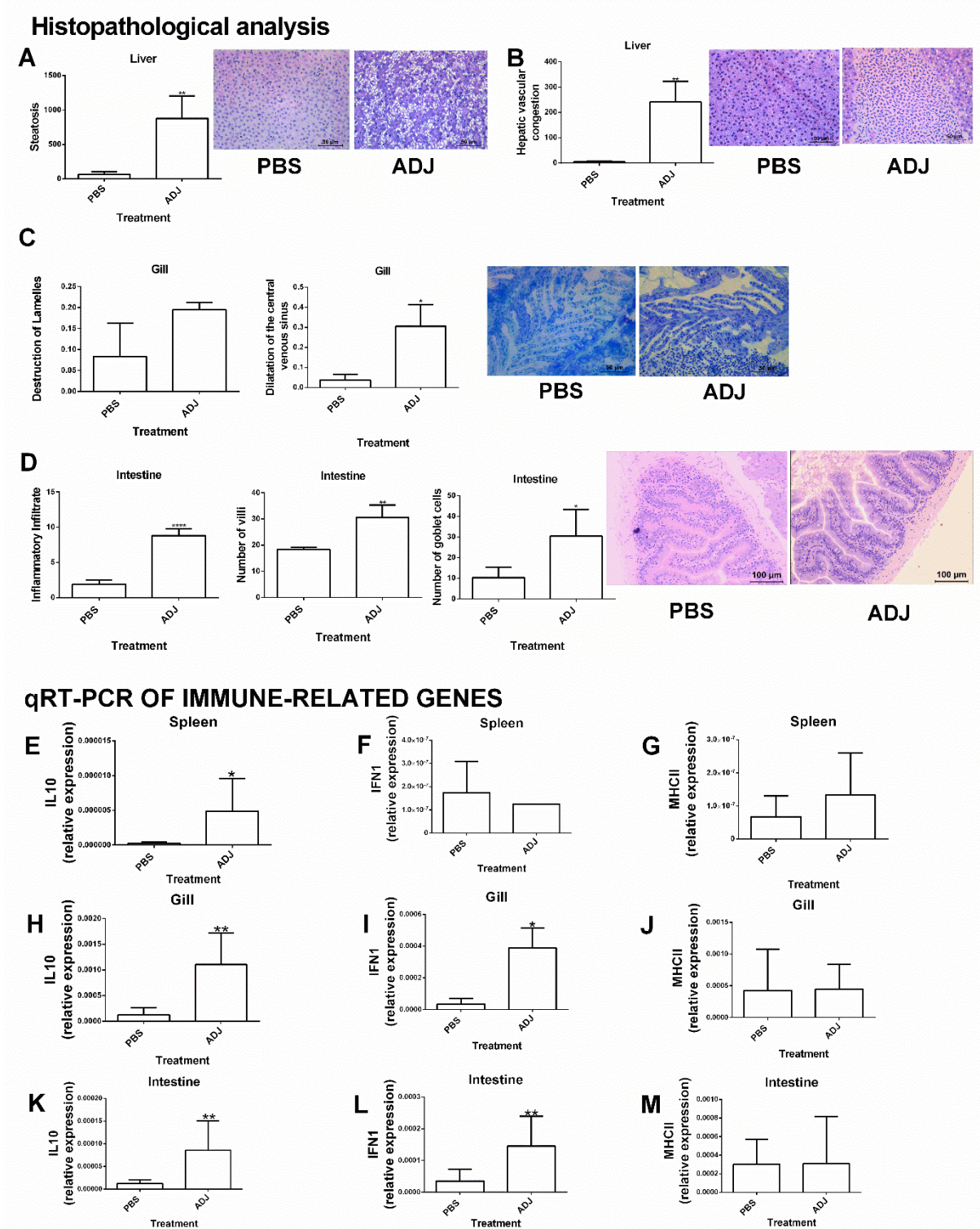


Figure 5: Histopathological and immune-related genes analysis of zebrafish ADJ group. (A to D), the histopathological and statistical analysis of zebrafish organs, liver with steatosis (A) and vascular congestion (B), gills with destruction of lamellae and dilatation of the central venous sinus (C) and intestine with inflammatory infiltrate and increased number of villi and number of goblet cells (D), after aluminum hydroxide (ADJ) intraperitoneally administration compared to saline buffer control group (PBS). (E to M), relative immune-related genes expression of IL10, IFN1 and MHCII after aluminum hydroxide (ADJ) intraperitoneally administration on spleen (E to G), on gills (H to J) and on intestine (K to M). The test-t unpaired was performed and p value <0,5

consider statistically significant evidenced by (*).

The histopathological analysis of liver (Fig 5 A and B), gills (Fig 5 C) and intestine (Figure 5 D) of zebrafish aluminum hydroxide group control was compared to PBS group control to understand how aluminum hydroxide compromise the fish homeostasis and tissues. Pathologies focus such as, steatosis and hepatic vascular congestion on liver, destruction of lamellas and dilation of central venous sinus on gills and Inflammatory infiltrate, number of villi and number of goblet cells on intestine were statistically increase on ADJ group then PBS group. Recombinant vaccines are recognized as strategies that need immunological enhancement and even been consider safe and antigen-specific response the lack of immune response stimulation the combination of adjuvant compounds is required (de Pinho Favaro et al., 2022; Nascimento and Leite, 2012). After the immunoinformatic emergence some recombinant vaccines are being designed with bioinformatic strategies such as the chimeric fish vaccine evaluated in this work, in intention to overcome the recombinant vaccines challenges, although, the use of some adjuvants in aquatic species still remain encouraged but their toxic effects need be carefully evaluated. Adjuvants are substances applied with vaccines to improve the immune response stimulating gradually the immune factors and cells (Petrovsky, 2015). Common adjuvants used in aquaculture are oils emulsions and salt based like aluminum derivates. The aluminum adjuvants are extensively used in human vaccines because they can induce the expression of some histocompatibility complex genes on surface of antigen presenting cells and enhance the adaptative response (Du et al., 2022d), Despite aluminum adjuvants are consider safe to use in human vaccines and some veterinary vaccines. The toxic profile accessed on this work alert for a better evaluation.

In addition the histopathological analysis, relative expression of immune-related genes was accessed on spleen (fig 5, E to G), gills (fig 5, H to J) and intestine (fig 5, k to M) and suggest a not controlled inflammation in all organs on the up-regulation of anti-inflammatory cytokine and factors, IL10 and IFN1, and no significant up-regulation of genes related to macrophages and cells T immune stimulation like MHCII. The high mortality rate is explained by the tissues damage and acute inflammation process observed only in the groups that received the aluminum hydroxide formulation. Supporting the results presented in this work (Galindo-Villegas et al., 2019) showed a immunotransitory toxicity of aluminum hydroxide in gilthead seabream immunized intraperitoneally with aluminum adjuvant with compromising of liver and spleen and also with u-regulation of IL10 and other anti and pro inflammatory cytokines, although the adjuvant role was successfully observed and fish immune response increased.

(Vinay et al., 2014) also showed a toxicity effect of aluminum hydroxide adjuvant in olive flounder fish, although, this toxic effect was observed only in concentrations above 1600 µg, however, the adjuvant and toxic role of aluminum hydroxide in leads to infiltration must be severally evaluated.

The zebrafish protection against *A. hydrophila* infection was also investigated and a similar survival percent observed on recombinant chimeric antigen group (PTN) of 90% was achieved for ADJ and ADJ+PTN groups and after the histopathological and immune-related genes analysis, the aluminum hydroxide adjuvant role was observed but with uncontrolled inflammation process resulting in high mortality rate when administered intraperitoneally.

4. CONCLUSION

Immunoinformatic emerged as a crucial tool for antigen enhancement and had been applied in humans and veterinary subunit vaccines. After immunoinformatic prediction of a murC gene enzyme that belongs a crucial gene family involved on cell wall biosynthesis, a subunit vaccine was constructed and had great immunogenic profile on fish against pathogenic bacteria. The aquaculture industry suffer with significant losses on fish production due to *A. hydrophila* infection. The results showed a great performance from chimeric recombinant antigen developed in this work in to protect zebrafish after a challenge with pathogenic strain of *A. hydrophila* and achieved 90 % of fish survival. The immunogenic response was splenic targeted with up-regulation of IL10, IFN1, MHCII and IL6 immunological factors and no related negative acute inflammatory process observed. In addition, the adjuvant and toxicity profile of aluminum hydroxide was accessed in adult zebrafish and surprisingly showed high toxicity and acute inflammatory process resulting in zebrafish mortality and liver, gills and intestine damage after intraperitoneal administration, however, more studies about the administration and molecular mechanism of aluminum hydroxide for fish need to be carefully explored.

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CHAPTER 2**RECOMBINANT CHIMERIC ANTIGEN NANOENCAPSULATION WITH
NANOSTRUCTURED LIPID CARRIER, PHYSIOCHEMICAL PARAMETERS,
RELEASE AND STABILITY EVALUATION****PAPER 2****FORMULATION DEVELOPMENT OF NANOSTRUCTURED LIPID CARRIERS FOR
ORAL DELIVERY OF RECOMBINANT PROTEIN-BASED VACCINE ANTIGENS IN
AQUATIC SPECIES**

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ABSTRACT

Recombinant proteins have emerged as a safe and effective method in the field of veterinary vaccines. However, the use of oral and stable recombinant antigen vaccines for aquatic species remains underexplored. To investigate the potential use of nanostructured lipid carriers (NLCs) as a strategy to protect recombinant proteins from gastrointestinal degradation and damage, and to provide good protection for fish against *Aeromonas hydrophila*, we employed high pressure homogenisation and ultrasonication methods for nanoencapsulation using glycerol distearate, propylene glycol dicaprylate as solid/liquid lipids, and polysorbate 80 as the surfactant, in varying ratios. The physicochemical properties of resulting nanostructured lipid carriers (size, homogeneity, charge, miscibility, shape) were assessed using dynamic light scattering (DLS), zeta potential, differential scanning calorimetry (DSC) and transmission electronic microscopy (TEM). The antigen loading efficiency and release in a gastric buffer mimicking fish conditions was measured by micro BCA assay. NLC colloidal stability was assessed at pH values encountered in the fish gastrointestinal tract and storage conditions evaluated at 4 and 42 °C. Our results showed that while ultrasonication time influenced NLC size and dispersity, neither high pressure homogenisation nor ultrasonication impacted protein integrity. Formulation composition (solid and liquid lipid ratio and surfactant concentration) was crucial to the resulting nanoparticle properties and influenced protein loading capacity with an increase in solid lipid content leading to the highest antigen incorporation ($58 \pm 10.9\%$). All formulations with 90:10, 80:20 and 70:30 ratios of glycerol distearate to propylene glycol dicaprylate and 5% polysorbate 80 resulted in NLCs with low dispersity, and sizes below 200 nm. However, *in vitro* release and stability improved with high higher solid lipid, suggesting that the balanced solid and liquid oil composition of the 80:20 is a promising formulation for vaccine delivery in aquatic environment.

1. INTRODUCTION

Global aquaculture has experienced unprecedented growth in recent decades, with Brazil emerging as a significant producer (Lucas, 2015b; Valenti et al., 2021). As the second-largest aquaculture market in Latin America, Brazil generates approximately one billion dollars in annual revenue, with fish production representing over two-thirds of the total output (Fiorella et al., 2021; Valenti et al., 2021). Despite this remarkable growth and recognition by the Food and Agriculture Organization of the United Nations (FAO) as an increasingly competitive international market, the sector continually faces significant economic loss due to persistent disease outbreaks (Irshath et al., 2023; Mondal and Thomas, 2022). Bacterial pathogens such as species of the genus *Aeromonas*, *Edwardsiella*, *Vibrio*, and *Pseudomonas* pose particular challenges to the industry. For example, *Aeromonas hydrophila* has been linked to substantial mortality events in farmed tilapia, with reported losses between 30-50% in Brazilian aquaculture farms (Tavares-Dias and Martins, 2017). Moreover, *A. hydrophila* represents a significant public health concern as a zoonotic pathogen, capable of causing severe gastric disease in humans and persisting in food processing and storage environments (Semwal et al., 2023; Vaz Rodrigues et al., 2019).

While disease treatment has conventionally relied on antibiotics and chemotherapeutics, these can lead to drug resistance and pose safety concerns (Haenen et al., 2023; Jensen et al., 2004). Fish immunization presents a more effective and sustainable solution for preventing bacterial and viral diseases (Adams, 2019). However, vaccinating fish populations is uniquely challenging compared to terrestrial livestock. The aquatic environment, with its variable water temperature, pH, and salinity, can degrade vaccine components and reduce their immunogenicity. Additionally, the gill and mucosal surfaces of fish act as physical barriers to effective antigen uptake (Gomez et al., 2013). Traditional injection-based vaccination methods are labour-intensive and stressful for the fish, limiting their scalability and practicality for large-scale aquaculture (Tamma et al., 2024). Although oral vaccination offers a promising alternative for non-invasive, mass administration, the harsh conditions of the fish gastrointestinal tract can degrade sensitive protein or peptide-based antigens before they can elicit an immune response (Radhakrishnan et al., 2023).

Nanostructured lipid carriers (NLCs) are an attractive delivery system for sensitive pharmaceutical and biological cargoes, including vaccine antigens (Mahor et al., 2023). NLCs can protect labile molecules from degradation, facilitate cellular uptake, and enable controlled release profiles. Additionally, these carriers can exhibit adjuvant properties, stimulating both

innate and humoral immune responses – key for subunit or peptide antigen vaccines. While recent studies have demonstrated successful NLC-based delivery of viral RNA and peptide vaccines *via* parenteral routes (Bayon et al., 2018; Courant et al., 2017), the potential for oral immunization, particularly in aquaculture applications, remains largely unexplored.

Building on previous work demonstrating the efficacy of an in-house produced chimeric antigen against *A. hydrophila* in zebrafish (manuscript under preparation), this study aims to optimise NLC formulations for the oral delivery of this recombinant protein-antigen for fish immunisation. Herein, we systematically investigated the influence of processing parameters and formulation composition on NLC physicochemical properties, including particle size, homogeneity, and surface charge. Optimised parameters and composition were used to encapsulate in-house produced chimeric protein-antigen and *in vitro* stability and release properties assessed under simulated gastrointestinal conditions. We further evaluated the storage stability of prepared NLCs to understand shelf-life capability for practical applications. This research represents a crucial step towards developing an effective oral delivery platform for fish immunisation, with potential implications for improving vaccine administration in aquaculture settings and contributing to enhanced animal health, food safety, and sustainable food production.

2. MATERIALS AND METHODS

2.1 MATERIALS

Glycerol distearate (Precirol ATO 5) and propylene glycol dicaprylate (Labrafac PG) were kindly donated from Gattefossé (Lyon, France). Polysorbate 80 (Tween 80) was purchased from Sigma-Aldrich. The recombinant protein used in this work was obtained by heterologous expression from Artic Express (DE3) strain (ATCC). Micro BCA[®] Protein Assay Kit was purchased from Thermo Fisher. Hydrophilic PTFE syringe filters (13 mm, 0.45 µm) were purchased from AllPure. NuPAGE[™] 4-12 %, Bis-Tris, protein gel was purchased from Invitrogen. Amicon[®] Ultra 100 kDa was purchased from Merck Millipore Ltd.

2.2 DESIGN AND RECOMBINANT PROTEIN EXPRESSION

The chimeric antigen sequence was designed using immunoinformatic tools and synthesized in a PET28a plasmid vector. The construct was used to transform *Escherichia coli*

BL21 and Artic Express (DE3) cells via heat shock. Transformants were selected on Luria-Bertani (LB) agar plates containing kanamycin (50 µg/mL) and gentamycin (20 µg/mL) where appropriate. Successful transformation was confirmed by polymerase chain reaction (PCR) using specific primers. Transformed cells were cultured in LB broth until reaching an optical density of 0.6. Protein expression was induced with 0.5 µmol/mL IPTG, with cultures incubated under agitation at either 37 °C or 12 °C. Following induction, cells were harvested by centrifugation and lysed by sonication. The insoluble fraction was collected and purified using a His-trap column. The insoluble fraction was analysed by SDS-PAGE and Western blotting. For SDS-PAGE, the samples were denatured at 95 °C for 10 minutes in the presence 2-mercaptoethanol and separated by gel electrophoresis. Proteins were visualized by Coomassie blue staining. For Western blot analysis, proteins were transferred to a nitrocellulose membrane, which was blocked with Molico for 1 hour. After washing three times with PBS- Tween buffer, the membrane was incubated with anti-His primary antibody, followed by peroxidase anti-IgG secondary antibody. Protein bands were visualized using 3,3-diaminobenzidine tetrahydrochloride (DAD) and hydrogen peroxide under dark conditions(Pereira et al., 2020).

2.3 PREPARATION OF NANOSTRUCTURED LIPID CARRIERS

NLCs were prepared by hot high-pressure homogenisation (HPH) technique followed by ultrasonication as described by Dumont and co-workers(Dumont et al., 2019). Glycerol distearate and propylene glycol dicaprylate were selected as the solid and liquid lipids, with polysorbate 80 as the surfactant. Their composition is given in Table 1.

Table 1 Composition of nanostructured lipid carriers (NLC) formulations showing the relative proportions of solid lipid (glycerol distearate), liquid lipid (propylene glycol dicaprylate), and surfactant (polysorbate) used in each preparation.

NLC formulation	Lipid ratio	Glycerol distearate (mg)	Propylene glycol dicaprylate (mL)	Polysorbate 80 (mg)	Polysorbate 80 (% w/v)
NLC ₁		193		200	10
NLC ₂	90:10		23	100	5
NLC ₃				40	2
NLC ₄				200	10
NLC ₅	80:20	171	46	100	5
NLC ₆				40	2
NLC ₇				200	10
NLC ₈	70:30	150	69	100	5
NLC ₉				40	2

Empty nanoparticles were prepared by melting glycerol distearate at 70 °C. Propylene glycol dicaprylate was added into the melted lipid phase and let to stir at 400 rpm for 10 minutes. Separately, the aqueous phase containing polysorbate 80 was heated to the same temperature. Posteriorly, 2 mL of the aqueous phase were dispersed in the melted lipid by homogenisation (6500-24000 rpm) using a Fisher PowerGen 500 homogeniser. The pre-emulsion was then sonicated for 3, 5 or 10 minutes (Qsonica ultrasonicator) after which the obtained dispersions were cooled to 4 °C, mixed at 400 rpm for 10 minutes. For protein-loaded NLCs, 100 mg of the recombinant protein antigen was added to 1.5 mL of the aqueous phase. Resulting nanoparticles were stored at 4 °C until further analysis.

2.4 CHARACTERISATION OF NANOSTRUCTURED LIPID CARRIERS (NLCS)

2.4.1 PARTICLE SIZE, SIZE DISTRIBUTION AND SURFACE CHARGE

Nanoparticle size, dispersity and surface charge were measured at 25 °C by dynamic light scattering (DLS) using Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK). Samples were appropriately diluted (1:150) with ultra-pure water and filtered using 0.45 µm membrane filter. Zeta potential (ZP) of nanoparticles were measured by laser Doppler micro-electrophoresis using a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK). All analyses were carried out on three samples for each formulation, and the mean value was calculated for each measured parameter.

NLC size was further confirmed by Transmission Electron Microscopy (TEM, Joel JEM -1400 Plus Transmission Electron Microscope). Formulations were diluted 50-fold to better analyse separate particles. Diluted formulations (5 µL) were placed on a carbon grid (carbon Type-B, 400 mesh, 42 mm copper grid), over 5 minutes and dried (Izham et al., 2021). A solution of 2 w/v % of uranyl was placed on carbon grid over 3 minutes then dried by 30 minutes until further analysis using an acceleration voltage between 80-120 kV.

2.4.3 EVALUATION OF PROTEIN ENCAPSULATION EFFICIENCY

The amount of recombinant protein loaded in nanostructured lipid carriers was determined using the indirect method. Non-encapsulated protein was separated from the nanoparticles by ultrafiltration (Amicon® Ultra 100 kDa). Briefly, 1 mL of the sample was kept in the upper chamber compartment of the ultracentrifuge tube and centrifugated at 13 000 rpm

for 40 minutes. The collected sample in the lower chamber was analysed by micro BCA assay, and the encapsulation efficiency (EE), calculated by equation 1.

Equation 1

$$\text{Encapsulation efficiency, \%} = \frac{(\text{Protein amount total} - \text{Protein amount Non encapsulated})}{(\text{Protein amount total})} \div$$

2.4.3 LIPID MISCIBILITY STUDIES USING DIFFERENTIAL SCANNING CALORIMETRY (DSC)

The thermal state and miscibility of the glycerol distearate and propylene glycol dicaprylate were assessed by differential scanning calorimetry (TA[®] DSC Q20), as previously described (Zhang et al., 2013). Briefly, 5 mg of a lyophilized mixture of the solid and liquid lipid in the ratios 90:10, 80:20 or 70:30 was placed on pre-weighed aluminium pans. Samples were heated from 10 - 100 °C at a 5 °C. min⁻¹ ramp rate. Glycerol distearate was used as control.

2.5 IN VITRO EVALUATION OF NANOSTRUCTURED LIPID CARRIERS

2.5.2 COLLOIDAL STABILITY

To investigate the colloidal stability of NLCs under conditions simulating gastric conditions, 100 µL of NLCs were diluted with 900 µL of 0.1 M phosphate buffer, pH 3.0, to represent the average stomach pH found in fish (Solovyev et al., 2015). The mixtures were incubated in a water bath at 37 °C. At 0, 10, 30 and 60 min, the average particle size and dispersity was determined using Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK) as described in section 2.4.1.

2.5.3 PROTEIN RELEASE STUDIES

Release studies for recombinant protein-loaded NLC formulations (90:10, 80:20, and 70:30 solid: liquid lipid ratios) was adapted from a previously described methodology (Oldham et al., 2023). Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF) were prepared to mimic fish gastrointestinal conditions (Siri et al., 2021). The base buffer solution for both SGF and SIF contained: NaCl (8819.6 mg/L), KCl (224.4 mg/L), MgSO₄ (108.8 mg/L), CaCl₂ (148.2 mg/L), galactose (912 mg/L) and sodium pyruvate (558.6 mg/L). For SGF, the pH was adjusted to pH 2.0 using 0.1 M HCl. For SIF, NaHCO₃ (0.84 mg/L) and 20 mL/mL HEPES 1

M solution were added, and the pH adjusted to 7.0. Equal volumes of NLC formulations in either SGF or SIF were incubated in glass vials at 37 °C with continuous agitation (200 rpm) for 18 hours. Samples were collected at 6 and 18 hours, to quantify released protein by micro BCA assay and assess NLC physicochemical properties.

2.5.3 STORAGE STABILITY STUDIES

Following production, NLCs were stored in closed 1.5 mL Eppendorf tubes at 4 and 42 °C for 1 month, after which NLCs were assessed for mean particle size, dispersity and zeta potential (Zetasizer Nano ZS, Malvern Instruments Ltd., Malvern, UK) and compared to freshly prepared NLCs. Three replicate analyses were done for each sample. Possible crystallization, precipitation or gelling were also evaluated by visual inspection.

2.6 STATISTICAL ANALYSIS

The results of all the above-described studies were statistically analysed by one-way analysis of variance (ANOVA) followed by the Tukey multiple comparison post-test using GraphPad Prism version 10.2.2. for Mac, GraphPad Software (Boston, Massachusetts, USA, www.graphpad.com). Differences were considered statistically significant when $p < 0.05$.

3. RESULTS AND DISCUSSION

The prevalence of pathogens in aquaculture, combined with ineffective oral vaccines, poses significant challenges to fish health and risks to human health. Although the number of oral vaccines for fish is increasing, their effectiveness is often compromised by antigen degradation, the tolerogenic gut environment, and suboptimal formulations. Here, we focused on the formulation development of nanostructured lipid carriers to provide effective and sustained oral delivery of a recombinant protein antigen against *Aeromonas hydrophila*, a major fish pathogen causing significant morbidity and mortality in aquaculture. The protein antigen was obtained through bioinformatic prediction and heterologous expression in the *E. coli* Arctic Express (D3) strain (Figure S1 in supporting information). Its immunogenic properties were evaluated in a zebrafish model, showing promising results (manuscript under preparation).

3.1 INFLUENCE OF PROCESSING PARAMETERS ON NLC PROPERTIES

Nanostructured lipid carriers (NLCs) were prepared using hot high-pressure homogenisation followed by ultrasonication. This approach is cost-effective, highly efficient, and does not require harmful organic solvents. However, the processing parameters, namely homogenisation power and ultrasonication time, can impact particle size and dispersity, which ultimately determine NLC stability, appearance, texture, and bioavailability. Therefore, it was important to evaluate the influence of these parameters on the nanoparticle properties to optimise NLC production. Particle size is a key property in the physical stability and gastrointestinal uptake of nanocarriers (Murugan et al., 2015). As such, part of the preliminary studies focused on the preparation of monodisperse NLCs, with dispersity values within 0.3, and particle sizes below 200 nm. Glyceryl distearate and propylene glycol dicaprylate, in a 93:7 ratio, were used as the solid and liquid lipid, respectively with 4% of polysorbate 80 as the surfactant (Dumont et al., 2019). Particle size, dispersity, and surface charge were evaluated as a function of homogeniser power speed range (6500-24000 rpm) and ultrasonication time (3, 5, or 10 minutes) (Figure 1).

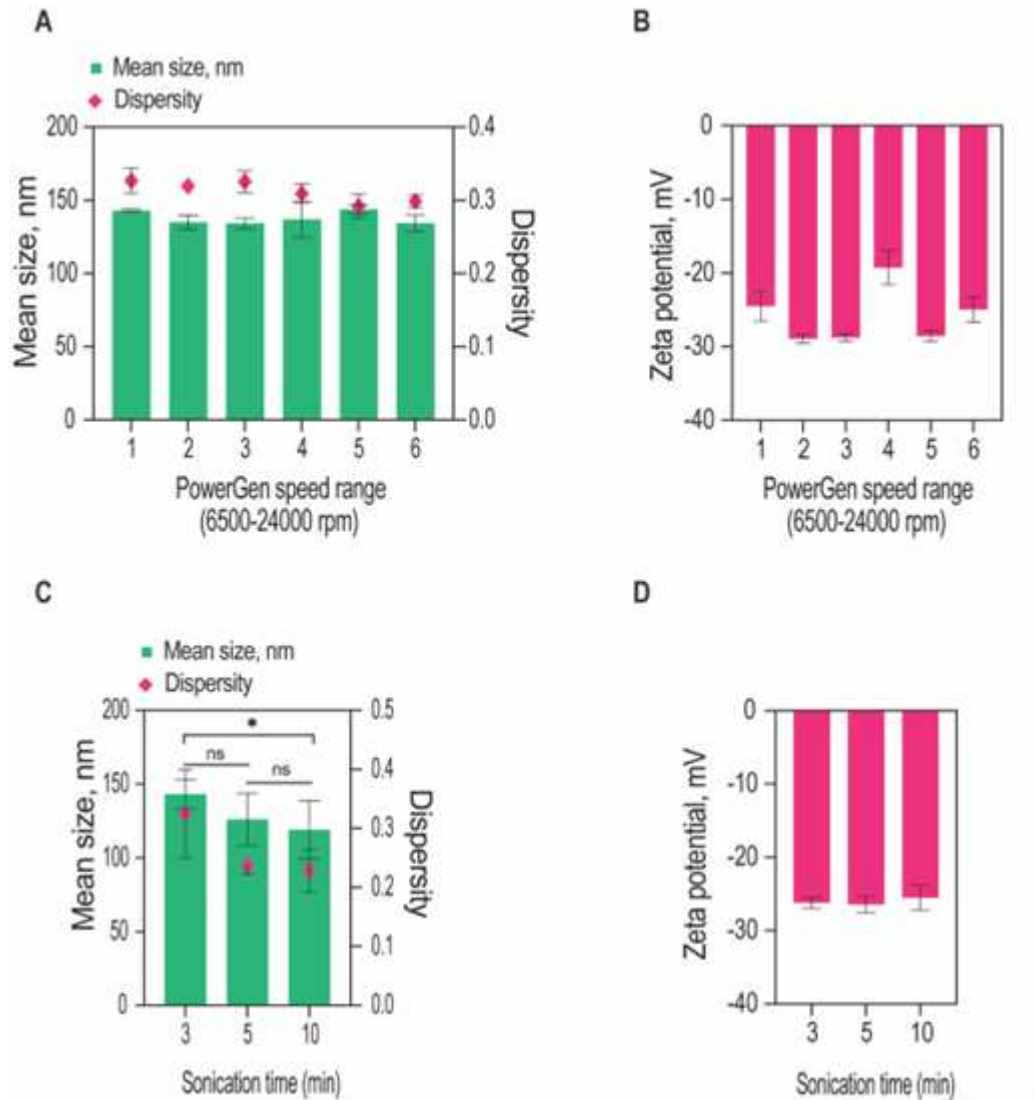


Figure 1 Influence of processing parameters on NLC properties. Plots represent the effect of high-pressure homogenizer speed (6500-24000 rpm) on **A** particle size, dispersity and **B** surface charge as determined by zeta potential. The effect of ultrasonication time (3, 5 and 10 minutes) on **C** particle size, dispersity and **D** surface charge was also determined. Experiments were conducted as independent triplicates using NLC formulation composed of 93:7 with 4% polysorbate 80. Results were analysed by one-way Anova, Tukey multiple comparison and considered statistically significant with p value ≤ 0.05 .

Analysis of the initial optimisation study revealed that particle size and dispersity were not affected by the homogenisation speed range, producing NLCs suitable for oral administration, with an average size of 138.0 ± 18.2 and a dispersity of 0.312 ± 0.042 (Figure 1A)(Murugan et al., 2015; Roger et al., 2010). The NLCs were negatively charge, due to their lipidic composition, with a surface charge between - 15 and - 30 mV, indicating adequate colloidal stability (Figure 1B). In contrast, ultrasonication time significantly impacted particle size and dispersity. Longer sonication times resulted in smaller and more homogenous nanoparticles, with 10 minutes of sonication producing significantly smaller and more uniform

NLCs compared to 3 minutes ($p < 0.05$, Figure 1C). These findings align with previous studies showing that increased energy input from longer sonication times yields smaller, monodisperse particles (Das et al., 2011). Notably, variations in sonication time did not affect the nanoparticle's surface charge (Figure 1D). Since these nanoparticles are intended to encapsulate a protein-based antigen, we aimed to select the mildest processing parameters that would produce NLCs with the optimal size, dispersity and stability without compromising the structural integrity of the encapsulated antigen. Therefore, subsequent studies were conducted using a homogenisation speed range 2 followed by 5 minutes of ultrasonication.

3.2 IMPACT OF FORMULATION COMPOSITION ON NLC PARTICLE SIZE, DISPERSITY AND SURFACE CHARGE

Having optimised the processing parameters for NLC production, we next focused on the nanocarrier formulation. Upon oral administration, the fate of NLCs is influenced by their interaction with the physiological environment. Key parameters such as particle size, and surface can determine the absorption process and can be optimised through formulation composition. To investigate this, we prepared a series of empty NLCs composed of glyceryl distearate, propylene glycol dicaprylate and polysorbate 80, and evaluated their effects on nanoparticle size, homogeneity and zeta potential. The percentage of solid lipid and liquid lipid in the lipid matrix was varied between 90 – 70 % and 10 – 30%, respectively, and surfactant concentrations of 2, 5 or 10% were used. (Table 1).

Particle size, dispersity and zeta potential measurements of the prepared NLCs showed that formulation composition influenced significantly NLC physical properties. (Figure 2). Surfactant concentration showed a pronounced effect on particle size. At 10% of polysorbate 80, particles formed were below 100 nm, albeit with high dispersity values (Figure 2A and C). Conversely, at 2% polysorbate 80, particle size increased with liquid lipid content. The low surfactant and high liquid lipid levels likely caused nanoparticles instability and aggregation, resulting in larger particles. This instability was also indicated by lower zeta potential values (Figure 2 D), suggesting poor colloidal stability.

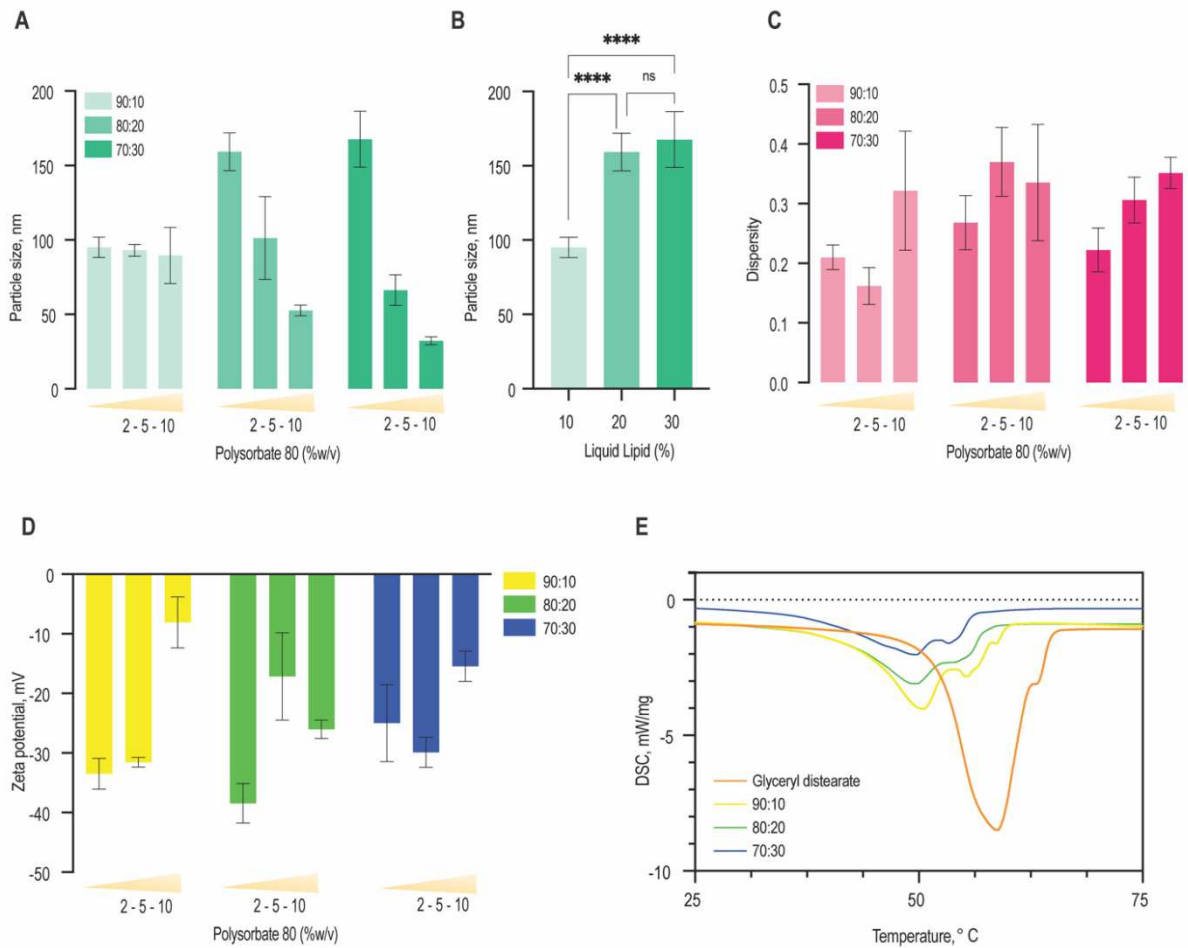


Figure 2 Impact of formulation composition on NLC properties. Plots represent the effect of surfactant concentration and lipid composition on **A** particle size, demonstrating a significant influence of the **B** liquid lipid content at the minimum surfactant concentration (polysorbate 2% w/v). The influence of surfactant concentration and lipid composition on **C** dispersity, and **D** surface charge was also determined. **E** The impact of liquid lipid content on the melting temperature was determined by differential scanning calorimetry. Experiments were conducted in independent triplicates using NLC formulations composed of glycerol distearate, and propylene glycol dicaprylate in a 90:10, 80:20, and 70:30 ratio, with 2, 5 and 10 % of polysorbate 80. Results were analysed by one-way ANOVA, multiple comparison and considered statistically significant with p value ≤ 0.05 .

To confirm the miscibility of solid and liquid lipids, formulations with ratios of 90:10, 80:20, and 70:30 were evaluated using differential scanning calorimetry (DSC). The thermal curve of pure glyceryl distearate exhibited a single endothermic peak at 59.1°C, corresponding to the melting of the stable β -polymorphic form (Saupe et al., 2005). In the binary mixtures (90:10, 80:20, and 70:30), a linear decrease in the peak temperature was observed, with values of 51.3°C, 50.2°C, and 50.1°C, respectively. This reduction in the melting point of glyceryl distearate suggests that propylene glycol dicaprylate had good miscibility with the solid lipid,

disrupting its crystalline structure and forming a less ordered α -polymorphic form (Kasongo et al., 2011; Shah et al., 2016). This transformation can be advantageous for the encapsulation of sensitive molecules, such as proteins and peptides, as the less ordered structure may enhance the encapsulation efficiency and stability of these molecules. Additionally, the broadening of the melting peak width with increasing liquid lipid content indicates a more imperfect crystal structure, further supporting the incorporation of the liquid lipid into the solid matrix (Zheng et al., 2013).

3.3 CHARACTERISATION OF PROTEIN-LOADED NLCS

3.3.2 STRUCTURAL PROTEIN INTEGRITY DURING NLC

An investigation into the effect of homogenisation parameters on protein stability revealed that the biomolecule remained stable under the harsh conditions of the high-pressure homogenisation process (Figure 3A). The protein antigen was subjected to the sequential steps of NLC preparation: 10 minutes of stirring at 70°C, 3 minutes of high-pressure homogenisation at speed range 2, and 5 minutes of ultrasonication. Samples were recovered after each step and evaluated using electrophoresis gel. Analysis by SDS-PAGE showed that the protein remained unchanged, with no alteration in the migration pattern compared to the control solutions. This indicated that the temperature, homogenisation speed, and ultrasonication did not cause any detectable damage to the antigen structure. While protein therapeutics and vaccines are often susceptible to degradation during bioprocessing due to factors such as temperature, pH, and mechanical stress, our results that both high-pressure homogenisation and ultrasonication are suitable techniques for protein nanoencapsulation in this system.

3.3.2 PROTEIN-LOADED NANOSTRUCTURED LIPID CARRIERS (NLCS)

Having confirmed that our optimised NLC preparation method did not affect antigen integrity, we next focused on protein encapsulation. We prepared NLC formulations with solid: liquid lipid ratios of 90:10, 80:20 and 70:30 solid: liquid ratios, incorporating 5% polysorbate 80, both with and without loading 100 mg of the protein antigen. Encapsulation efficiency (EE) was measured using the method described in section 2.4.2, commonly employed for nanoparticles below 100 nm (Lv et al., 2018), followed by quantification with a micro BCA assay.

NLCs with the highest solid lipid content (90:10) exhibited the highest encapsulation efficiency ($58.3 \pm 10.9 \%$), while those with the highest liquid lipid content (70:30) showed the lowest encapsulation efficiency ($52.6 \pm 9.5 \%$). The 80:20 NLC formulation had an encapsulation efficiency of ($58 \pm 5.2 \%$). These findings indicate a trend of decreasing protein encapsulation with increasing liquid lipid content, suggesting that the protein may have a higher affinity to the solid lipid.

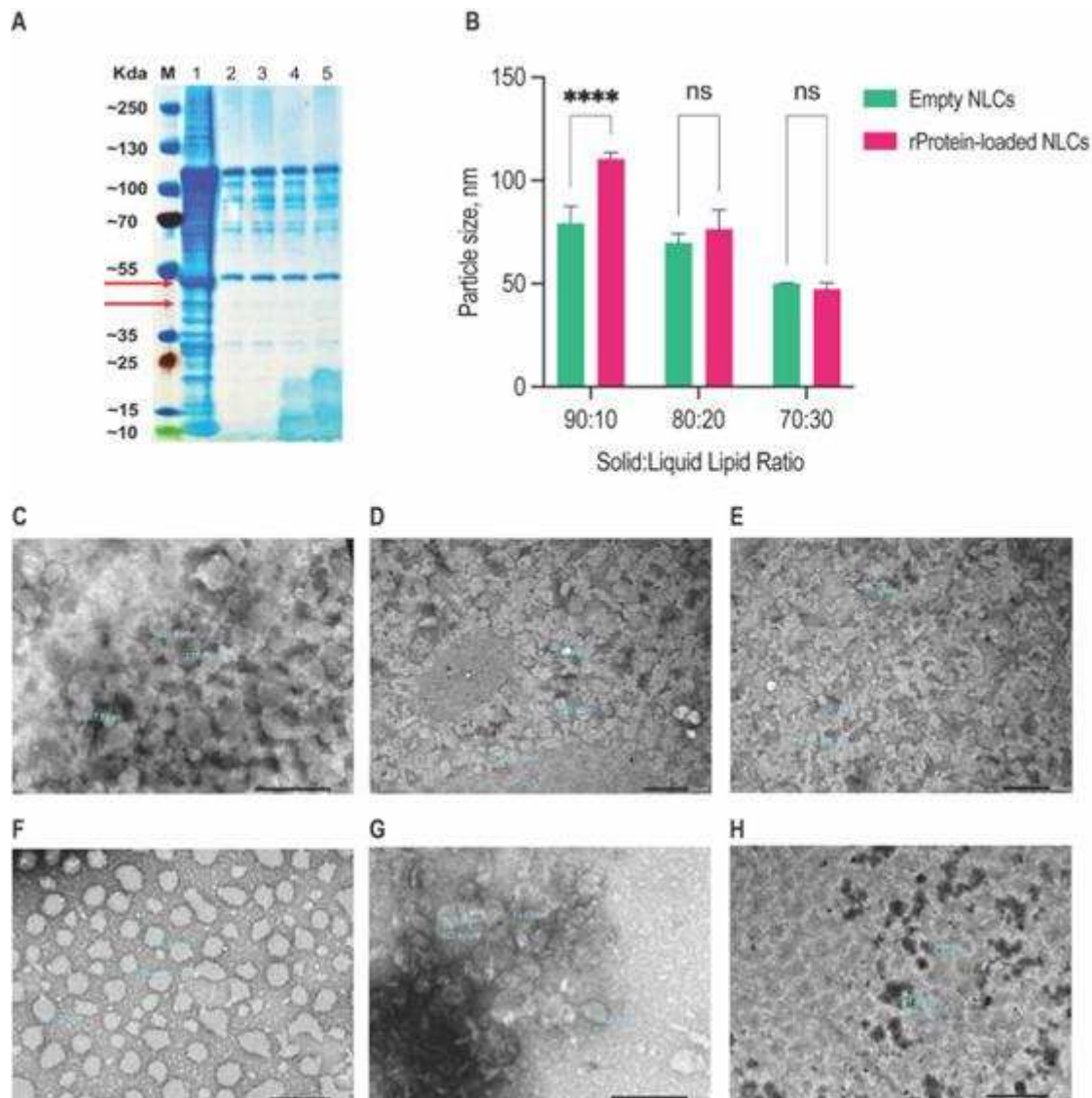


Figure 3 Characterisation of protein-encapsulated NLCs. **A** SDS PAGE gel showing antigen integrity throughout NLC processing steps: *Lane M* – molecular weight standards (kDa), *Lane 1* – concentrated protein (~45-50 kDa), *Lane 2* – protein (100 μ g) control, *Lane 3* – protein following incubation at 70 $^{\circ}$ C, with stirring after 10 minutes, *Lane 4* – protein after 3 minutes of high pressure homogenisation on speed range 2, *Lane 5* – protein after 5 minutes of ultrasonication (20 seconds on and 10 seconds off). Red arrows indicate the protein location on the gel. **B** Comparison of NLC particle sizes following protein encapsulation, with 90:10, 80:20 and 70:30 formulations, as determined by dynamic light scattering. Transmission electron microscopy (TEM) was also used to characterise

the morphology and particle size of empty NLCs with **C** 90:10, **D** 80:20 and **E** 70:30 formulations compared to protein-loaded NLCs with **F** 90:10, **G** 80:20 and **H** 70:30 formulations.

The particle size characteristics of empty and protein-loaded NLC were also compared (Figure 3B). According to DLS measurements, both set of NLCs were within the 100 nm range size. A significant increase in size was observed for the 90:10 protein-loaded NLCs. suggesting that antigen encapsulation impacted particle size. This effect was not observed for NLCs with increasing liquid lipid content, further indicating the higher affinity of the protein towards glyceryl distearate.

All NLCs in this study presented a dispersity below 0.3, indicating suitable polydispersity for drug delivery (Figure S2, supporting information). Comparison between empty and protein-loaded NLCs showed that protein encapsulation increased NLC polydispersity. Statistical analysis further confirmed that this increase was significant with 90:10 and 70:30 formulations, highlighting the impact that solid or liquid lipid content had on NLC characteristics.

Transmission electron microscopy (TEM) was also used to confirm nanoparticle size and examine structural characteristics (Figure 3C-H). TEM analysis revealed uniform, spherical nanoparticles, consistent with previous findings by other researchers. The images supported the hypothesis that lipid composition influences particle morphology, with increasing liquid lipid content affecting spatial arrangement and particle size. Both blank and protein-loaded nanoparticles showed comparable sizes when measured by TEM and DLS, with minor variations attributable to the different measurement principles of each technique (Izham et al., 2021). Importantly, both methods confirmed particle sizes below 200 nm, indicating successful formulation characteristics.

3.4 IN VITRO EVALUATION OF NANOSTRUCTURED LIPID CARRIERS

3.4.2 NLC COLLOIDAL STABILITY UNDER ACIDIC CONDITIONS

To ensure effective fish immunisation it was crucial to evaluate NLC colloidal stability under conditions resembling the fish gastrointestinal tract. Stability in the hostile stomach environment is key to preventing antigen degradation and allowing passage to the intestine for absorption and uptake (Mutoloki et al., 2015). Empty NLCs with 90:10, 80:20 and 70:30 solid:liquid lipid ratios with 5% polysorbate were incubated at pH 3.0. Samples were analysed for particle size, dispersity and surface charge at 0, 10 and 60 minutes, as can be seen in Figure 4

A. We selected pH 3.0 to represent fish stomach conditions, which typically range between 1-3, depending on species and diet.

Regardless of lipid composition, no significant changes in particle size were observed over 1 hour under acidic conditions, indicating that NLCs were physically stable *in vitro*. NLC zeta potential, a measure of the electrostatic forces between nanocarriers, is important for evaluating colloidal stability (Neves et al., 2013). Generally, an absolute zeta potential value above 15 mV indicates strong electrostatic repulsion and stability (Yu et al., 2011). NLCs with 90:10 and 80:20 compositions maintained an absolute zeta potential above 15 mV throughout the 1 hour incubation at pH 3.0, suggesting they could remain stable without aggregation in the fish stomach. In contrast, the 70:30 NLCs showed a lower absolute zeta potential value over time, indicating some instability under acidic conditions.

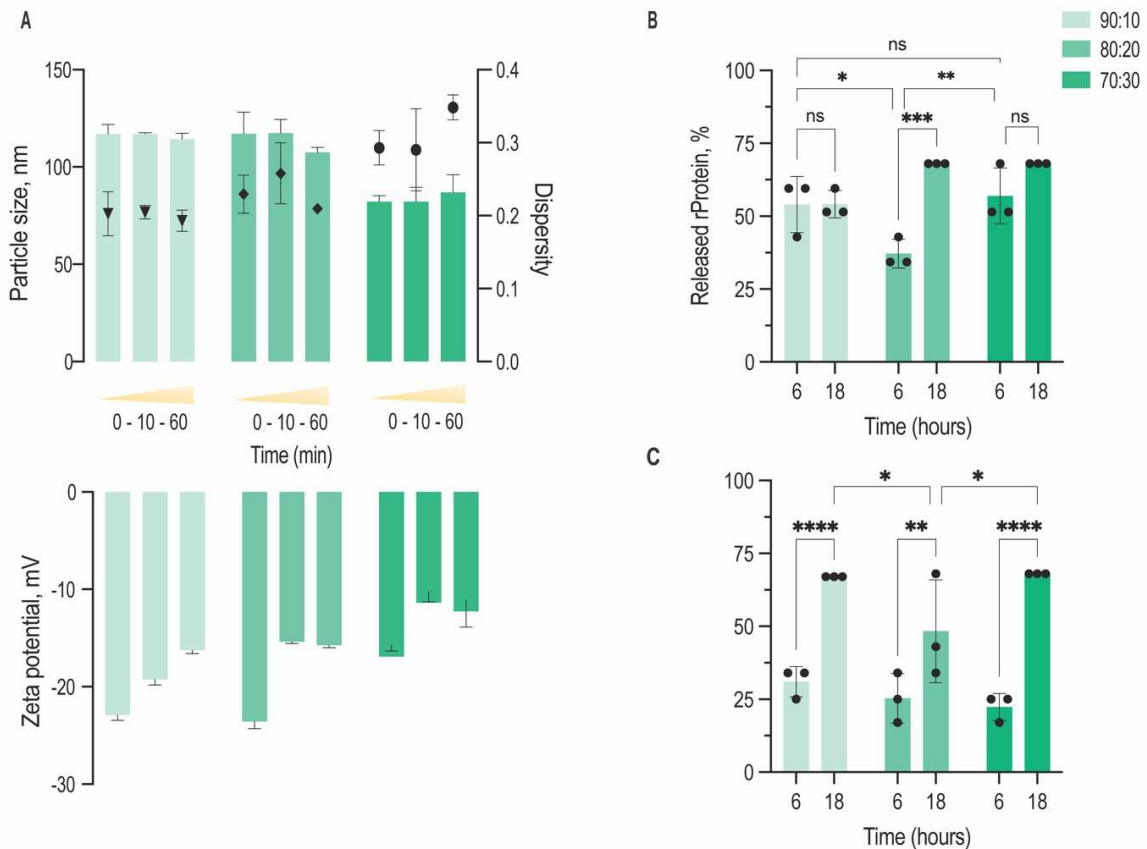


Figure 4 *In vitro* evaluation of nanostructured lipid carriers with 90:10, 80:20 and 70:30 solid: liquid lipid ratios with 5% polysorbate 80 **A** Particle size, dispersity and zeta potential of NLCs after incubation in pH 3.0. Samples were taken at 0, 10 and 60 minutes. Cumulative protein release (%) from NLCs after incubation in **B** simulated gastric fluid (SGF, pH 2, 37 °C stirring at 200 rpm) and **C** simulated intestinal fluid (SIF, pH 6.5, 37 °C stirring at 200 rpm). All experiments were conducted as independent triplicates. Results were analysed by two-way ANOVA, Tukey's multiple comparison and considered statistically significant with p value ≤ 0.05 .

3.4.3 RECOMBINANT PROTEIN RELEASE STUDIES

Having evaluated NLC stability at low pH, we next sought to understand protein release characteristics from each NLC formulation under *in vitro* conditions similar to the fish gastrointestinal tract. Drug release from NLCs is influenced by factors such as lipid type, composition, surfactant concentration and production temperature (Khosa et al., 2018). Release can be controlled by drug diffusion, and matrix erosion, depending on whether the drug is entrapped in the core, shell or matrix of the NLC. Typically, drug release is governed by the slow dissolution rate in the aqueous environment and the degradation rate of lipids.

Figure 4B and C show the cumulative release profile of the recombinant protein antigen from NLC formulations with 90:10, 80:20 and 70:30 solid: liquid lipid ratio with 5% polysorbate 80. Figure 4B depicts the release under simulated gastric fluid conditions (pH 2.0 and 37 °C) while Figure 4C shows the release under simulated intestinal fluid conditions (pH 6.5 and 37 °C). Aliquots were taken at 6 and 18 hours, and released protein was quantified by micro BCA assay.

Statistical analysis suggested that lipid composition was key for obtaining a sustained release profile. Under low pH conditions (Figure 4B), NLCs with the lowest liquid lipid content (90:10) released a maximum of $54.02 \pm 9.63\%$ of the protein within the first 6 hours, with no significant increase over the 18-hour incubation. This immediate, high release may be due to non-encapsulated protein, or antigen adsorbed on the NLC surface. In contrast, NLCs with the highest liquid lipid content (70:30) released nearly 70% of the protein within 18 hours, likely due to the poor stability of this formulation under acidic conditions. NLCs with an 80:20 solid: liquid lipid ratio exhibited a more moderate and sustained release profile, with $37.18 \pm 4.95\%$ protein release after 6 hours.

When NLCs were incubated under more alkaline conditions (Figure 4C), all formulations showed a more controlled release profile. Interestingly, increasing the liquid lipid content led to a progressively lower initial burst release, with the 90:10 NLCs releasing $31.0 \pm 5.2\%$, and the 70:30 NLCs releasing only $22.33 \pm 4.62\%$. However, this trend was not maintained at the 18-hour timepoint, where 80:20 NLCs showed a slower overall release compared to the other formulations. These observed differences in protein release profiles among formulations likely stem from variations in lipid matrix organisation, particularly at low pH. Higher solid lipid content (90:10) may create a more ordered crystal structure, potentially leading to surface adsorption rather than matrix incorporation of the antigen. In contrast, increased liquid lipid

content (70:30) creates imperfections in the crystal lattice, possibly allowing better protein incorporation but potentially compromising stability (Apostolou et al., 2021).

3.4.3 STORAGE STABILITY STUDIES

Evaluating storage stability is a critical aspect in nanomedicine-based vaccine formulation. For successful vaccine development, maintaining the physical integrity and structural characteristics of nanocarriers is essential. Changes in particle size, homogeneity, and surface charge can impact key factors such as cellular uptake, biodistribution, and immunogenicity of the antigen. For example, particle aggregation can lead to altered pharmacokinetics and suboptimal delivery to the target immune cells.

The physical stability of the prepared NLC formulations was monitored after 1 month of storage at 4 and 42 °C (Figure 5). The physicochemical properties of the NLC dispersions, including particle size, homogeneity (dispersion) and surface charge (zeta potential), were evaluated and compared to freshly prepared NLCs. An increase in particle size and dispersity (values > 0.3 - 0.5) during storage indicates agglomeration and poorly homogenous systems.

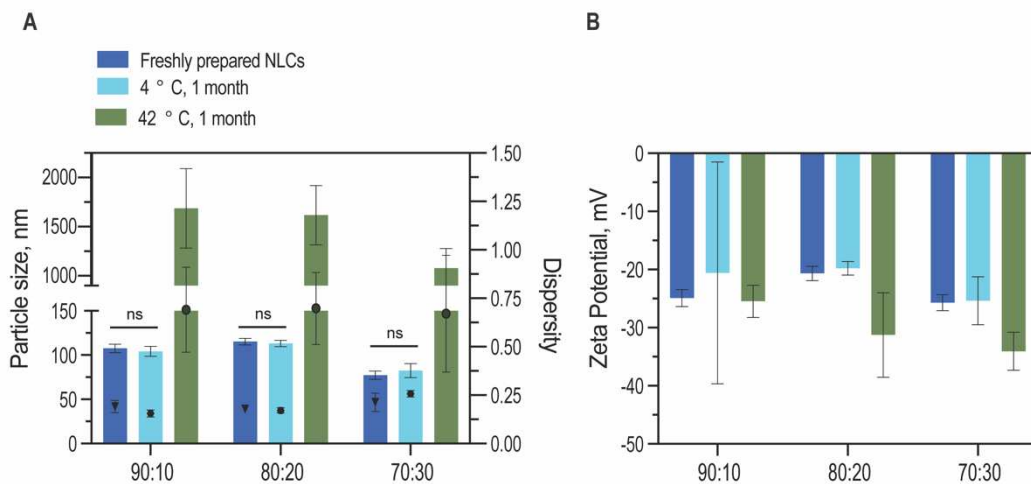


Figure 5 Storage stability of nanostructured lipid carriers with 90:10, 80:20 and 70:30 solid: liquid lipid ratios with 5% polysorbate 80. Impact of temperature storage conditions (4 ° and 42 °C) on **A** particle size, dispersity and **B** surface charge of empty NLCs as a function of NLC lipid composition.

As shown in Figure 5A, NLCs stored at 4°C showed no significant changes in their physicochemical properties, indicating robust stability under refrigerated conditions. This is an important finding, as maintaining the cold chain is a common challenge in the distribution and administration of many vaccine products, particularly in resource-limited settings.

In contrast, NLCs stored at 42 °C displayed clear signs of instability, including particle aggregation and changes in dispersity. This was also evident by the increase in zeta potential for 42 °C samples, indicating poor stability of the nanoparticle suspension and a propensity for aggregation (Figure 5B). To address the lack of thermostability observed at elevated temperatures, further studies could explore the use of cryoprotectants or lyophilization, coupled with longer-term studies to further safeguard NLC stability. These strategies may enhance the shelf-life and ease of handling of these nanoparticle-based vaccine candidates, ultimately facilitating their successful clinical translation.

Having established the formulation parameters, *in vitro* release and stability characteristics of our NLC formulations, it is important to consider these findings in the context of current fish vaccination approaches. As previously mentioned, current fish vaccination methods primarily rely on injection, requiring individual handling and causing significant stress. Oral vaccination is a promising alternative; however, their efficacy can be compromised by gastrointestinal degradation. Our study in NLC formulation development demonstrate several advantages, namely controlled release properties that can protect antigens through the gastrointestinal tract, particle sizes below 200 nm, and thus suitable for cellular uptake along with scalable production methods using food-grade materials. However, several challenges must be addressed before practical implementation, including cost considerations for large-scale production, long-term stability requirements, and possible regulatory approval pathways. Moreover, while this work demonstrates the potential of NLCs for oral vaccine delivery, further studies including the influence of real-world environmental conditions (e.g., temperature fluctuations, and humidity), and *in vivo* immune responses and protection efficacy are required to advance this technology toward practical application in aquaculture settings.

4. CONCLUSIONS

Nanostructured lipid carriers, an advanced class of solid lipid nanoparticles, are an attractive approach to protect therapeutic and vaccine antigen from degradation and damage in gastric and aquatic environments. In this study, we report that NLC preparation was affected by ultrasonication time, yielding smaller and more homogenous particles with time, forming NLCs with 120 nm in size and low dispersity (~ 0.2) after 10 minutes. On the other hand, protein integrity was not impacted negatively by the NLC manufacturing methods used in this work. Variation in the solid: liquid lipid ratio and surfactant concentration influenced the resulting NLC physicochemical properties, with low surfactant concentrations and high liquid lipid

content producing unstable and aggregated particles. The antigen loading efficiency was above 50 % for all NLCs tested (90:10, 80:20, 70:30 ratios of solid: liquid lipid and 5 % surfactant), and TEM showed desirable spherical and uniform nanoparticles. However, we noted that protein loading efficiency increased with higher solid lipid. *In vitro* protein release and stability in gastric fluids was dependent on lipid composition. Higher liquid lipid content (70:30 solid: liquid lipid ratio) showing a less controlled release and instability in acidity, with possible occurrences of aggregation in comparison to NLCs with higher solid lipid content (90:10 and 80:20 solid: liquid ratios). NLC formulations stored at 4 °C for up to one month showed equivalent properties of the freshly prepared NLCs, although, this was not observed with the NLCs stored at 42 °C during the same time. Overall, our findings suggest that a balance of solid: liquid content, namely the 80:20 NLC formulation with a 5 % surfactant is an optimal formulation that can be explored further in future *in vitro* and *in vivo* studies.

SUPPORTING INFORMATION

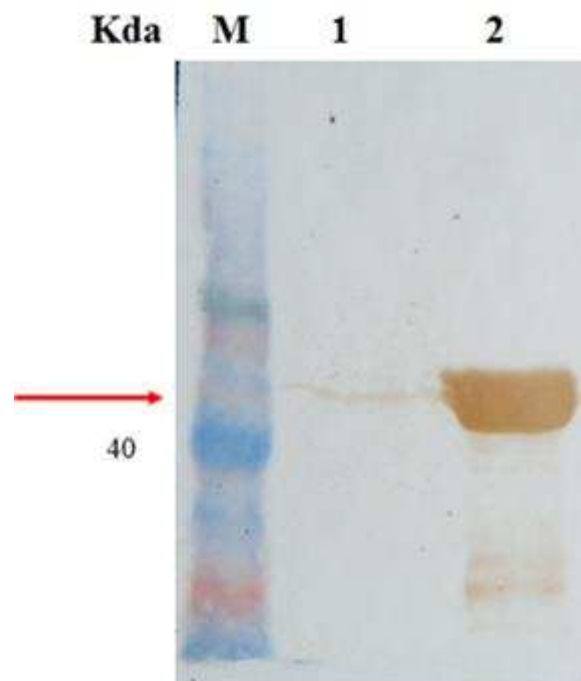


Figure S1 Analysis of chimeric protein expression. Western blotting of chimeric protein in (1) soluble and (2) insoluble fraction. Molecular marker is in lane M.

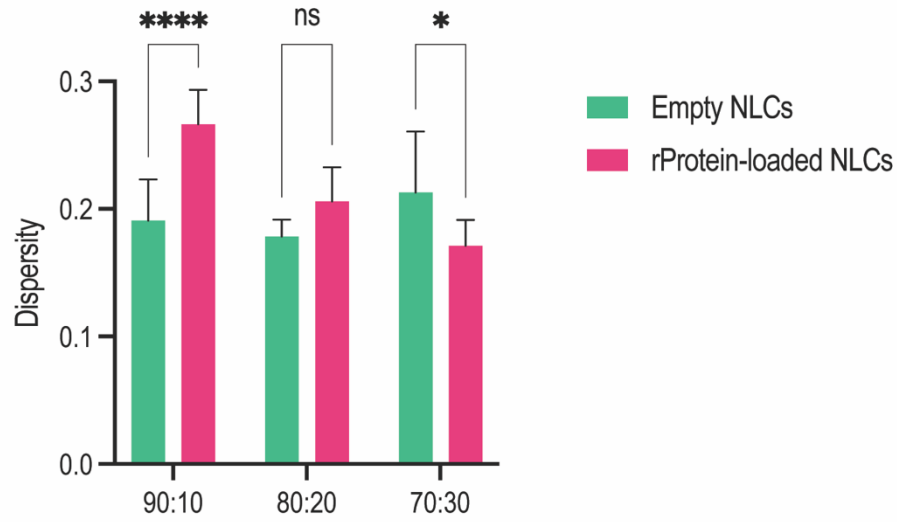


Figure S2 Impact of particle dispersity following encapsulation of recombinant protein.

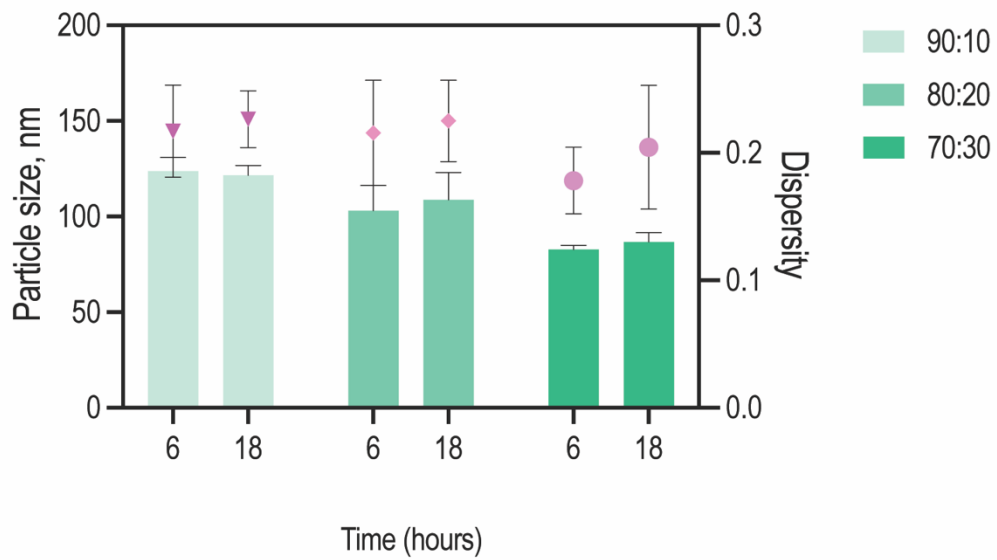


Figure S3 Changes in particle size and dispersity of NLCs after incubation in simulated gastric fluid (SGF, pH 2, 37 °C stirring at 200 rpm).

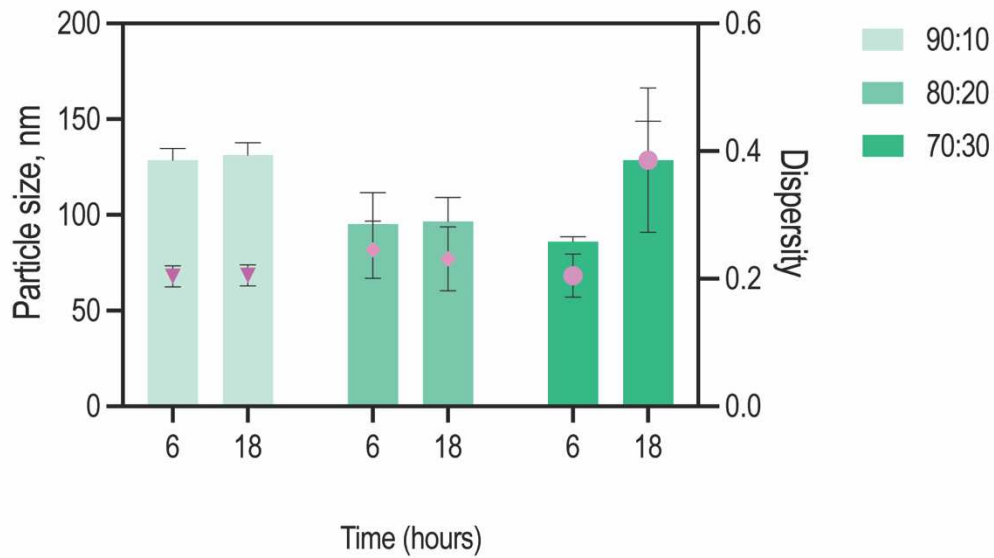


Figure S4 Changes in particle size and dispersity of NLCs after incubation in simulated intestinal fluid (SIF, pH 6.5, 37 °C stirring at 200 rpm)

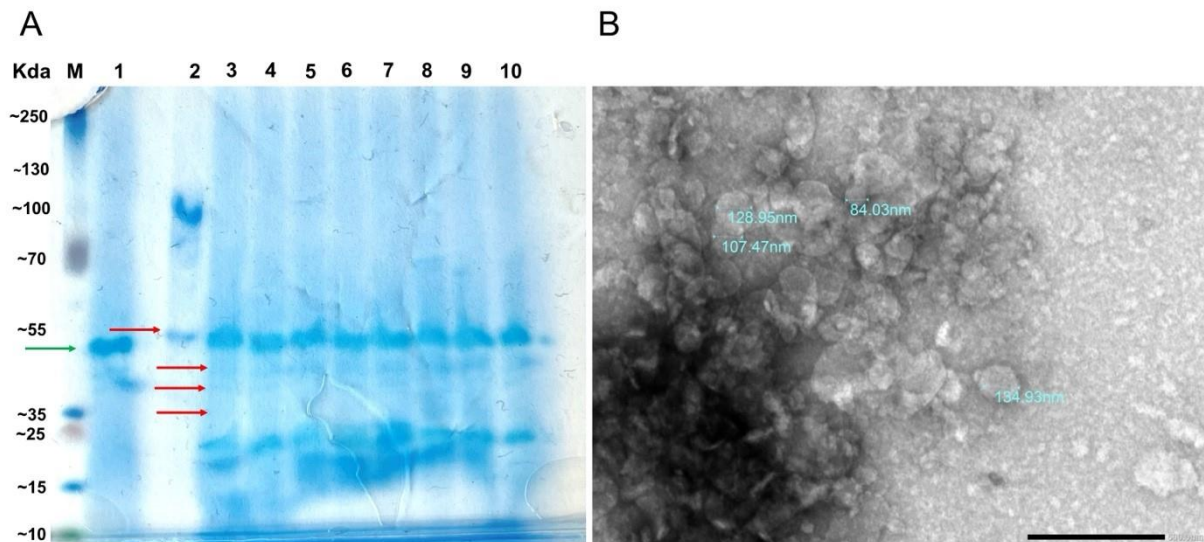


Figure S5 **A** SDS PAGE showing recombinant protein antigen when incubated with trypsin: *Lane M* – molecular weight marker; *Lane 1* – recombinant protein without trypsin, *Lane 2* – recombinant protein incubated with trypsin after 5 minutes, *Lane 3* – recombinant protein incubated with trypsin after 10 minutes, *Lane 4* – recombinant protein incubated with trypsin after 15 minutes, *Lane 5* – recombinant protein incubated with trypsin after 20 minutes, *Lane 6* – recombinant protein incubated with trypsin after 25 minutes, *Lane 7* – recombinant protein incubated with trypsin after 30 minutes, *Lane 8* – recombinant protein incubated with trypsin after 40 minutes, *Lane 9* – recombinant protein incubated with trypsin after 50 minutes, *Lane 10* – recombinant protein incubated with trypsin after 60 minutes. After each time the reaction was stopped with 4N NaOH. **B** Recombinant protein-loaded NLC formulation 80:20 with 5 % surfactant following 1 hour incubation with trypsin was also analysed by TEM and showed no change in nanoparticle shape and size.

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CHAPTER 3

NANO-ENCAPSULATED CHIMERIC RECOMBINANT PROTEIN: ZEBRAFISH PROTECTION, CYTOTOXICITY AND OXIDATIVE STRESS EVALUATION

PAPER 3

NANOSTRUCTURED LIPID FISH VACCINE BASED ON CYTOPLASMATIC BACTERIA CELL WALL BIOSYNTHESIS ENZYME: IN VITRO AND IN VIVO TOXICITY EVALUATION, ZEBRAFISH PROTECTION AND ADJUVANT RESPONSE AGAINST AEROMONAS HYDROPHILA

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ABSTRACT

Nanostructured lipid carrier (NLC) is a second generation of lipid nanoparticles composed by solid and liquid lipids and stabilized by surfactants with interesting properties for fish vaccine adjuvant and delivery. The aim of this work was to evaluate the protection and adjuvant profile of a nanostructured lipid fish vaccine against a virulent strain of *Aeromonas hydrophila*, as well as their toxicity. Fish nano vaccine was made by nano encapsulation of a chimeric recombinant protein with glyceryl distearate as solid lipid, propylene glycol dicaprylocaprate as liquid lipid, and polysorbate 80 as surfactant using high pressure homogenizer method. The nano vaccine was physically and morphologically characterized by dynamic light scattering (DLS) and transmission electronic microscopy (TEM). The zebrafish protection was performed by vaccinating intraperitoneally adult fish with 250 ng, 1 µg or 3 µg of chimeric recombinant protein or with a nano dose of chimeric recombinant protein nano encapsulated and posteriorly challenged with a lethal dose of *Aeromonas hydrophila*. The percentage of survival assessed over 5 days. The toxicity profile was accessed using the MTT assay *in vitro* as well as *in vivo* with adult zebrafish submitted to intraperitoneal administration of blank nano formulations (90:10, 80:20 and 70:30 % of solid:liquid lipids ratios) or aluminum hydroxide (0.2, 0.5 and 1%). The oxidative status was evaluated in liver, gills and intestine. The results showed a zebrafish protection dose depending, with 3 µg of chimeric recombinant protein resulting in a 90% of survival rate after challenge with lethal dose of *A. hydrophila*, however, when fish receive nano dose of chimeric protein nano encapsulated (250 ng), the protection was significantly high, showing a crucial role of the nano formulation as adjuvant factor. The safe status of different nano formulation proportions (90:10, 80:20 and 70:30 % of solid:liquid lipids) was accessed under Vero cells and the cytotoxicity concentration that decrease cell viability in 50 % was 7.5, and 35 mg, respectively. Based on catalase, and superoxide dismutase activity, total antioxidant activity, lipid peroxidation, and protein carbonylation levels no oxidative stress was observed when fish received 100 mg of the nanoformulation. On the other hand, the oxidative stress biomarkers were statistically higher when fish received 0.2, 0.5, or 1 % of aluminum hydroxide. In conclusion, the results indicate a promising application of lipid nanoparticles for aquatic vaccine, but further analysis must be conducted to elucidate the molecular mechanism of how nanostructured lipid carriers stimulate fish immune system and then the oral delivery be performed.

1. INTRODUCTION

Fish diseases outbreaks is a massive challenge for aquaculture development and sustainable production with a significant role of *Aeromonas hydrophila* in fish mortality, economic losses and zoonotic spread. The etiologic agents from aquaculture disease also include: virus, parasite and fungi, however, bacteria can be considered a principal responsible to fish illness. *Aeromonas hydrophila* is a gram-negative, widespread, aquatic, zoonotic and highly virulent bacteria related to severe hemorrhage, skin ulcers and ascites in infected fish (Dong et al., 2024; Ofek et al., 2023; Pereira et al., 2024). The screening of antimicrobial agents and vaccines to aquatic species have been explored, and few of them are available on the market, despite vaccines being considered by far the best control method against microbial infection, the aquatic delivery, vaccine strategy, and fish immune-stimulation are still challenges to overcome (Kumar et al., 2024).

The advances in biology, biotechnology, chemistry and physics allowed the development of nanotechnology, a revolutionary science based on nanoscale processes that physically, biologically and chemically change the size, composition and nature of nanomaterials to produce nanoparticles range of 10 – 1000 nm (Malik et al., 2023). Currently, nanotechnology has been applied in the vaccine field as a delivery vehicle, protecting and guiding antigens to specific sites, and adjuvant agents, enhancing the vaccine antigenicity, and for this reason the term Nano vaccines was consoled. Lipid nanoparticles, nanoemulsion, virus-like particles (VLP), polymeric nanoparticles, liposome, polymeric micelle, and micelle are known as nano vaccine platforms (Ebrahim et al., 2024; Huesmann and Mayer, 2022; Nooraei et al., 2023).

Aquatic nano vaccine properties can mitigate and overcome the antigen harshness, delivery and lack of immunogenicity of recombinant vaccines. Fish nano vaccines are already in trial and the significant results have gained attention. Kitiyodom (2019) developed a chitosan nano vaccine with mucoadhesive properties and showed significant protection against *Flavobacterium columnare* in infected Tilapia, *Oreochromis sp*, as well as Ruyra et al., (2014) showed immunostimulant role of LPS/dsRNA loaded nanoliposomes in zebrafish and protection against bacterial and viral infection, although, different for a human vaccine, reports about the adjuvant and delivery role of nano vaccine for aquatic species remains lack of information and a new class of lipid nano vaccine not explored.

Lipid nanoparticles (LNPs) are a nano-delivery system approved by Food and Drug Administration (FDA) for drug and vaccine use. The first generation of LNPs was established in the 1990s using a lipid core made of solid lipids at room temperature and stabilized by surfactants, without the use of organic solvent during preparation, defined as Solid Lipid Nanoparticles (SLN). The LNPs present stability against drug hydrolysis and the possibility of large-scale industrial production; however, it has disadvantages such as low hydrophilic drug loading efficiency, alteration in drug release, and drug expulsion during storage, although these disadvantages have been overcome by the application of Nanostructured Lipid Carrier (NLC), the second generation of LNPs (Alfutaimani et al., 2024; Garg et al., 2022; Javed et al., 2022).

The NLC comprise a homogenous mixture of solid lipid, liquid lipid, water and surfactant to produce tiny nanoparticles with imperfect, amorphous or multiple structure types and all components classified as “generally recognized as safe (GRAS)” (Ghasemiyeh and Mohammadi-Samani, 2018; Haider et al., 2020). The lack of information about the use of NLCs for aquatic species encourage the exploration of this system to improve the delivery and immune-stimulation of antimicrobial and antigens to deal with the aquaculture limitations. So, for the first time, this work related the immunogenic and adjuvant role of a fish nano vaccine based on nanostructured lipid carrier of a cytoplasmatic enzyme involved in cell wall biosynthesis against *Aeromonas hydrophila* infection.

2. MATERIAL AND METHODS

2.1 BACTERIA STRAIN AND CULTURE CONDITIONS

The *A. hydrophila* ATCC 7966 was genteelly donated by Enterobactérias Laboratoty from Fundação Oswaldo Cruz (FIOCRUZ) and aerobic cultured in Trypticase Soy Broth (TSB) medium at 30 °C under agitation of 180 rpm when necessary.

2.2 ANIMAL ETHICS APPROVAL AND MAINTENANCE

The experiments with zebrafish, *Danio rerio*, were conducted after approval of Animal Production Ethic Committee (CEUAP) from Federal University of Viçosa (UFV) (Protocol 39/2023). The zebrafish were obtained from pisciculture market and manipulated in Laboratory of Physiology Applied to Pisciculture and Laboratory of Proteomic and Bioinformatic from Instituto de Biotecnologia Aplicada a Agropecuária (BIOAGRO-UFV). The fish manipulation

and maintenance respected the Zebrafish Breeding Manual (Dammski et al., n.d.). Briefly, fish were kept in tank of 20 L (30 N) or 10 L (10 N) with aerated water quality guaranteed, water temperature of 25-30 °C, light and dark cycle by 12h/12 h and fed twice by day *ad libitum*. Fish health was evaluated by behavior (movement/ appetite) and clinical signs diseases were also evaluated during the experiments.

2.3 ANTIGEN CONSTRUCTION AND NLCS PREPARATION

The murC gene product: UDP-N-acetylmuramate-L-alanine ligase was chosen as the backbone to construct the chimeric by immunoinformatic tools and posteriorly synthesized in a PET28a plasmid vector and expressed in Artic Express (DE3) cells via heat shock. The steps of antigen construction can be accessed on previous work. The recombinant chimeric antigen was used to nanoencapsulation process. The nanostructured lipid carriers were produced by high pressure homogenizer using glyceryl distearate and propylene glycol dicaprylocaprate as the solid and liquid lipids, respectively, with polysorbate 80 as the surfactant. The proportions of solid-liquid lipids evaluated were: 90:10, 80:20 and 70:30 with 5 % of surfactant. The steps of the nano formulation process and characterization can be accessed in Rodrigues et al., 2025 work: **“Formulation development of nanostructured lipid carriers for oral delivery of recombinant protein-based vaccine antigens in aquatic species”**. The recombinant chimeric antigen was nano encapsulated only in the 80:20 formulation in zebrafish protection assay, but all proportions of solid-liquid lipids were used to access the toxicity profile *in vitro* and *in vivo*.

2.4 TRANSMISSION ELECTRONIC MICROSCOPY

The chimeric protein NLC size was further confirmed by Transmission Electron Microscopy (TEM, Joel JEM -1400 Plus Transmission Electron Microscope). The nano formulation with or without chimeric protein were diluted 50-fold to better analyse separate particles. Diluted formulations (5 µL) were placed on a carbon grid (carbon Type-B, 400 mesh, 42 mm copper grid), over 5 minutes and dried (Izham et al., 2021). A solution of 2 w/v % of uranyl was placed on carbon grid over 3 minutes, then dried by 30 minutes until further analysis, using an acceleration voltage between 80-120 kV.

2.5 ZEBRAFISH PROTECTION ASSAY

Adult zebrafish were vaccinated intraperitoneally with 20 μ l of different concentrations of chimeric protein (250 ng, 1 μ g and 3 μ g) or with nanostructured lipid chimeric protein (formulation 80:20 with each fish receiving 250 ng of nano encapsulated chimeric protein) or with empty nanostructured lipid nano formulation or with PBS (control group) and randomly divided and placed in tanks of 20 L, with 30 fish in each. A second dose of all treatments was administered after 10 days of the first dose. Posteriorly 10 days of the second dose, 40 fish of each group were challenged with 10 μ l of a lethal dose of *Aeromonas hydrophila* ATCC 7966 (6×10^8) intraperitoneally and the survival percent evaluated during 4 days. The spleen, liver, gills and intestine were dissected before and after challenge and submitted to histopathological analysis or RT-qPCR of immune-related genes expression (Guo et al., 2018).

2.6 NLC FORMULATIONS CYTOTOXICITY ASSAY

The cytotoxicity of all NLC formulations (90:10, 80:20 and 70:30) were assessed by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay to evaluate the cellular viability by determination of the cytotoxic concentration that decreases viability by 50% (CC_{50}). Prior to the experiment, a suspension of Vero CCL-81 cells containing approximately 1×10^4 cells/mL, was seeded in a 96-well plate (100 μ L/well). After 24 hours or upon reaching confluence, the cells were treated with NLC solutions, diluted in incomplete DMEM medium, starting with a concentration of 100 mg/mL and incubated for 24 hours at 37 $^{\circ}$ C under a 5% CO_2 atmosphere. Subsequently, the medium was removed and replaced with 100 μ L of MTT solution (at 0.5 mg/mL), followed by incubation of 3 hours under the same conditions as previously described. After this, the medium was aspirated and 100 μ L of DMSO per well was added to dissolve the formazan crystals. The 96-well plate was shaken for 20 minutes and absorbance measured at 540 nm. CC_{50} values were obtained by non-linear regression analysis of the percentages of inhibition of cell viability related to different concentrations of compounds and represent the average of three independent experiments.

2.7 OXIDATIVE STATUS EVALUATION

The intestine, liver and gills of adult zebrafish were collected to analyze oxidative stress biomarkers from fish that were submitted to a single dose of 100 mg of nano-formulations with

90:10, 80:20 and 70:30 % of solid-liquid lipid ratios and 5 % of polysorbate 80; as well as 0.2, 0.5, and 1 % of aluminum hydroxide; and saline solution (PBS) as control group, administered intraperitoneally in 40 fish. Fish mortality was accessed and the organs described above of the survivals were collected 24 hours and 48 hours after the treatment administration and the activity of the enzymes superoxide dismutase (SOD), catalase (CAT) and the ferric reduction capacity (FRAP), lipid peroxidation by malondialdehyde (MDA) and protein peroxidation by carbonyl proteins (PC) were analyzed. The samples were homogenized in the Mixer Mill MM 400, centrifuged at 10.000 rpm for 10 minutes and the supernatant harvested and used in the tests. The protein concentration was determined according Bradford method. SOD activity was measured by the quantification of superoxide anion production due to autoxidation of pyrogallol. CAT activity was evaluated by undecomposed of hydrogen peroxide with ammonium molybdate. The ferric reducing antioxidant power (FRAP) method was used to measure the total antioxidant capacity. Protein oxidation was evaluated by quantification of protein carbonyls using DNPH (2,4-dinitrophenylhydrazine) method and the lipid peroxidation measured by malondialdehyde (MDA) quantification. The SOD and CAT results were expressed in U/mg of protein, FRAP in $\mu\text{mol}/\text{mg}$ of protein, protein and lipid peroxidation in nmol/mg of protein.

2.8 STATISTICAL ANALYSIS

The results of all the above-described studies were statistically analysed by one-way analysis of variance (ANOVA,) followed by the Tukey multiple comparison post-test using GraphPad Prism version 10.2.2. for Mac, GraphPad Software (Boston, Massachusetts, USA, www.graphpad.com). Differences were considered statistically significant when $p < 0.05$.

3. RESULTS AND DISCUSSION

The major strategies for fish nano vaccine are based on polymers, metals and liposomes with trial experiments showing satisfactory results against microbial pathogens that treats aquaculture. Nanostructured lipid carries can be formulated from different solid and liquid lipids as well as stabilized with different surfactants and, depending on their chemical nature, can show different loading, protection and adjuvant properties. Beside been established in the end of 90s, the NLCs, are a new technology that must be better understood. Currently, the main propose for the use of NLCs is focus on antimicrobial and cancer therapy delivery, their use for

fish nano vaccine still remains unexplored. The advantages of NLCs to aquatic environmental species include antigen loading efficiency, antigen protection against harsh conditions of aquatic conditions and fish internal barriers, storage stability, fish immune-stimulation, target delivery and can be applied for different routes of administration. Firstly, this work had an intention to investigate the adjuvant role and toxic profile of a possible fish nano vaccine based on NLC. A chimeric antigen based on cytoplasmatic enzyme involved on the cell wall biosynthesis showed in previous report that can protect zebrafish after a challenge with LD₅₀ of *A. hydrophila* and our hypothesis was that after a nanoencapsulation process with small amount of chimeric protein, the chimeric antigen NLC can have a great response against a lethal dose of *A. hydrophila* and no induce toxic process *in vivo* achieving satisfactory results to explore the oral administration.

3.1 ZEBRAFISH SURVIVAL VACCINATED WITH CHIMERIC ANTIGEN AND CHALLENGE WITH LETHAL DOSE OF A. HYDROPHILA

After a immunization period with 1 µg or 3µg of recombinant chimeric antigen and a boost dose after 10 days of the first immunization, adult zebrafish were challenged with a lethal dose of *A. hydrophila* and interesting results were reported (Figure 1 A and B). The control group did not resist the acute infection, with no live fish registered, the *A. hydrophila* lethal dose resulted in the 100 % of mortality for this group, however, the vaccinated groups showed a survival percent depending on the chimeric protein concentration. Fish vaccinated with 1 µg of chimeric recombinant protein had a survival of 18 %, although, when fish received 3 µg the survival rate significantly increased achieving 94% (Fig. 1 B). Recombinant protein has the disadvantage of low immunogenicity when compared to attenuated and inactivated vaccines and for this reason the use of high concentrations of recombinant protein for vaccine is necessary to achieve a satisfactory protection.

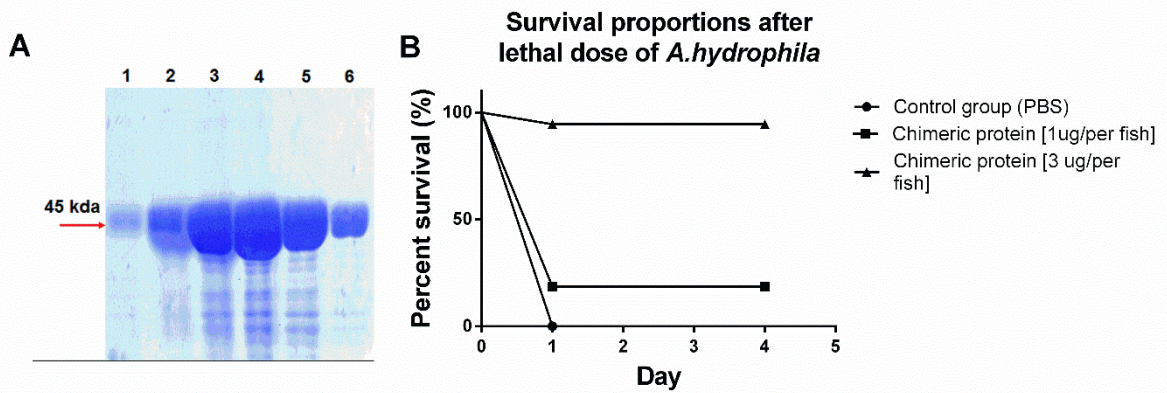
The antigen chosen in this work is not a conventional strategy based on Outer membrane protein (OMP), usually classified as antigen determinant for vaccine development, although, the use of immunoinformatic and reverse vaccinology emerged to analyze and enhance the non-conventional antigen strategies to fish vaccines. Beside the satisfactory response of fish protection vaccinated with 3 µg of a non-conventional antigen immunoinformatic enhanced, new strategies to potentialize the immune response must be applied such as the adjuvant role of nanomaterials, less explored in the context of fish vaccine field.

3.2 ZEBRAFISH SURVIVAL VACCINATED WITH NANO VACCINE AND CHALLENGED WITH LETHAL DOSE OF *A. HYDROPHILA*

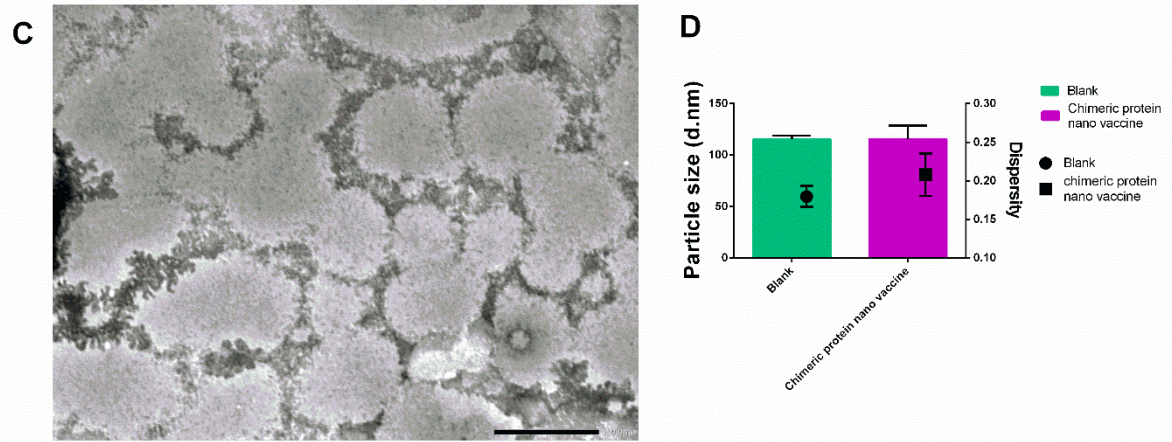
In intention to show the immune response of low concentration of chimeric protein nano encapsulated with nanostructured lipid carrier, adult zebrafish were vaccinated intraperitoneally with 20 µl of the nano vaccine resulting in a delivery of 250 ng of antigen per fish. A second dose was administrated after 10 days of the first immunization and past 10 day a challenge with a lethal dose of *A. hydrophila* was intraperitoneally administrated and zebrafish survival percent registered. The nano-formulation development and characterization can be accessed in Rodrigues et al., 2025 “ Formulation development of nanostructured lipid carriers for oral delivery of recombinant protein-based vaccine antigens in aquatic species”, briefly, the nano-formulation chosen to antigen nano encapsulation was the 80:20 proportion of solid-liquid lipid, stabilized with 5 % of surfactant. The reason of this chosen was based on the stability and peptide release profile reported. The nano vaccine achieved a particle size and dispersity bellow 200 nm and 0.3 respectively, considered great characteristics of nano carriers as well as expected spherical and uniformed shape (Fig 1 C and D), furthermore, the encapsulation efficiency was above 50 %.

After the nano vaccine immunization period and the *A. hydrophila* challenge, interesting results were reported as can be seen in the figure 1 (E), Fish vaccinated with nano vaccine and receiving a final concentration of 250 ng of chimeric recombinant vaccine had a survival percent of 51%, result of the nanoparticles adjuvant properties proved by the protection of zebrafish that received only nano-formulation and achieved a survival percent of 40 %. The control groups, chimeric protein 250 ng and PBS achieved a survival percent of 16 and 10 % respectively. The results presented in this section indicate a chimeric antigen protection depending of concentration, however, after nanostructured lipid encapsulation the satisfactory protection can be accessed even in low concentrations dose of the chimeric protein.

Chimeric protein expression and Zebrafish protection against lethal dose of *A. hydrophila*



Morphology and characterization of nano vaccine



Nano vaccine immunization and Zebrafish protection against lethal dose of *A. hydrophila*

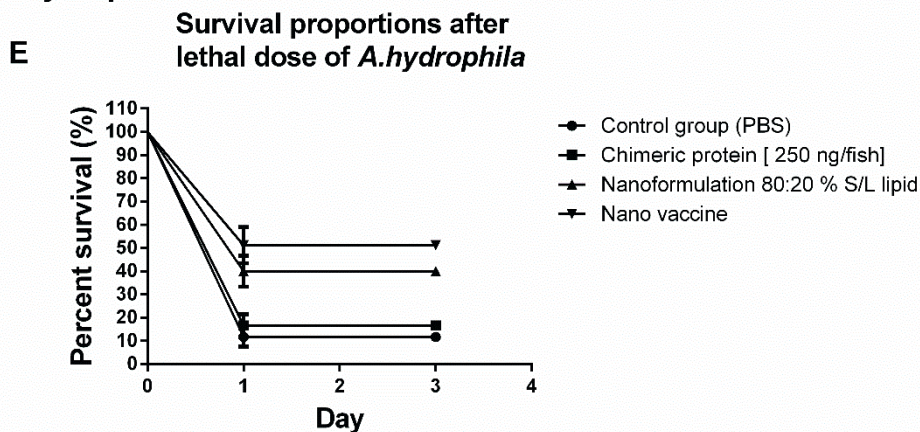


Figure 1: Chimeric recombinant protein nano vaccine characterization and zebrafish protection. (A) SDS-page of purified chimeric recombinant protein. (B) Challenge with lethal dose of *Aeromonas hydrophila* against vaccinated fish with 1 µg or 3 µg of chimeric recombinant protein and survival curve reported during 5 days. (C) Transmission electronic microscopy of chimeric recombinant antigen nanostructured lipid nano vaccine. (D) Nano vaccine size and dispersity evaluation. (E) Challenge with lethal dose of *Aeromonas hydrophila* against vaccinated fish with

250 ng of chimeric recombinant protein or chimeric recombinant protein nano encapsulated and survival curve reported during 4 days.

3.3 NANO-FORMULATION IN VITRO CYTOTOXICITY

The access of the chemical cytotoxicity is crucial to prove the safe status for use in animal or human's finality. The nano-formulations evaluated in this work were made by combination of glyceryl distearate as solid lipid, propylene glycol dicaprylocaprate as liquid lipid and tween 80 as surfactant in the following proportions: 90: 10, 80:20 or 70:30 % of solid-liquid lipid and 5 % of surfactant. Depending on the proportion, the particle size, dispersity, drug loading, stability and protein release parameters can change and those results can be accessed in previous work as cited above. Beside all components already considered safe and present in many medicines approved and available on market, the cell viability assay was performed to understand the toxic profile of the nano-formulations.

The MTT assay was performed on Vero cells, a mammalian cell lineage from Green Monkey Kidney, that were submitted a previous treatment with 100 mg of the formulations at 24 hours. The MTT named (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) is a compound that can penetrate live cells and be metabolized in the mitochondria by oxidoreductases enzymes to formazan crystals that can be solubilized and show a purple color which is measured by absorbance and a relative cell viability can be determined based on the colorimetry assay, then, as result, the more purple more live cells. The normalized absorbance was used to access the cytotoxic concentration that decrease cell viability in 50% (CC₅₀), as can be seen in figure 2 A. The maximum concentration (100 mg) decreased the cell viability when compared to the dilutions, however, even in high concentrations, the purple color is strongly present. Interestingly, the CC₅₀ of the formulations 90:10 (fig 2 B and C), 80:20 (fig 2 D and E) and 70:30 (fig 2 F and G) were respectively, 7, 55 and 35 mg, suggesting a high cytotoxicity profile depending on the amount of the solid lipid.

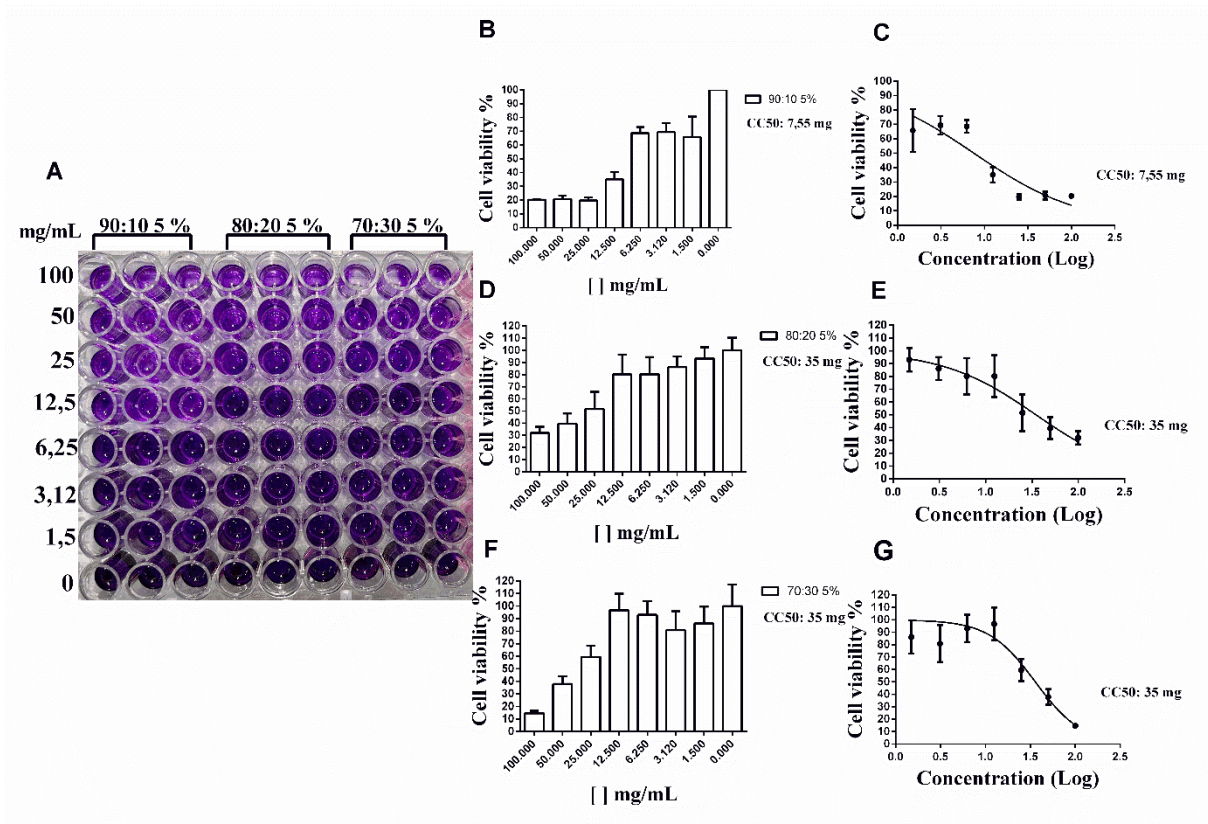


Figure 2: Nanostructured lipid formulations cytotoxicity. (A) Cell viability assay by MTT assay method using Vero cells and serial dilutions of nano formulations (100, 50, 25, 12,5, 6,25, 3,12, and 1,5 mg), the nano formulations proportion were 90:10, 80:20 and 70:30 of glyceryl ditearate: propylene glycol dicaprate 5 % of polysorbate 80, cells treated with DMEM medium were used as control. Cytotoxicity concentration that decrease cell viability in 50 % (CC_{50}) of nano formulation 90:10 (B and C). Cytotoxicity concentration that decrease cell viability in 50 % (CC_{50}) of nano formulation 80:20 (D and E). Cytotoxicity concentration that decrease cell viability in 50 % (CC_{50}) of nano formulation 70:30 (D and E).

3.4 OXIDATIVE STATUS

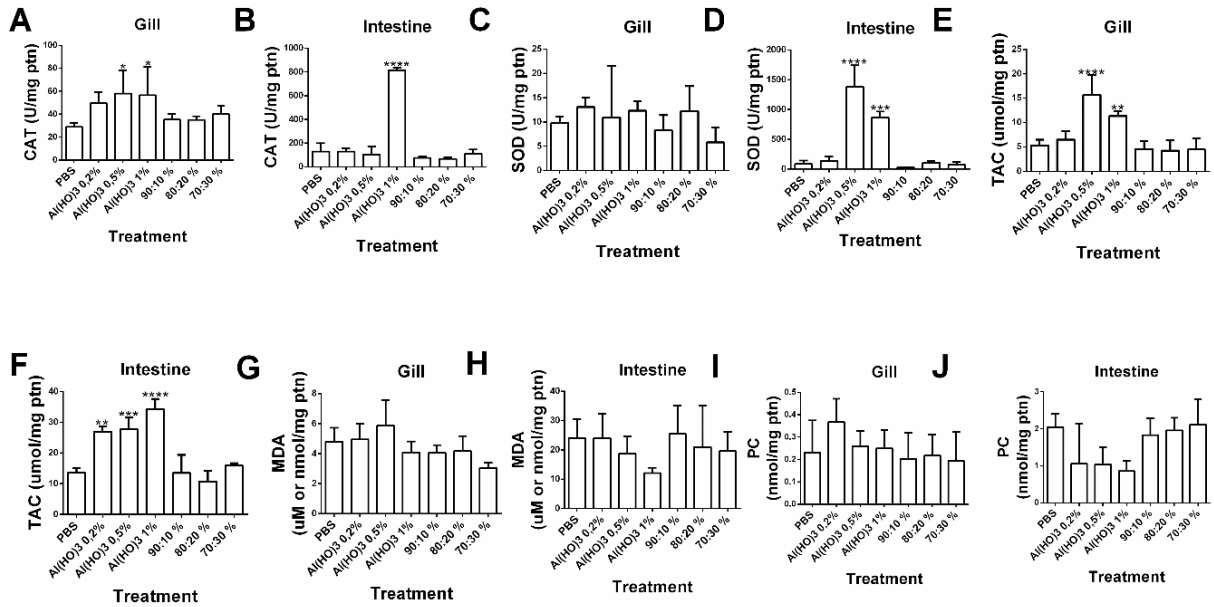
The adjuvant profile of chimeric antigen nanostructured lipid encapsulated was accessed and based on the zebrafish protection showed a effective response against *A. hydrophila* infection. In our previous reports, the aluminum hydroxide was used as adjuvant, although, showed high toxic profile in vivo, resulting in fish mortality and tissue damage. To continuous chowing the safe use of the nanostructured lipid nanoparticles proposed in this work, all blank nano-formulations (90:10, 80:20 and 70:30) were administered intraperitoneally in adult zebrafish, as well as different concentrations of aluminum hydroxide (0.2, 0.5 and 1 %). After 24 hours, the gills and intestine were harvested and after 48 hours the liver, gills and intestine

were harvested and all organs were processed and the supernatant was used to access the oxidative biomarkers profile. The catalase (CAT), superoxide dismutase (SOD), antioxidant activity (TAC), lipid peroxidation (MDA) and protein carbonyl (PC) were measured. Fish treated with saline solution (PBS) were used as a control group and samples with high concentration of the oxidative biomarkers, compared to control group, were considered indicative to of the toxicity of the nanoparticles or the adjuvant. No oxidative stress biomarker was statistically significant for fish treated with the nano-formulations in 24 hours, showing biomarkers levels similar to those found for the fish control group in gills (Fig 3 A, C, E, G, I) and intestine (Fig 3 B, D, F, H and J). Fish treated with all concentrations of aluminum hydroxide showed high levels of the oxidative stress biomarkers (Fig 3).

The liver, gill and intestine oxidative stress profile was accessed in 48 hours after the treatment with the nano-formulations and aluminum hydroxide concentrations and as expected only fish treated with aluminum hydroxide 0.2, 0.5 and 1 % obtained high levels of CAT, SOD, TAC, MDA and PC biomarkers when compared to control group (PBS) in all of organs, liver (fig 3 K, N, Q, T and W), gills (fig 3 L, O, R, U and X) and intestine (fig 3 M, P, S, V and X). Curiously, only a lipid peroxidation (MDA) high level was observed for nano-formulation 80:20 and 70:30 in gills (fig 3 U) and maybe the high level of liquid lipid can be related to this oxidative activity. Discutir os efeitos desses resultados (aumento dos danos em lipídios e proteínas e aumento das defesas antioxidantes) no sucesso da obtenção de reposta imune sem efeitos tóxicos para os peixes.

The oxidative status results corroborate the previous reports that aluminum hydroxide can cause toxicity for fish, and a molecular mechanism needs to be explored better to understand if aluminum hydroxide is safe for all fish species. The high oxidative biomarkers levels indicate an imbalance between pro-oxidative and antioxidative activity resulting in high levels of reactive oxygen species leading to cell malfunction by DNA damage, genetic mutations, protein function changes and apoptosis induction (“Oxidative Stress - an overview | ScienceDirect Topics,” n.d.).

Oxidative Stress (24 hours)



Oxidative Stress (48 hours)

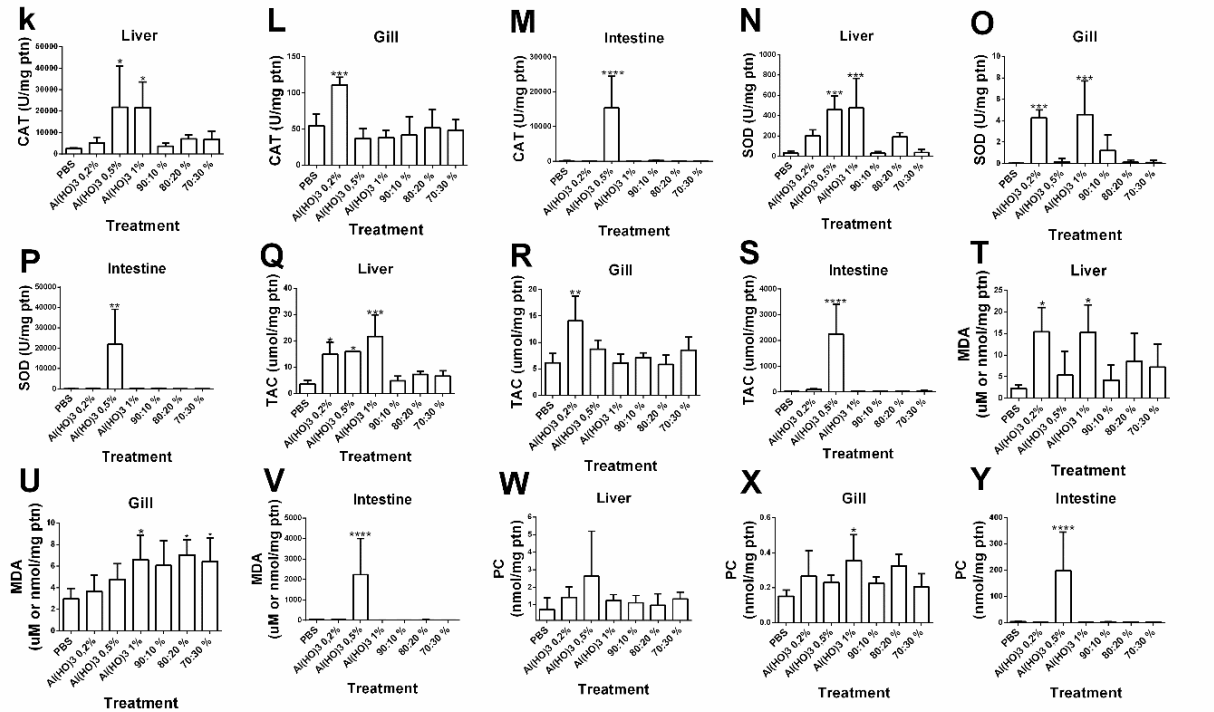


Figure 3: Oxidative stress evaluation. Evaluation of catalase (CAT), superoxide dismutase (SOD), total antioxidant capacity (TAC), lipid peroxidation (MDA) and protein carbonyl (PC) levels on Gills (A, C, E, G and I) and intestine (B, D, F, H and J) 24 hours after fish treatment with 100 mg of nano formulations (90:10, 80:20 and 70:30) and aluminum hydroxide (0.2, 0.5 and 1%) or saline solution (PBS). Evaluation of catalase (CAT), superoxide dismutase (SOD), total antioxidant capacity (TAC), lipid peroxidation (MDA) and protein carbonyl (PC) levels on liver (K, N, Q, T and W), gills (L, O, R, U and X) and intestine (M, P, S, V and X) 48 hours after fish treatment with 100 mg of nano formulations (90:10, 80:20 and 70:30) and aluminum hydroxide (0.2, 0.5 and 1%) or saline solution (PBS).

1 %) or saline solution (PBS). The statistic significance was performed by Graph Prism 7.0 and the results were submitted to ANOVA, multiple comparisons, and only results with $p < 0,5$ was considered significative.

4. CONCLUSION

The nano vaccine proposed in this work based on nanostructured lipid carrier showed satisfactory results in potentialize fish immune response against the virulent strain of *Aeromonas hydrophila* and no exhibit apparent toxic profile. The safety status was also evaluated by cytotoxic and oxidative stress assays and how expected, no acute toxic profile was observed in the mammalian cells and zebrafish organs, therefore, the results indicate an strong potential use of lipids nanoparticles for fish vaccine delivery and further analysis of molecular mechanisms of how this protection occur, the histopathological analysis and new routes of administration with focus in the oral route must be explored. The commercialized adjuvant based on aluminum hydroxide in all proportions tested here presented toxic profile in vivo with the increase of oxidative stress biomarkers level in all zebrafish organs, call an attention for their use as animal adjuvant.

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CHAPTER 4

PROBIOTIC EVALUATION OF *LACTOCOCCUS LACTIS* AND *LACTOBACILLUS ACIDOPHILUS* AGAINST *AEROMONAS HYDROPHILA* AND *PSEUDOMONAS FLUORESCENS*.

PAPER 4

THE *LACTOCOCCUS LACTIS* AND *LACTOBACILLUS ACIDOPHILUS* PROBIOTIC EVALUATION IN VITRO AND FISH ANTIMICROBIAL ACTIVITY AGAINST *AEROMONAS HYDROPHILA* AND *PSEUDOMONAS FLUORESCENS*?

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ABSTRACT

The *Lactobacillus acidophilus* and *Lactococcus lactis* are Lactic Acid Bacteria (LAB) found in human and animal intestinal microbiome with probiotic properties. Probiotics are live microorganisms with a beneficial role in host nutritional metabolism, immunological and physiological systems. The antimicrobial mechanism observed in probiotics is considered an important approach to the aquaculture field. The aquaculture industry is threatened by pathogenic microorganisms such as bacteria, viruses, fungi, and parasites, resulting in fish mortality and economic loss. Probiotics combined with vaccination are the gold standard for fish infection disease control. Therefore, we aim to evaluate if the strains of *L. lactis* and *L. acidophilus* isolated from human probiotic supplements can act like fish probiotics against fish pathogens *Aeromonas hydrophila* and *Pseudomonas fluorescens*. The LAB in vitro antimicrobial activity, cell-free supernatant antimicrobial activity, and antioxidant profile were assessed. The in vivo toxicity role was assessed in adult zebrafish. The probiotic role was evaluated in juvenile zebrafish with 3 months of age after treatment with commercial fish feed mixed with *L. lactis* or *L. acidophilus* suspension and a challenge assay was performed using *Aeromonas hydrophila* and *Pseudomonas fluorescens*. The results showed an in vitro antimicrobial activity of both LAB strains only against *A. hydrophila* IOC23116 strain and a slightly better performance against *P. fluorescens* 07A but no antimicrobial activity was observed against the *A. hydrophila* ATCC 7966. The in vitro results indicate that fermentation acidity is the main antimicrobial mechanism involved, however, a non-significant probiotic response was observed in vivo. The mechanism of probiotic bacterial activity is dependent of bacterial strains and isolates, fish age, fish development, and host colonization.

1. INTRODUCTION

Fish is a good source of proteins, vitamins and minerals that exert important role in human nutrition and health (Fiorella et al., 2021). With high demands of fish, the chain production is threatened by infectious bacteria, fungi and virus that take advantage of the decrease of fish immune response caused by stress factors originated from handling, transport and high stocking density. Among the strategies to minimize such effects, the use of probiotics in feed stands out. Probiotics are living microorganisms that can be ingested by the host and confer beneficial properties to host digestive, immune system and gut-brain axis. Bacterial strains with probiotic role have been used in aquaculture to mitigate the damage caused by pathogen microorganisms. Probiotics can establish in the host gut and prevent the growth of harmful pathogens acting in the gut acidification or producing antimicrobial molecules, in addition, they can favor a better absorption of nutrients, as well vitamin synthesis, increasing the host growth performance (Martínez Cruz et al., 2012; Simón et al., 2021a).

The use of probiotics as an alternative control method to those traditionally used (antibiotics) is based on their ability to generate defenses against pathogens and modulate the immune system competing for binding sites and nutrients with undesirable bacteria, producing antimicrobial metabolites and carrying antigens shared by groups of pathogens (Balcázar et al., 2006; Parra et al., 2024; Rahayu et al., 2024; Verschuere et al., 2000). Currently, engineered probiotic that can express antimicrobial peptides (AMPs) under surface and promote dual strategy to deal with infectious diseases is a new control method class of interests (Laguna et al., 2024; Mathipa and Thantsha, 2017).

The most important groups of bacteria probiotics comprise, lactic acid bacteria (LAB), *Bacillus*, *Lactococcus*, *Streptococcus*, *Enterococcus*, *Alteromonas*, *Arthrobacter*, *Bifidobacterium* and *Streptomyces* as well as some eukaryotic microorganisms such as microalgae *Tetraselmis* and yeast from genera *Debaryomyces*, *Phaffia* and *Saccharomyces* (Mandal et al., 2014; Parra et al., 2024).

Lactococcus lactis and *Lactobacillus acidophilus* are gram positive lactic acid bacteria with extensive use in food supplementation. *Lactobacillus acidophilus* naturally exists in the gastrointestinal tract and vagina of humans, and produces organic acids, hydrogen peroxide, diacetyl, ammonia and bacteriocins, generally accepted as human probiotic (Neish, 2017; Ozogul et al., 2021; Ozogul and Hamed, 2016). *Lactococcus lactis* is an important member of industrial and artisanal starter cultures with a crucial role in dairy fermentation carrying the generally recognized safe (GRAS) status. There are several subspecies of *L. lactis*, such as

cremosis, lactis, and lactis biovar diacetylactis. *L.lactis* genome is recognized to carry plasmids genes involved in lactose utilization, casein breakdown, bacteriophage resistance, bacteriocin and exopolysaccharide production (Cavanagh et al., 2015; Kazou, 2021; Mills et al., 2011). Both probiotics strains showed antimicrobial activity in aquaculture, evidenced by many works that treated fish and challenged them with pathogenic strains of *Aeromonas hydrophila*, *Aeromonas veronii*, *Enterococcus faecalis*, *Aeromonas salmonicida*, *Vibrio anguillarum* and *Pseudomonas fluorescens*, and resulted in resistant fish against infectious disease and great growth performance (Cano-Lozano et al., 2022; Hoseinifar et al., 2015; Nguyen et al., 2018; Qiu et al., 2024; Wu et al., 2023).

Zebrafish is extensively used as animal model to evaluate the probiotic potential of bacterial species and pathogen infection mechanisms due to handling facility and host immune response characterization. *Aeromonas hydrophila* and *Pseudomonas fluorescens* are gram-negative pathogenic species with massive reports of their infection in fish (AlYahya et al., 2018; Boonthai et al., 2018; Vaz Rodrigues et al., 2019; Wang et al., 2019). Based on the *L. lactis* and *L. acidophilus* fish probiotics properties evaluated in many studies and the harmful fish pathogenicity of *A. hydrophila* and *P. fluorescens*, we aimed to evaluate the in vitro and in vivo antimicrobial activity of *Lactococcus lactis* and *Lactobacillus acidophilus* against *Aeromonas hydrophila* and *Pseudomonas fluorescens*, and their potential as growth promoters for fish.

2. MATERIAL AND METHODS

2.1 BACTERIA STRAIN AND CULTURE CONDITIONS

The *Lactobacillus acidophilus* and *Lactococcus lactis* (lactic acid bacteria - L.A.B) strains were donated by Genetic and Molecular Laboratory from Federal University of Minas Gerais and cultivated in Man, Rogosa, Sharpe Broth (MRS Broth) at 37°C under aerobic or anaerobic conditions. The *A. hydrophila* ATCC 7966 and IOC23116 strains were genteelly donated by Enterobactérias Laboratoty from Fundação Oswaldo Cruz (FIOCRUZ) and the *Pseudomonas fluorescens* (07A) strain was genteelly donated by Microbiologia Industrial Laboratory from Universidade Federal de Viçosa (UFV) and were aerobic cultured in Trypticase Soy Broth (TSB) medium at 30 °C under agitation (180 rpm) when necessary.

2.2 LACTIC ACID BACTERIA ANTIMICROBIAL ASSAY

The spot-on-lawn assay was performed to evaluate the antimicrobial activity of *Lactobacillus acidophilus* and *Lactococcus lactis* against *Aeromonas hydrophila* ATCC 7966, IOC 23116 and *Pseudomonas fluorescens* 07A. Briefly, 20 μ L of *A. hydrophila* or *P. fluorescens* were placed in Erlenmeyer's flasks containing 5 mL of TSB medium at 30° C under agitation to achieve an Optical Density (OD) of 0.6, then, 50 μ L of each strain was added in test tubes containing 6 mL of semi-solid TSB. Posteriorly, the tubes were homogenized and poured under petri dishes already containing previously MRS agar inoculated with 1 μ L of each LAB strain (spot) incubated at 24 hours under aerobic or anaerobic conditions. The petri dishes were incubated in aerobic conditions at 30 °C by 24 hours to promote the *A. hydrophila* or *P. fluorescens* growth and the antimicrobial activity was evaluated by formation of inhibition zone (no visible microbial growth around the LAB spot) (De Martinis and Franco, 1998; Muñoz-Atienza et al., 2014).

2.3 CELL-FREE SUPERNATANT ANTIMICROBIAL ASSAY

The antimicrobial potential from cell-free supernatant of *L. lactis* and *L. acidophilus* was evaluated as following steps. Firstly, MRS medium containing *L. lactis* or *L. acidophilus* cultured by 24 hours was added in Eppendorf tubes of 1,5 mL and centrifuged for 5 minutes, 4000g at 4 °C and the supernatant harvested and filtered in membrane of 0.22 μ m for posterior use. Petri dishes containing agar TSB were inoculated with 100 μ L of *A. hydrophila* or *P. fluorescens* suspension and spread on all surface. Posteriorly, wells of 8mm were made in the medium by a sterile sharp and 50 μ L of the cell-free supernatant was inoculated in each well to diffusion process and incubated at 30 °C. Non-inoculated MRS medium was used as control. The antimicrobial potential was evaluated before and after pH correction and inhibition zone around the wells were considered positive (Kaewchomphunuch et al., 2022).

2.4 ANTIOXIDANT AND BIOCHEMISTRY CONTENT

2.4.1 THE ABTS ASSAY

To access the antioxidant effect of probiotic cell-free supernatant, the ABTS (2,2'-azino-

bis (3-ethylbenzothiazoline-6-sulfonic acid)) assay was performed. The ABTS+ was produced by mixing 7mM of ABTS and 88 μ l of potassium sulfate 140mM. The ABTS+ solution was stored in the dark at room temperature. The ABTS solution was diluted in water to reach an absorbance of 0.70 ± 0.05 and posteriorly 10 μ l of cell-free supernatant diluted 10x were mixed with 190 μ l of ABTS+ solution and incubated for 30 minutes and the percent inhibition was accessed by absorbance at 734 nm used in the following equation: % Scavenging activity = $100 \times (\text{Abs of control} - (\text{Abs of sample} - \text{Abs of blank}))/\text{Abs of control}$. The experiments were done in triplicate and the control was prepared mixing 190 μ l of ABTS+ solution and 10 μ l of M.R.S medium diluted 10x (Khatri and Chhetri, 2020; Sornsenee et al., 2021a; Zhang et al., 2022).

2.4.2 THE DPPH ASSAY

To access the antioxidant effect of probiotic cell-free supernatant, the DPPH (2,2-Diphenyl-1-picrylhydrazyl) assay was performed. The probiotic cell-free supernatant was diluted 10x and 25 μ l was mixed with 175 μ l of DPPH 0.1 mM and incubated in the dark by 30 minutes and the percent inhibition was accessed by absorbance at 517 nm and used in the following equation: % Scavenging activity = $100 \times (\text{Abs of control} - (\text{Abs of sample} - \text{Abs of blank}))/\text{Abs of control}$. The experiments were done in triplicate and the control was prepared mixing 175 μ l of DPPH 0.1 mM solution and 25 μ l of M.R.S medium diluted 10x (Khatri and Chhetri, 2020; Sornsenee et al., 2021a; Zhang et al., 2022).

2.4.4 THE FLAVONOIDS CONTENT

The total flavonoids content (TFC) of the probiotic cell-free supernatant was determined using the aluminum chloride colorimetric method incubating 24 μ l of cell-free supernatant diluted 25x or quercetin (5-200 μ g/mL) with 9.5 μ l of sodium nitrate 10% and 157.7 μ l of water for 6 minutes at room temperature. Posteriorly, 18.2 μ l of 5% AlCl_3 solution was added and incubated for 6 minutes at room temperature and then 60 μ l of NaOH 1M was mixed and the absorbance was measured at 425 nm. The TFC was calculated from a calibration curve and the results was expressed as mg of quercetin equivalents (QE) per mL of cell-free supernatant (Khatri and Chhetri, 2020; Sornsenee et al., 2021a; Zhang et al., 2022).

2.4.4 THE PHENOLICS CONTENT

The Folin-Ciocalteu method was used to perform the total phenolic content (TPC) assay with some modification. Briefly, 10 µl of probiotic cell-free supernatant diluted 2x was mixed with 165 µl of 10% Folin-Ciocalteu reagent (Sigma-Aldrich, St.Louis, USA) in a 96-well plate and incubated for 3 minutes. Posteriorly, 100 µl of Na₂CO₃ 20% (m/v) was added and incubated for 30 minutes and the absorbance read at 650 nm. A standard curve was plotted using gallic acid with a concentration range of 1-100 µg/mL. The TPC was determined by gallic acid equivalence (GAE) in mg/mL of cell-free supernatant (Khatri and Chhetri, 2020; Sornsenee et al., 2021a; Zhang et al., 2022).

2.5 ANIMAL ETHICS APPROVAL AND MAINTENANCE

The experiments with zebrafish, *Danio rerio*, were conducted after Animal Production Ethic Committee (CEUAP) approval (Protocol 39/2023). The zebrafish were obtained from Fisiologia Aplicada a Piscicultura Laboratory (LAFAP) and manipulated in LAPAF and Proteômica e Bioinformática Laboratory of Biotecnologia Aplicada a Agropecuária (BIOAGRO) institute from UFV. The fish manipulation and maintenance respected the Zebrafish Breeding Manual (Dammski et al., n.d.). Briefly, fish were kept in tanks of 20 L (30 N) or 10 L (10 N) with water aeration, mechanical and biological filters, temperature of 27 °C through thermostats. The laboratory was kept in light and dark cycles by 12h/12h, using a timer. Fish were fed twice a day with the experimental rations, and health was evaluated by behavior (movement/appetite), physiology profile, and clinical diseases signs.

2.6 INFECTION AND TOXICITY EVALUATION

The *in vivo* toxicity of *L. lactis* and *L. acidophilus* strains was evaluated by injection of 10µl of each strain in fish peritoneum. Adult zebrafish were injected with bacteria suspension at 0,5 Mcfarland standard (1,5 X 10⁸ cells) and randomly distributed in tanks of 10 L (10N). The control group received 10 µl of saline solution (NaCl 0,9%). In addition, 10 µl of fish bacteria pathogen *Pseudomonas fluorescens* 07A or 10 ul of *A. hydrophila* 7966 was also administered to compare the toxicity profile with the probiotic strains. The toxicity were evaluated by clinical signals, symptoms and mortality during 7 days ((Pacheco et al., 2021a; Yi et al., 2019).

2.7 PROBIOTIC FISH FEED FORMULATION

The L.A.B strains were cultivated in MRS medium during 24 hours in Erlenmeyer's of 50 mL and in the following day the cell suspension was centrifuged for 10 minutes, 5000g and washed twice with saline solution. The cells pellet were resuspended in 5 mL of PBS and each *L.lactis* or *L.acidophilus* suspension was inoculated under the fish feed at 10^8 UFC/g and let dry by 24 hours. The probiotic fish feed formulation was prepared every day and administered twice for day. The cells viability was evaluated during 60 days under 4°C and 42°C by spread plate, briefly, 1g of each probiotic formulation was added in 9mL of PBS and after serial dilutions 100 µl of each dilution was spread under MRS medium, incubated during 24 hours at 37 °C and the viability evaluated (Abomughaid, 2020; Pacheco et al., 2021a).

2.8 PROBIOTIC FISH FEED TREATMENT

Juveniles zebrafish with 3 months of life and in growth stage were cultivated in tanks of 20 L and randomly distributed (N: 30 per tank) in 9 tanks (3 per group) an maintained as described above. Fish weight was measured before the beginning of the treatments with probiotic fish feed formulation. The treatment consisted in three groups: fish treated with commercial feed mixed with saline solution (G1) or mixed with *L. lactis* suspension (10^8) (w/v) (G2) or mixed with *L. acidophilus* suspension (10^8) (w/v) (G3). All treatments were administered twice per day during 60 days and posteriorly the fish weight was measured and the weight gain was accessed by the following formula: final weight – initial weight (Davis et al., n.d.; Mani et al., 2021; Pacheco et al., 2021b).

2.9 FISH CHALLENGE WITH AEROMONAS HYDROPHILA AND PSEUDOMONAS FLUORESCENS

The *A. hydrophila* ATCC 7969 and *P. fluorescens* 07A strains were cultured as described above and 10 µl of each bacteria was administrated intraperitoneally in fish after experimental time of 60 days with commercial feed mixed with saline solution (G1) or mixed with *L. lactis* suspension (10^8) (w/v) (G2) or mixed with *L. acidophilus* suspension (10^8) (w/v) (G3). The infected fish with clinical signs were observed daily. The mortality was observed during 7 days after challenge and the survival rate was analyzed by Graph Prism 7.0 (Yi et al.,

2019).

2.10 HISTOPATHOLOGIC EVALUATION

The intestine of six zebrafish of each experimental group was collected to histopathologic evaluation. The samples were fixed with 10 % of formaldehyde and dehydrated in a graded series of ethanol and xylene and posteriorly embedding in paraffin wax. The samples were cut into sections of 5 µm thickness and stained with hematoxylin and eosin (H&E) posteriorly observed under light microscope. The results were submitted to ANOVA and considered statistically significant with p value <0,05 (He et al., 2017).

3. RESULTS AND DISCUSSION

3.1 THE *LACTOCOCCUS LACTIS* AND *LACTOBACILLUS ACIDOPHILUS* ANTIMICROBIAL ACTIVITY IN VITRO

3.1.1 DIRECTED ANTIMICROBIAL ACTIVITY

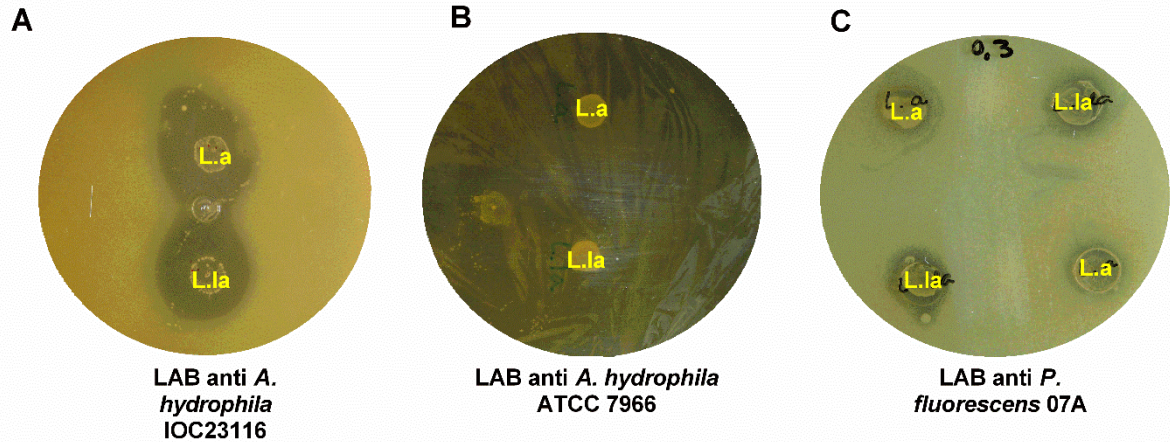
The *in vitro* antimicrobial activity of *L. lactis* and *L. acidophilus* against *A. hydrophila* and *P. fluorescens* was accessed by the spot-on-law or cell-free supernatant assay (Fig 1). In case of a directed antimicrobial activity, the spot of LAB strains would inhibit the growth of pathogenic bacteria around their spot. Probiotic bacteria can secret antimicrobial compounds in the medium such as bacteriocins, organic acids and H₂O₂, *L. acidophilus* for example is recognized to produce lactacin B and acidolin bacteriocins as well as the bacteriocin nisin by *L. lactis* (Fijan, 2023). Therefore, in case of antimicrobial compounds production, the cell-free supernatant would inhibit the growth of the pathogenic bacteria by the diffusion of the supernatant under the medium.

The results showed a significant directed antimicrobial activity against *A. hydrophila* IOC 23116 strain (Fig 1 A), evidenced by the presence of an prominent inhibition zone around the spot of *Lactococcus lactis* and *Lactobacillus acidophilus*. Curiously, antimicrobial activity against *A. hydrophila* ATCC 7966 (Fig 1 B) was not observed. Interestingly, the strain IOC 23116 was isolated from infected fish but did not show virulent profile for zebrafish and for this this strain could not be used for *in vivo* assay. The same results were observed when L.A.B was cultivated in aerobic or anaerobic conditions, therefore, the presence or lack of oxygen did not affect the antimicrobial activity. The antimicrobial activity against *P. fluorescens* 07A (Fig

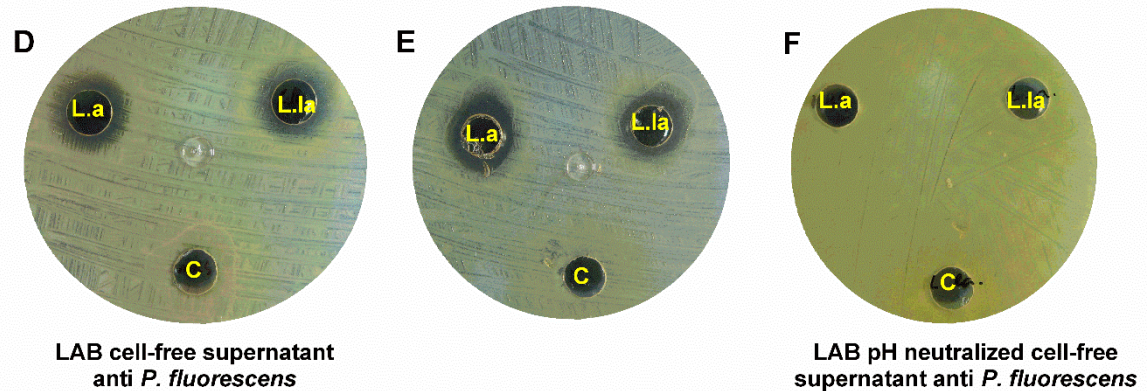
1 C) was observed for *L. acidophilus* and *L. lactis*, although, the inhibition zone was less prominent and possibly the assay was affected by the difficulty to perform the spot-on-lawn assay for *P. fluorescens* and some modifications were required since *P. fluorescens* wasn't able to growth under M.R.S medium. The modifications consisted in a medium transplant of M.R.S comprising the LAB spots inside of some wells made in the agar TSB.

The in vitro antimicrobial activity of lactic acid bacteria isolated from fish were evaluated by Muñoz-Atienza et al. (2014) and, as expected, showed a significant activity against *Tenacibaculum maritimum* and *Vibrio splendidus*, both fish pathogens associated with high mortality of turbot fish, *Psetta maxima*. The major studies use to isolate potential probiotic bacteria from their host, this is not different in aquaculture field, bacteria strains that showed significant antimicrobial activity commonly were founded colonizing the host intestine. The LAB strains evaluated in this work was not isolated from fish and maybe this is the reason of less effectivity.

Spot-on-law assay



Cell-free supernatant assay



Antioxidant assay of cell-free supernatant

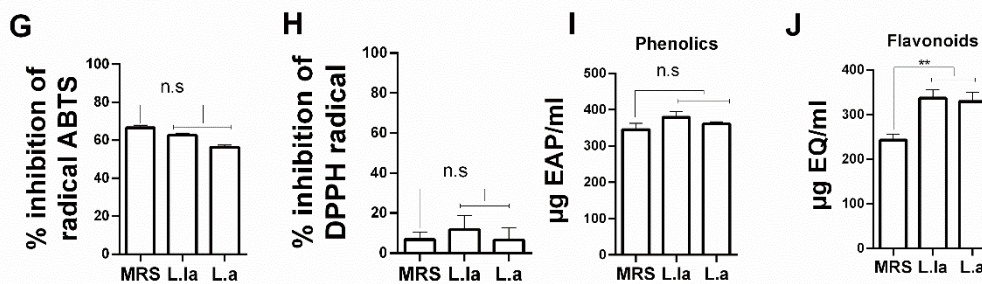


Figure 1: L.A.B antimicrobial and antioxidant activity. (A) *L. lactis* (L.la) and *L. acidophilus* (L.a) antimicrobial activity against *Aeromonas hydrophila* IOC23116. (B) *L. lactis* (L.la) and *L. acidophilus* (L.a) antimicrobial activity against *Aeromonas hydrophila* ATCC 7966. (C) *L. lactis* (L.la) and *L. acidophilus* (L.a) antimicrobial activity against *Pseudomonas fluorescens* 07A. The antimicrobial activity was considered significant by the presence of inhibition zone around the L.A.B spot. Antimicrobial activity of cell-free supernatant from *L. lactis* (L.la), *L. acidophilus* (L.a) and MRS medium (control) without pH neutralization (7.0) against *Pseudomonas fluorescens* (D) and *Aeromonas hydrophila* (E) and after pH neutralization (F). Antioxidant and compound content of probiotic cell-free supernatant. (G) ABTS inhibition percent from *L. lactis* (L.la) and *L. acidophilus* (L.a) and only medium (MRS). (H) DPPH inhibition percent from *L. lactis* (L.la) and *L. acidophilus*

cell-free supernatant (L.a) and only medium (MRS). **(I)** Total Phenolic content of *L. lactis* (L.la) and *L. acidophilus* cell-free supernatant (L.a) and only medium control (MRS). **(J)** Total flavonoids content of *L. lactis* (L.la) and *L. acidophilus* cell-free supernatant (L.a) and only medium control (MRS). The statistical analysis was made in GraphPrism 7.0 and the results was considered significant with p value <0,05. The one-way ANOVA test was chosen to evaluate the significance and only flavonoids content showed a p value of ** (0,001).

3.1.3 CELL-FREE SUPERNATANT ANTIMICROBIAL ACTIVITY

Biological process such as fermentation is a strong characteristic of L.A.B strains and products originated from this process can act as an antimicrobial agent. The cell-free supernatant from *L. lactis* and *L. acidophilus* was harvested, filtered and the antimicrobial activity accessed before and after pH correction. As can be seen in figure 1, both *L. lactis* and *L. acidophilus* cell-free supernatant were able to inhibit the *Pseudomonas fluorescens* (Fig 1 D) and *Aeromonas hydrophila* growth (Fig 1 E), resulting in inhibition zone around the wells only in acidity conditions, however, the antimicrobial activity was not observed when the pH of the cell-free supernatant was neutralized (Fig 1 F). These results indicate as expected a strong organic acid production and no bacteriocins antimicrobial activity.

Mechanisms associated with probiotic antibacterial activity comprise production of inhibitory substances and is not knew that acid compounds such as acetic, butyric, lactic, propionic acids have a crucial role in to control the pH of gastrointestinal sites of the host and avoid the establishment of pathogenic bacteria (El-Saadony et al., 2021; Simón et al., 2021b), Scillato et al. (2021) showed antimicrobial proprieties of cell free supernatant from LAB strains founded in vaginal microbiota of health females and the results were significantly positive against multidrug urogenital pathogens *Enterococcus faecium* and *Enterococcus faecalis* and the enzymatic activity of lactic dehydrogenase with D- and L- lactic acid production was the main point of the bactericidal mechanism.

3.1.3 CELL-FREE SUPERNATANT ANTIOXIDANT EVALUATION

The probiotic cell-free supernatant showed an anti *A. hydrophila* and *P. fluorescens* activity only in acidity conditions, and to certify that the antioxidant activity did not contribute with the bactericidal activity, the ABTS and DPPH assays were performed, as well as the phenolic and flavonoids content accessed (Fig 1). The results corroborate the cell-free supernatant anti *A. hydrophila* and *P. fluorescens* activity only in acidity conditions since the

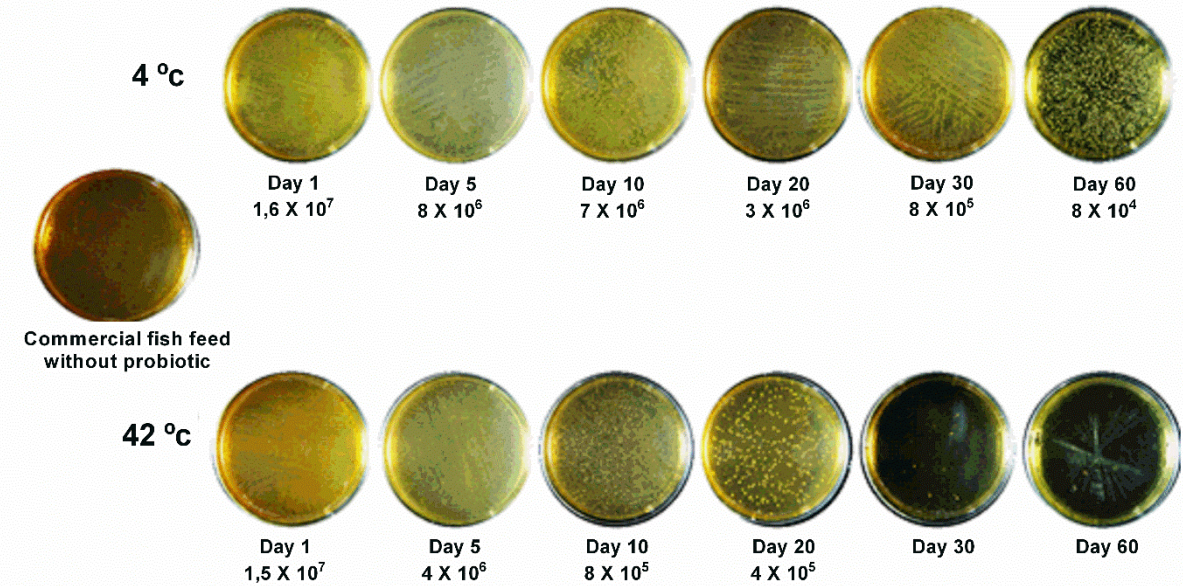
inhibition of DPPH and ABTS radicals did not show significant difference when compared with control, composed by fresh M.R.S medium (Fig 1 G and H, respectively). Oxidative stress is defined as an imbalance between prooxidant-antioxidant factors, contributing with cell damage by the DNA hydroxylation, protein denaturation, lipid peroxidation and apoptosis. It is not known if probiotic bacteria produce many antioxidant compounds related to the anti-bacterial mechanism (Wang et al., 2017; Zhou et al., 2022). As can be seen in figure 1, the phenolic content (Fig 1 I) wasn't significantly present in the cell-free supernatant when compared to M.R.S medium control, only flavonoids content (Fig 1 J) presented significant value, however, flavonoids content did not contribute with the anti-bacterial activity of *L. lactis* and *L. acidophilus* probiotics evaluated in this work.

During lactic acid bacteria fermentation process, phenolic and flavonoids compounds are produced as final products. Sornsenee et al. (2021b) showed a significant antioxidant activity related to high phenolics and flavonoids content from 10 *Lactobacillus* strains and all of them presented antimicrobial and antibiofilm properties against *S. aureus*, *A. baumannii* and *E. coli*. Curiously, the LAB strains evaluated in this work presented significant flavonoids content, but with no antioxidant properties.

3.2 PROBIOTIC STORAGE CONDITIONS

Probiotics are already available in some commercial fish feed, and the probiotic storage condition is a crucial factor to analyze. The viability of *L. lactis* and *L. acidophilus* strains used in this work were evaluated by plate counting under 4 °C and 42 °C degrees during 60 days (Fig 2). The bacteria suspension at 1×10^8 concentration was mixed with commercial fish feed presenting only probiotics in their composition. The *L. acidophilus* has long time viability evidenced by the presence of bacteria colonies under 4 °C and 42 °C with long viability in 4 °C degrees than 42 °C degrees. The *L. lactis* temperature resistance also showed great results, although, the viability was less evidenced than *L. acidophilus* in both temperature conditions. Aly et al. (2008), also evaluated the viability of probiotic bacteria mixed with fish feed and founded better viability when *B. subtilis* and *L. acidophilus* were mixed together at 4 °C then at 25 °C. Not only probiotic role of LAB is important but also food preservation, the application of microorganisms with bio preservation role can be an alternative of chemical treatment, since their compounds such organic acids can extend the shelf life and increase the food safety (Zapašnik et al., 2022).

Probiotic formulation with commercial feed and *L. acidophilus*



Probiotic formulation with commercial feed and *L. lactis*

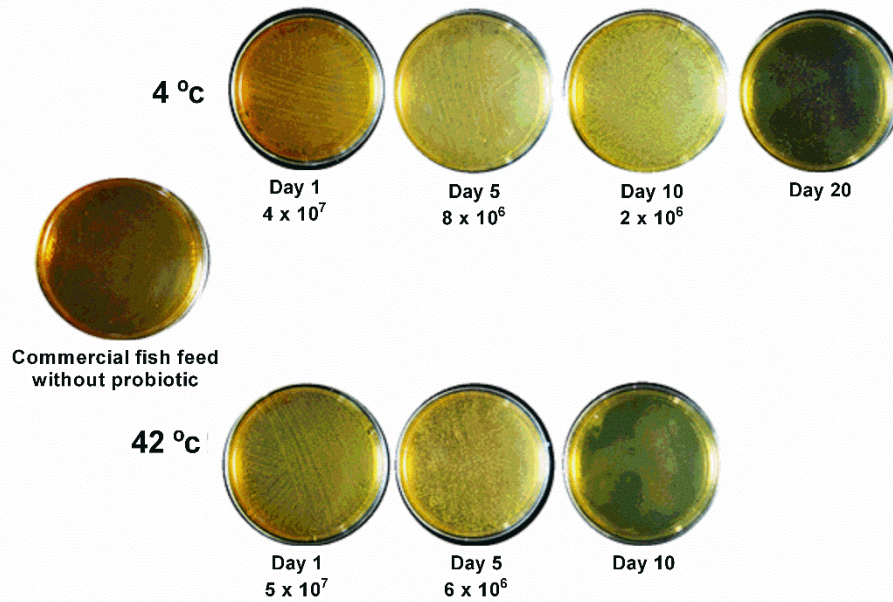


Figure 2: LAB temperature resistance evaluation. The viability of *L.lactis* probiotic mixed with commercial fish feed with only prebiotic in composition was evaluated by plate counting during 60 days under 4 °C and 42 °C storage conditions .The viability of *L.lactis* probiotic mixed with commercial fish feed with only prebiotic in composition was evaluated by plate counting during 60 days under 4 °C and 42 °C storage conditions .

3.3 THE *LACTOCOCCUS LACTIS* AND *LACTOBACILLUS ACIDOPHILUS* IN VIVO EVALUATION

3.3.2 LAB TOXICITY

The *L. lactis* and *L. acidophilus* toxicity was evaluated to assess their potential pathogenicity in fish, and their safety status as probiotic control method (Fig 3). The toxicity assay was performed inoculating 10 µl of each bacteria suspension intraperitoneally and mortality and clinical symptoms observed during 7 days (fig 3 A). The results showed a non-toxic role of the *L. lactis* and *L. acidophilus* with no mortality reported, keeping the percent survival of zebrafish in 100%. High mortality was registered only for fish infected with *Pseudomonas fluorescens* and *Aeromonas hydrophila*, as well as clinical symptoms such as skin depigmentation, skin hemorrhage, organ necrosis and organ hemorrhage (Fig 3 B). Corroborating the results of survival rate, no histopathological damage was founded in the intestine of juvenile fish treated orally with commercial fish feed mixed with *L. lactis* or *L. acidophilus* (Fig 3 C, D, E, F, G) and no intestine damage was found in the intestine of juvenile fish treated with commercial fish feed mixed with *L. lactis* (I) or *L. acidophilus* (J), resulting in a intestine structure similar those fish without treatment (H).

Fish toxicity evaluation of *L. lactis* and *L. acidophilus*

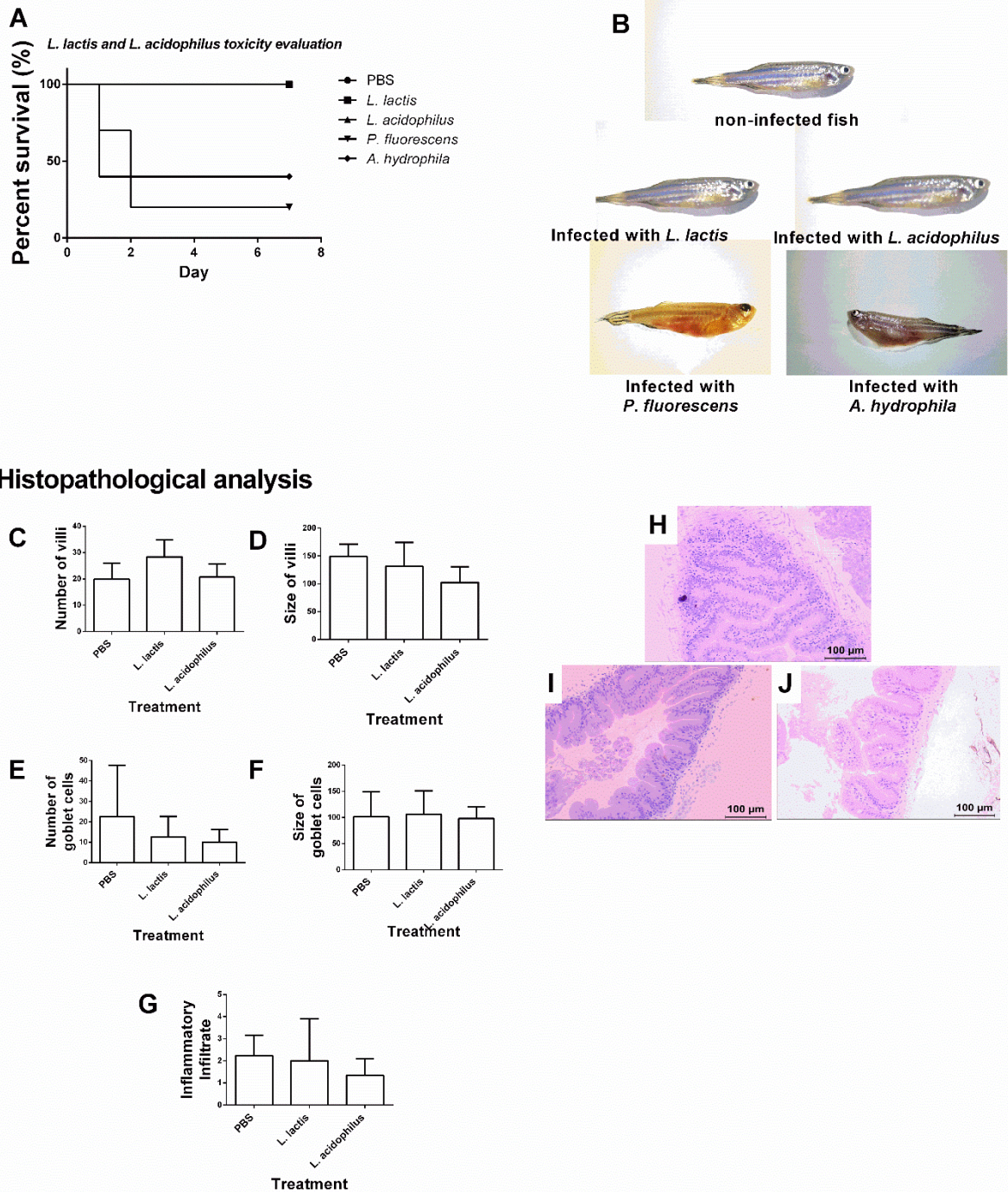


Figure 3: L.A.B toxicity in vivo evaluation. (A) Percent survival of adult zebrafish intraperitoneally infected with *L. lactis* (100%), *L. acidophilus* (100%), saline solution (PBS) (100%), *P. fluorescens* (20%) and *A. hydrophila* (40%) after 7 days of challenge. (B) Clinical symptoms of infected fish with saline solution, 10^8 of *L. lactis*, 10^8 of *L. acidophilus*, 10^8 of *P. fluorescens* and 10^8 of *A. hydrophila*. Histopathological analysis of the intestine juvenile zebrafish treated with commercial fish feed mixed with saline solution (PBS), *L. lactis* or *L. acidophilus* suspension, number of villi (C), size of villi (D), number of goblet cells (E), size of goblet cells (F), inflammatory infiltrate (G). Intestine micrography of fish without treatment (H) or treated with commercial fish feed mixed with *L. lactis* (I) or *L. acidophilus* (J). The statistical analysis were made in GraphPrism 7.0 and the

survival percent was considered significant with p value <0,05. The Lon-rank test was chosen to evaluate the significance and the p value was **** (<0,0001).

3.3.3 INFLUENCE OF LAB IN FISH GROWTH

The *L. lactis* and *L. acidophilus* evaluated in this work did not present benefits in fish body weight gain when compared with fed with fish fed commercial feed mixed with saline solution (Table 1 and Figure 4). Curiously, during the experiment time, mortality rate was observed in treated groups, and fish that were fed with probiotic bacteria *L. lactis* and *L. acidophilus* presented a mortality percent of 23% and 14,4% respectively while the control group the mortality rate was 8%.

Some factors can explain the reason why this probiotics bacterium did not present significative nutritional benefits to fish treated. The age of zebrafish used in this experiment for example, juvenile's zebrafish with 3 months of life possess the immunological system in development and no completely able to deal with massive bacteria administration, in addition, their maturation is also related to fish size and body weight gain and the stress may have negatively influenced fish maturation and homeostasis (Singleman and Holtzman, 2014). The age factor can be evidenced since the tanks with smaller (0.05 g) fish the mortality rate was intensified. Differently from the results obtained in this work, Dawood et al. (2016) reported that a mixing of 10^6 cell/g of *Lactobacillus rhamnosus* and *Lactococcus lactis*, together or alone, with basal fish feed, had great positive influence on red sea bream growth performance when compared with fish without probiotic supplementation. Hoseinifar et al. (2015) also showed a significant weight body gain in black swordtail fish supplemented with *L. acidophilus* mixed in fish feed. In both works the body gain seems to be related with beneficial modulation of intestine microbiota and with the activation of genes related with nutritional metabolism. The intestine colonization is essential to probiotic bacteria activity and ally with the results obtained in this work, another factor that *L. lactis* and *L. acidophilus* did not present a probiotic activity for fish could be based on the non-effective fish intestine colonization because this strains were not obtained from intestine of fishes.

Table 1: Zebrafish growth parameters

Groups: Fish feed formulation			Starting fish group weight	Finishing fish group weight	Body Growth Percent
		1	0,08 g	0,22 g	175 %
PBS + Commercial fish feed	Tank	2	0,08 g	0,21 g	162 %
		3	0,08 g	0,23 g	187 %
<i>L. lactis</i> + Commercial fish feed	Tank	1	0,08 g	0,22 g	175 %
		2	0,11 g	0,25 g	127 %
		3	0,05 g	0,16 g	220 %
<i>L. acidophilus</i> + Commercial fish feed	Tank	1	0,08 g	0,18 g	125 %
		2	0,11 g	0,24 g	118 %
		2	0,05 g	0,20 g	300 %

*Body growth percent = (Finishing fish group weight – Starting fish group weight)/Starting fish group weight X 100

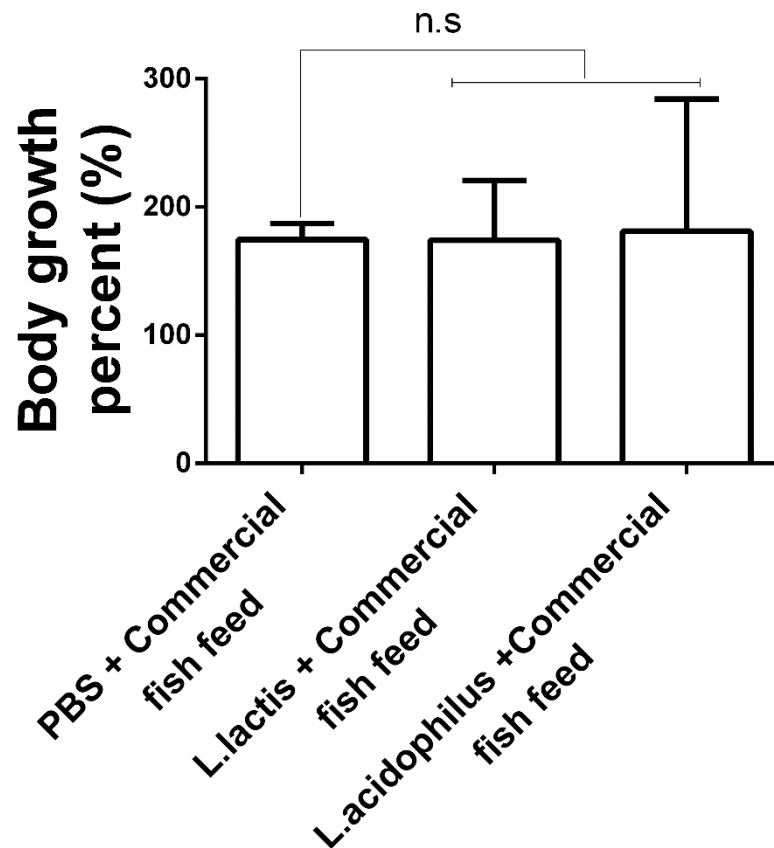


Figure 4: Zebrafish weight gain after probiotic formulation treatment. Juveniles zebrafish were treated with fish feed mixed with saline solution (PBS) or *L. lactis* suspension (10^8) or *L. acidophilus* suspension (10^8) during 60 days, twice per day. The body weight was measured before and after treatment and the average was used to calculate the body growth percent based on the following formula: (Final weight – Initial weight)/Initial weight X 100. The statistical analysis was made in GraphPrism 7.0 and the results was considered significant with p value <0,05. The one-way ANOVA test was chosen to evaluate the significance and no statistical significance was observed for all groups with p value of (0,9).

1. IN VIVO LAB ANTIMICROBIAL ACTIVITY AGAINST AEROMONAS HYDROPHILA AND PSEUDOMONAS FLUORESCENS

The juveniles zebrafish mortality was accessed after challenge with pathogenic bacteria *A. hydrophila* ATCC 7969 and *P. fluorescens* 07A (Fig 5). The results showed a non-significant anti-*A. hydrophila* and anti-*P. fluorescens* activity of *L. lactis* and *L. acidophilus* in vivo. The juveniles zebrafish mortality treated with *L. lactis* or *L. acidophilus* mixed with fish feed were the same observed for juveniles zebrafish non-treated. The percent survival consisted in 56 % 64% and 54% respectively for *A. hydrophila* challenge and 9% 4% and 4% respectively for *P. fluorescens*. The challenge was performed with 10µl of pathogenic bacteria administrated

intraperitoneally and wasn't consider the best method to evaluate the fish infection and mortality since the treatment was oral and the fish age and maturation didn't contribute with the immunological system strength.

The protective in vivo effect from LAB strains against pathogenic bacteria was reported in the literature several times as Aly et al. (2008) showed high protective effected of *B. subtilis* and *L. acidophilus* when administered for Nile tilapia, *Oreochromis niloticus*, the in vivo antimicrobial activity against *P. fluorescens* and *A. hydrophila* was observed and the relative level of protection was approximately 36% after 1 month and 52% after two month of treatment against both pathogenic bacteria. The in vivo probiotic effect of *L. lactis* BFE920 strain was reported by Kim et al. (2013) in olive flounder challenged by *Streptococcus iniae* showing 68-77% of survival rate.

It is more than proved that probiotic bacteria have antimicrobial activity in vitro and in vivo against a huge spectrum of pathogenic bacteria such as a diversity of strains of *L. lactis* and *L. acidophilus* with probiotic spectrum action and benefits for fish supplementation, however , the bacteria stirpes and identification, fish species, fish age, intestine colonization and bacteria culture seems to play a crucial role for effective probiotic activity.

Juveniles zebrafish survival percent after challenge with *A. hydrophila* and *P. fluorescens*

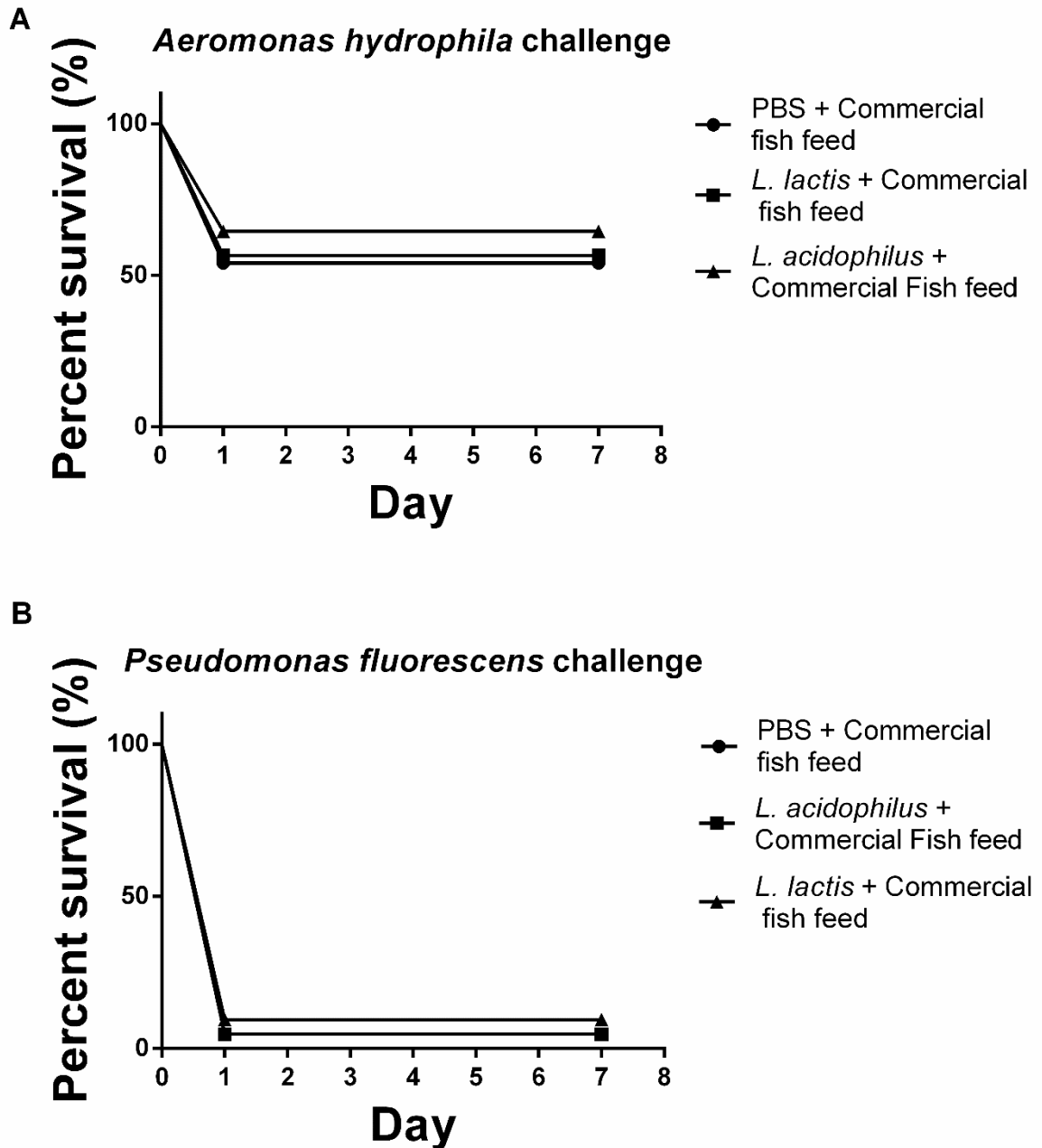


Figure 5: Probiotic bacteria *L. lactis* and *L. acidophilus* in vivo protection against fish pathogenic bacteria.

(A) Percent survival of Juveniles zebrafish treated with commercial fish feed mixed with *L. lactis* (10^8) or *L. acidophilus* (10^8) suspension or saline solution (PBS) against *A. hydrophila*. (B) Percent survival of Juveniles zebrafish treated with commercial fish feed mixed with *L. lactis* (10^8) or *L. acidophilus* (10^8) suspension or saline solution (PBS) against *P. fluorescens*. The statistical analysis was made in GraphPrism 7.0 and the survival percent was considered significant with p value <0,05. The Lon-rank test were chosen to evaluate the significance and for *P. fluorescens* 07A the p value was (0,7) and for *A. hydrophila* ATCC 7966 the p value was (0,6).

4. CONCLUSION

Probiotic bacteria mechanism action against fish pathogen have been explored in aquaculture for decades and are present as fish additives in commercial fish feed. Probiotic bacteria combined with vaccine is consider the gold standard to deal with fish pathogen infection. The results presented in this work did show antimicrobial activity in vitro for *L. lactis* and *L. acidophilus* strains against *Aeromonas hydrophila* IOC23116 and *Pseudomonas fluorescens* 07A and this mechanism was based on acid production, although, same results was not founded in vivo and fish that were fed with fish feed mixed with *L. lactis* or *L. acidophilus* did not resist the challenge with pathogenic bacteria, *P. fluorescens* and *A. hydrophila*, as well as did not present weight body gain. The probiotic intestine colonization is the key for an efficient antimicrobial response and analysis to evaluate if the probiotic strains tested here were able to colonize the juvenile zebrafish intestine must be performed to understand the lack of response in vivo, therefore, zebrafish age, fish species, bacteria isolates, bacteria stirpes, mature immune response and intestine colonization are the key for probiotic screening.

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