POLITÉCNICO DE LEIRIA | ESCOLA SUPERIOR DE TURISMO E TECNOLOGIA DO MAR

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In vivo assessment of recombinant antibodies against Nervous Necrosis Virus (NNV) in European seabass (*Dicentrarchus labrax*)

Henrique da Cruz Batista

Dissertation for a Master's Degree in Aquaculture

Dissertation carried out under the supervision of Specialist Professor Teresa Baptista

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Abstract

Viral nervous necrosis (VNN) is one of the most significant diseases in aquaculture, mainly affecting larvae and juveniles of several fish species, including the European sea bass (*Dicentrarchus labrax*), and it's associated with high mortality rates and substantial economic losses. This study aimed to evaluate the effect of oral administration of recombinant antibodies (Ccombodies) on the immune response of juvenile sea bass experimentally infected with nervous necrosis virus (NNV). In a preliminary LD_{50} trial, both viral doses tested (10^5 and 10^6 $TCID_{50}$/fish) resulted in 50% mortality at 5 days post-infection, accompanied by typical VNN clinical signs such as loss of orientation, spiral swimming, hyperactivity and anorexia. Due to the similarity of results the lowest dose was selected for the main trial. The passive immunization trial included four treatments: feed without antibodies (control) and feed supplemented with 1, 10 and 100 mg Ccombodies/100g diet. Cumulative mortality ranged from 36.6% (control) to 53.3% (10mg/100g), with no statistically significant differences between groups ($p>0.05$). Clinical signs were similar across treatments, suggesting that oral administration of recombinant antibodies did not provide effective protection against NNV. Enzymatic degradation in the digestive tract and dispersion of antibodies in the water may have compromised efficacy. In conclusion, under the tested conditions, recombinant antibodies did not confer significant protection against NNV infection in juvenile European sea bass. Future studies should explore alternative delivery strategies, such as microencapsulation, to enhance stability and improve the effectiveness of oral antibody administration. It is also essential in future studies to review the development of antibodies so that they confer protection against the virus.

Keywords: Aquaculture; *Dicentrarchus labrax*; Viral nervous necrosis; Recombinant antibodies; Passive immunization.

Resumo

A necrose nervosa viral (NNV) é uma das doenças mais significativas em aquacultura, afetando principalmente larvas e juvenis de várias espécies de peixes, incluindo o robalo europeu (*Dicentrarchus labrax*), e está associada a elevadas taxas de mortalidade e a perdas económicas substanciais. Este estudo teve como objetivo avaliar o efeito da administração oral de anticorpos recombinantes (Ccombodies) na resposta imunitária de juvenis de robalo experimentalmente infetados com o vírus da necrose nervosa (VNN). No ensaio preliminar de LD_{50}, ambas as doses virais testadas (10^5 e 10^6 $TCID_{50}$/peixe) resultaram em 50% de mortalidade aos 5 dias após a infeção, acompanhada por sinais clínicos típicos de NNV, como perda de orientação, natação em espiral, hiperatividade e anorexia. Devido à semelhança dos resultados, foi selecionada a dose mais baixa para o ensaio principal. O ensaio de imunização passiva incluiu quatro tratamentos: ração sem anticorpos (controlo) e ração suplementada com 1, 10 e 100 mg de Ccombodies/100g de dieta. A mortalidade cumulativa variou de 36,6% (controlo) a 53,3% (10 mg/100 g), não havendo diferenças estatisticamente significativas entre os grupos ($p > 0,05$). Os sinais clínicos foram semelhantes entre os tratamentos, sugerindo que a administração oral de anticorpos recombinantes não proporcionou uma proteção eficaz contra o VNN. A degradação enzimática no trato digestivo e a dispersão de anticorpos na água podem ter comprometido a eficácia do tratamento. O RNA extraído das amostras de cérebro e rim cranial apresentou pureza e concentração adequadas, mas a análise de integridade revelou degradação, impedindo a quantificação da carga viral por RT-qPCR. Em conclusão, nas condições testadas, os anticorpos recombinantes não conferiram proteção significativa contra a infeção por VNN em juvenis de robalo-europeu. Estudos futuros devem explorar estratégias de administração alternativas, como a microencapsulação, para aumentar a estabilidade e melhorar a eficácia da administração oral de anticorpos. É também essencial em estudos futuros rever o desenvolvimento de anticorpos para que estes confiram proteção contra o vírus.

Palavras-chave: Aquacultura; *Dicentrarchus labrax*; Necrose nervosa viral; Anticorpos recombinantes; Imunização passiva.

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List of abbreviations

AMPs: Antimicrobial peptides

BEI: Binary ethylenimine

BFNNV: Barfin flounder nervous necrosis virus

Ccombodies: Recombinant antibodies used in this study

cDNA: Complementary deoxyribonucleic acid

CIIMAR: Centro Interdisciplinar de Investigação Marinha e Ambiental

CNS: Central nervous system

CPA-LDF: Isothermal cross-priming amplification with lateral flow dipstick

CPEs: Cytopathic effects

DGAV: Direção Geral da Alimentação e Veterinária

DNA: Deoxyribonucleic Acid

dpi: Days post-infection

ELISA: Enzyme-Linked Immunosorbent Assay

EU: European Union

FAO: Food and Agriculture Organization

FEAP: Federation of European Aquaculture Producers

IFAT: Indirect Fluorescent Antibody

IFN- α: Interferon type I

IFN- γ: Interferon type II

IFNs: Interferons

Ig: Immunoglobulin

IgM: Immunoglobulin M

IHC: Immunohistochemistry

IL-1: Interleukins

LAMP: Loop-mediated isothermal amplification

LD50: Lethal dose 50%

LFB: Lateral flow paper biosensors

mAb: Monoclonal antibody

NASBA: Nucleic acid sequence-based amplification

NNV: Nervous Necrosis Virus

PBS: Phosphate-buffered saline

RAS: Recirculating Aquaculture System

RdRp: RNA-dependent RNA polymerase

RGNNV: Red-spotted grouper nervous necrosis vírus

RNA: Ribonucleic acid

rpm: Revolutions per minute

RT-PCR: Reverse Transcriptase Polymerase Chain Reaction

RT-qPCR: Real-time Quantitative polymerase chain reaction

SJNNV: Striped jack nervous necrosis vírus

ssRNA: Single-stranded RNA viruses

TCID50: 50% Tissue Culture Infective Dose

TMB: Tetramethylbenzidine

TNF- α: Tumour necrosis factor α

TNF: Tumour necrosis factor

TNV: Turbot nodavirus strain

TPNNV: Tiger puffer nervous necrosis vírus

T-TBS: Tris- Buffered Saline with Tween 20

UV: Ultraviolet Radiation

VER: Viral Encephalopathy and Retinopathy

VLPs: Virus-like particles

VLR: Variable lymphocyte receptor

VNN: Viral Nervous Necrosis

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Introduction

Aquaculture

In recent years, global fish consumption has increased, reaching 20.7 kg *per capita* in 2022. This represents an average annual growth of 1.4% since 1961, when per capita consumption was 9.1 kg. This increase can be attributed to several factors, including the greater availability of products, technological advancements in conservation and distribution, changes in consumption patterns and rising incomes (FAO, 2024).

Given this rise in consumption, a corresponding increase in production, mainly in aquaculture, has been inevitable. In 2022, of the 185 million tonnes of aquatic animals produced, 94 million tonnes originated from aquaculture, representing 51% of total production. This marked the first time that aquaculture production exceeded capture fisheries, which accounted for 91 million tonnes (Figure 1) (FAO, 2024).

	1990s	2000s	2010s	2020	2021	2022
	Average per year					
	(million tonnes, live weight equivalent)					
Production						
Capture fisheries:						
Inland	7.1	9.3	11.3	11.5	11.4	11.3
Marine	81.9	81.6	79.8	78.3	80.3	79.7
Total capture fisheries	**88.9**	**90.9**	**91.1**	**89.8**	**91.6**	**91.0**
Aquaculture:						
Inland	12.6	25.6	44.8	54.5	56.4	59.1
Marine	9.2	17.9	26.7	33.2	34.7	35.3
Total aquaculture	**21.8**	**43.4**	**71.5**	**87.7**	**91.1**	**94.4**
Total world fisheries and aquaculture	**110.7**	**134.3**	**162.6**	**177.5**	**182.8**	**185.4**

Figure 2 - Production of marine animals in aquaculture and fish catches. (Source: FAO, 2024 - The State of World Fisheries and Aquaculture).

One species that has experienced increased production is the European sea bass (*Dicentrarchus labrax*), with over 236,000 tonnes produced through aquaculture worldwide (FEAP, 2024). It is mainly farmed in the Mediterranean region. This species is euryhaline (tolerant of salinities from 0 to 40 ppt) and eurythermal (tolerant of temperatures from 2 to 32ºC), can be co-cultured with other species, and exhibits relatively rapid growth. These traits contribute to lower production costs, making sea bass a favorable species for aquaculture (Vandeputte *et al*., 2019).

The expansion in aquaculture production is essential, as capture fisheries have become increasingly unsustainable over the years. The proportion of fish stocks within biologically sustainable levels decreased to 62.3% in 2021, representing a 2.3% decline compared to 2019. Meanwhile, the proportion of stocks exploited at biologically unsustainable levels increased from 10% in 1974 to 37.7% in 2021 (FAO, 2024).

However, while increasing aquaculture production is necessary, it must be undertaken responsibly to minimize stressors that compromise fish health, as stressed fish are more susceptible to disease. In the main aquaculture-producing countries diseases can cause up to 30% of production losses, with some studies suggesting that these losses may reach up to 50% in certain cases (Lafferty *et al*., 2015; Subramani & Michael, 2017; Mohd-Aris *et al*., 2019). These diseases can be classified as non-infectious – such as environmental, nutritional, and genetic disorders - and infectious, including parasitic, bacterial, fungal and viral infections. Among the viral pathogens, the nervous necrosis virus (NNV) is particularly noteworthy (Alfred *et al*., 2020).

Nervous Necrosis Virus (NNV)

Aetiology

Nervous necrosis virus (NNV), the causative agent of viral nervous necrosis (VNN) - also known as viral encephalopathy and retinopathy (VER) - belongs to the family *Nodaviridae* genus *Betanodavirus*, (Mori *et al*., 1992). It is a small, non-enveloped, single-stranded RNA (ssRNA) virus with icosahedral symmetry and a diameter of approximately 25-30nm. The viral genome consists of two RNA segments: RNA1, which encodes the RNA-dependent polymerase, and RNA2, which encodes the capsid protein (Nishizawa *et al*., 1997; Yong *et al*., 2017). To date, five genotypes of this virus have been identified: RGNNV (red-spotted grouper nervous necrosis virus), the most widely distributed; SJNNV (striped jack nervous necrosis virus), BFNNV (barfin flounder nervous necrosis virus); TPNNV (tiger puffer nervous necrosis virus); and TNV (turbot nodavirus) (Nishizawa *et al*., 1995; Nishizawa *et al*., 1997; Johansen *et al*., 2004).

Geographical distribution

The disease caused by NNV was first described in the late 1980s in Australia and the Caribbean (Glazebrook & Campbell, 1987; Bellance & Gallet de Saint-Aurin, 1998). It has since been documented on all continents, with notable prevalence in South and East Asia, Oceania, North America and Europe – particularly in Mediterranean countries and Norway. To date, the disease has not been reported in South America. (Nakai *et al*., 2009).

The distribution of different NNV genotypes appears to be associated with their thermotolerance. The BFFNV genotype is primarily found in colder waters, such as those surrounding Japan, northern Europe and North America.

The RGNNV genotype is the most geographically widespread, mainly prevalent in the Mediterranean region and along the coasts of Asia and Australia. In contrast, the TPNNV, SJNNV and TNV genotypes have more limited distributions. TPNNV has been reported in only one species in Japan, SJNNV was initially identified in Japan and more recently in the Iberian Peninsula and TNV has been described in Norway (Figure 2) (Bandín & Souto, 2020).

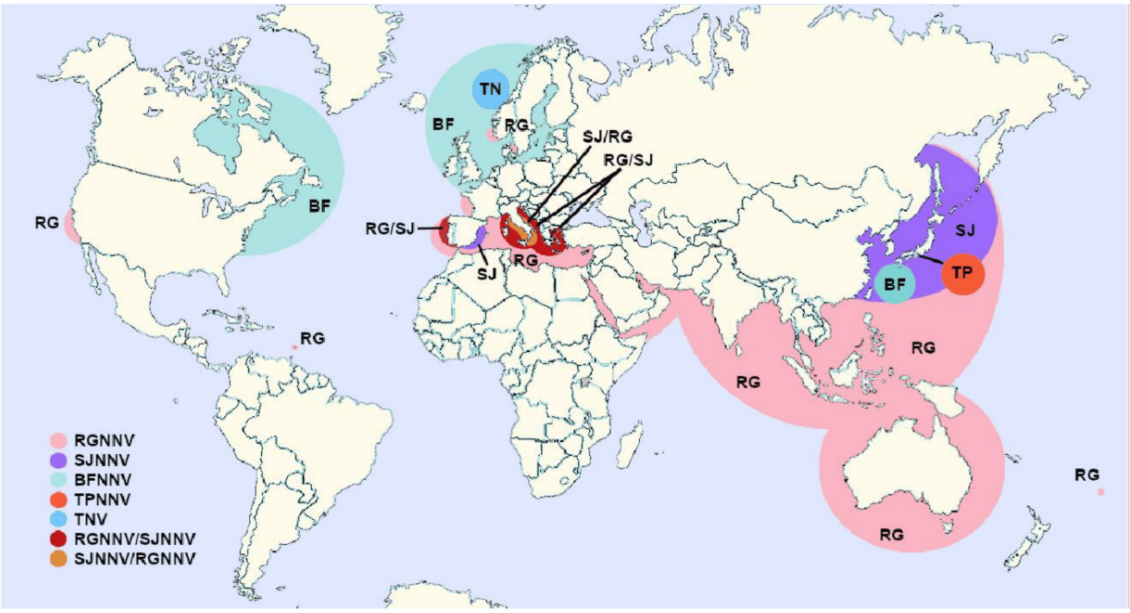

Figure 2- Distribution of nervous necrosis virus (NNV) genotypes (Source: Bandín & Souto, 2020).

Hosts

Originally, 19 fish species were reported as susceptible to NNV (Munday & Nakai, 1997); currently, approximately 40 species are known to be vulnerable, including: Atlantic cod (*Gadus morhua*), sturgeon (*Acipenser sp.*), turbot (*Scophthalmus maximus*), catfish (*Parasilurus asotus*), guppy (*Poecilia reticulata*), bluefin tuna (*Thunnus thynnus*), three species of grouper (*Epinephelus lanceolatus*, *Epinephelus septemfasciatus* and *Epinephelus akaara*), and European sea bass (*Dicentrarchus labrax*) (Patel *et al*., 2007; Kokawa *et al*., 2008; Gomez *et al*., 2009; Nakai *et al*., 2009; Hick *et al*., 2010). Freshwater and marine species are both susceptible (Chi *et al*., 2003; Johansen *et al*., 2003; Furusawa *et al*., 2006; Hasoon *et al*., 2011).

Genotypic susceptibility varies: SJNNV and TPNNV infect only *Pseudocaranx dentex* and *Takifugu rubripes*, respectively. BFNNV is isolated from cold-water species like *Verasper moseri* and *Gadus macrocephalus*. In contrast, the RGNNV genotype - the most widely distributed- infects various warm-water species, mainly sea bass and groupers (Aspehavg, 1999; Chi *et al*., 1999).

Epidemiology

Clinical signs

NNV typically affects larvae and juveniles (Munday & Nakai, 1997), but adults can also be infected (Fukuda *et al*., 1996; Le Breton *et al*., 1997; Ransangan *et al*., 2011). Signs include abnormal swimming behaviors such spiralling, vertical or horizontal loops, abrupt turns, sinking and corkscrew motion, along with lethargia, anorexia, color change, floating belly-up, and bottom-dwelling (Glazebrook *et al*., 1990; Yoshikoshi & Inoue, 1990; Bloch *et al*., 1991; Mori *et al*., 1991; Nguyen *et al*., 1994; Grotmol *et al*., 1997; Munday & Nakai, 1997).

Histopathological examination reveals extensive necrosis in the central nervous system (CNS), characterized by vacuolation and neural degeneration in the brain, and vacuolation of the retina (Glazebrook *et al*., 1990; Nguyen *et al*., 1996; Le Breton *et al*., 1997). Larvae show extensive damage than juveniles and adults (Mladineo, 2003).

Non-neural organs such as the liver, kidney, stomach, heart, spleen, intestines, gonads, fins and gill may also harbor the virus, though with limited viral replication (Comps *et al*., 1996; Nguyen *et al*., 1997; Gomez *et al*., 2004; Starkey *et al*., 2004; Kuo *et al*., 2011; Lopez-Jimena *et al*., 2011).

Transmission

Nervous necrosis virus (NNV) is transmitted from infected to healthy fish primarily through cohabitation or direct contact (Munday *et al*., 2002). Several factors increase susceptibility to infection by compromising immune function, including suboptimal water quality, inadequate nutrition, poor management practices, inadequate nutrition, high stocking densities, and transport - related injuries, and water temperatures conducive to viral replication (Mushiake *et al*., 1994; Tanaka *et al*., 1998; Johansen *et al*., 2004; Yuasa *et al*., 2007). The virus can persist in a subclinical state for extended periods, with disease outbreaks and high mortality often triggered under extreme environmental stress (Samuelsen *et al*., 2006).

Horizontal transmission occurs throughout the fish´s life via exposure to infected individuals, virus contaminated water, or feed (Chérif *et al*., 2009). Cannibalistic nature, such as that seen in Asian sea bass (*Lates calcarifer*), facilitates transmission (Manin & Ransangan, 2011). The virus can enter the host through gills and skin, as shown in Senegalese sole (*Solea senegalensis)* (Souto *et al*., 2018), and it can survive for up to one month outside a host. During this period, it remains transmissible via water or biological vectors like crabs, mussels and brine shrimp (Gomez *et al*., 2010).

Vertical transmission has also been confirmed. The virus is passed from broodstock to offspring via infected gonadal tissues or sperm (Arimoto *et al*., 1992; Breuil *et al*., 2002; Valero *et al*., 2015a). Moreover, the intestine may

play a role in viral shedding, potentially leading to contamination of fertilized eggs (Kuo *et al*., 2012; Souto *et al*., 2018).

Host-Pathogen Interaction

Nervous necrosis virus enters host cells via micropinocytosis and macropinocytosis. Following entry, the capsid proteins are removed, and the viral genome is released for transcription and translation. Viral RNA1 translates the RNA-dependent RNA polymerase (RdRp), directing it to the mitochondrial membrane to initiate viral RNA synthesis (Liu *et al*., 2005; Wu *et al*., 2010). The host´s Mx protein interacts with the RdRp protein and targeting it for degradation via autophagy and lysosomal pathways, thereby inhibiting viral replication (Lin *et al*., 2006; Chen *et al*., 2008; Scapigliati *et al*., 2010). In European sea bass, Mx protein expression is significantly higher following infection with the RGNNV strain compared to the SJNNV strain, suggesting strain-specific immune responses (Carballo *et al*., 2016).

During replication, viral RNA is translated into non-structural proteins, while capsid proteins mediate the assembly of new viral particles. This process induces apoptosis and necrosis in host cells and activates cell death pathways, which in turn trigger innate immune responses including cytokine activation, cytotoxic T cell formation, and antibody production (Chen *et al*., 2007; Reyes-Cerpa *et al*., 2012).

The host immune responses involve key signalling molecules such as interferons (IFNs), interleukins (IL-1) and tumor necrosis factor (TNF). Type I IFNs (IFN-α) are central to innate immunity, while type II IFNs (IFN-γ) are crucial for adaptive immune responses (Costa & Thompson, 2016). TNF-α also contributes to antiviral defense by activating the STAT3 signalling pathway, which has been associated with brain vacuolation (Poisa-Beiro *et al*., 2008; Huang *et al*., 2015).

The antibody response, particularly through immunoglobulin M (IgM) production, is essential for viral neutralization and reducing host mortality (Grove *et al*., 2006; Yamashita *et al*., 2009). Although the central nervous system is the primary site of infection, the gonads are also targeted. Infected European Sea bass and gilthead sea bream (*Sparus aurata*) exhibit elevated expression of interferons and antimicrobial peptides (AMPs) in gonadal tissues (Valero *et al*., 2015b; Valero *et al*., 2015c).

Diagnosis

Since the emergence of NNV, several diagnostic methods have been developed to detect and confirm infection in fish populations. Initially, diagnosis relied on clinical signs and histopathological analysis.

Light microscopy can reveal lesions such as the characteristic vacuolization in the spinal cord, brain, and retina, and necrosis of neural cells, especially in larvae and juveniles (Glazebrook *et al*., 1990; Le Breton *et al*., 1997). Additional lesions may occur in the liver and spleen. However, light

microscopy alone is insufficient for a definitive diagnosis of NNV infection (Munday *et al*., 2002). For definitive diagnosis, immunofluorescence microscopy using virus-specific antibodies is commonly employed (Qin *et al*., 2006).

Immunological assays, such as enzyme-linked immunosorbent assay (ELISA), have been used since early outbreaks to detect virus specific antibodies in eggs, larvae and broodstock (Arimoto *et al*., 1992; Chi *et al*., 2005). However, ELISA often lacks the sensitivity required to detect persistent or latent infections. Therefore, more reliable immunological methods include the fluorescent antibody technique (IFAT) and immunohistochemistry (IHC), which offer higher specificity and sensitivity (Munday & Nakai, 1997; Shetty *et al*., 2012).

Molecular diagnostics are now widely used due to their high sensitivity and specificity. Reverse transcription polymerase chain reaction (RT-PCR), which targets the viral RNA2 (capsid protein gene), has become a standard method for NNV detection (Grotmol *et al*., 2000; Barker *et al*., 2002; Azad *et al*., 2005). The development of quantitative RT-PCR (RT-qPCR) in 2005 further improved diagnostic precision, allowing for quantification of viral loads with detection limits as low as 10 $TCID_{50}$/mL (Dalla Valle *et al*., 2005; Panzarin *et al*., 2010). Innovative isothermal amplification methods have been introduced for rapid and field-applicable diagnosis. These include loop-mediated isothermal amplification (LAMP), lateral flow biosensors (LFB), nucleic acid sequence-based amplification (NASBA), and cross-primer isothermal amplification with lateral flow strips (CPA-LDF). These emerging innovative tools offer rapid, sensitive, and equipment-free alternatives suitable for point-of-care or field settings, enhancing early detection and control efforts (Starkey *et al*., 2004; Notomi *et al*., 2015; Su *et al*., 2015; Toubanaki *et al*., 2015).

Cell culture remains a crucial diagnostic and research tool, facilitating the study of viral mechanisms, as well as the characterization, propagation and quantification of nervous necrosis virus (NNV). In 1999, virus isolation in fish was standardized using the SSN-1 cell line, originally derived from whole tissues of *Ophicephalus striatus* fry. This line successfully propagated NNV isolated from diseased juvenile sea bass (Frerichs *et al*., 1996). The SSN-1 cell line was later cloned into the E-11, which shows stable cytopathic effects (CPEs) and is now widely used for both quantitative and qualitative viral analysis. Other cell lines, such as GF-1 (derived from *Epinephelus coioides*) and SB (*Lates calcarifer*), were initially considered unsuitable but were later shown to support viral replication, potentially due to variation in receptor expression specific to NNV strains (Chi *et al*., 1999; Iwamoto *et al*., 1999; Tan *et al*., 2001).

Vaccines

Due to the lack of effective treatments and the highly contagious nature of NNV, vaccine development is essential for disease prevention and control

(Costa & Thompson, 2016; Doan *et al*., 2017). A variety of vaccination strategies have been investigated: one of the earliest successful

approaches involved recombinant capsid proteins, which conferred significant protection in Senegalese sole when challenged two months post-vaccination at approximately 5 grams body weight (Húsgar *et al*., 2001). A DNA vaccine based on the capsid protein was tested in flounder but failed to induce protective immunity (Sommerset *et al*., 2005). In contrast, a DNA vaccine encoding the G protein from a fish rhabdovirus provided short-term but effective protection against NNV (Sommerset *et al*., 2003). Vaccination with inactivated virus vaccines has also shown promise. For instance, in *Epinephelus coioides* larvae, bath immunization using nanoencapsulated virus inactivated with formalin or binary ethylenimine (BEI) lead to protective immune responses (Kai & Chi, 2008). Oral and bath administration of such inactivated vaccines also induced expression of genes related to humoral and cellular immunity (Kai *et al*., 2014). Despite their promise, the commercial use of inactivated vaccines is limited by factors such as high production costs and the genetic diversity of NNV genotypes. Live attenuated vaccines have been evaluated in *Epinephelus septemfasciatus*, demonstrating enhanced viral control at lower temperatures (17ºC) (Nishizawa *et al*., 2012). Protective immune responses were also at natural seawater temperatures (Oh & Nishizawa, 2013). Another promising strategy involves the use of virus-like particles (VLPs). To date, NNV VLPs expressed in *E. coli* have been tested in *Epinephelus malabaricus*, *Epinephelus lanceolatus* and *Epinephelus coioides*, demonstrating high protective efficacy against NNV (Liu *et al*., 2006; Lai *et al*., 2014).

Antibodies

The development and application of vaccines against viruses in aquaculture have proven challenging. One major limitation arises from the ectothermic nature of fish, which leads to slower immune system development, particularly during early life stages. As a result, vaccines are often ineffective during the initial period of vulnerability (Uribe *et al*., 2011; Rajan *et al*., 2017). Additionally, the antibody response in teleost fish has been described as poorly anamnesic, with a tendency to favor quantity over specificity (Parra *et al*., 2015; Rajan *et al*., 2017).

In this context, passive immunization offers a promising alternative. Unlike vaccination, passive immunization involves the administration of exogenous antibodies, providing temporary protection or therapeutic effects against the pathogen. This strategy has gained increasing interest in aquaculture due to its potential use in both disease prevention and treatment (Hedegaard & Heegaard, 2016; Rajan *et al*., 2017). Passive immunity can occur naturally through maternal transfer of immune components, including antibodies. In teleost fish, maternally derived antibodies are transferred to the egg yolk, contributing significantly to early functional protection (Swain & Nayak, 2009; Hedegaard & Heegaard, 2016; Rajan *et al*., 2017).

In aquaculture, antibodies can also be delivered orally, through antibody-enriched diets. This method provides greater flexibility for repeated

dosing, is cost-effective and is less invasive compared to intraperitoneal injections, thereby improving fish welfare (Toranzo *et al*., 2005; Rajan *et al*., 2017). In addition, technological advancements have enabled the *in vitro* production of recombinant antibodies, offered greater specificity and potentially improved efficacy against pathogens such as NNV.

Some studies have reported mAbs from seabream (*Sparus aurata*) and giant grouper (*Epinephelus lanceolatus*) but Erwyn Bio mAb against NNV was produced from hagfish (*Eptatretus burger*). A recombinant monoclonal was produced by Jung *et al*. (2020) VLRB from hagfish immunized with NNV, in which authors describe the characteristics of this VLRB (e.g. the specificity and binding ability to NNV). CCombody is a recombinant antibody developed by genetic manipulation of the hagfish variable lymphocyte receptor (VLR, antibody). VLRB is analogous to mammalian Ig antibodies while having a completely different structure. Ccombodies can be applied where Ig antibodies have been the only available solution. The advancement of molecular engineering and biotechnology has allowed the production of these molecules in recombinant systems, which may facilitate their therapeutic or prophylactic application in aquaculture species.

This study is conducted within the framework of the Cure4Aqua project, funded by the European Union´s Horizon Europe programme. The project spans 4.5 years, with a total budget of 4.8 million euros, and is dedicated to developing innovative strategies for the prevention of infectious diseases in aquaculture species. Cure4Aqua focuses on the early detection of pathogens and the development of alternative treatments, aiming to reduce or replace the use of conventional pharmaceuticals in disease management.

The objective of this study was to evaluate the effect of recombinant antibody administration (Ccombody) on the immune response of juvenile European sea bass (*Dicentrarchus labrax*) following infection with nervous necrosis virus (NNV). This study aimed to assess the efficacy of this immunotherapeutic strategy in mitigating clinical symptoms and reducing mortality, thereby contributing to the development of more sustainable and effective disease control measures in aquaculture.

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Material and methods and Ethical Statement

All experimental procedures described in this dissertation were conducted in full compliance with the ethical standards for the use of animals in scientific research, as outlined in European Directive 2010/63/EU. The experimental work was supervised by a qualified professional certified by the Directorate-General for Food and Veterinary Affairs (DGAV) and formally trained in Laboratory Animal Sciences. All trials were carried out at CETEMARES – Centre for Research, Education and Dissemination of Knowledge in Marine Sciences, located in Peniche, Portugal.

The European Sea bass used in this study originated from a commercial aquaculture facility in the Algarve. Upon arrival at the Cetemares facilities (Polytechnic of Leiria, Peniche), the fish were acclimated in a recirculation aquaculture system (RAS), comprising three cylindroconical tanks and a sump. The system was equipped with a mechanical filter (mesh filter), a biological filter (bioballs), a UV sterilizer, and a protein skimmer.

The fish were kept at a water temperature of approximately 18±1ºC, and environmental parameters - including temperature, dissolved oxygen, salinity, and pH - were monitored daily. Ammonia levels were also measured regularly, and nitrite levels were assessed when ammonia exceeded 1 ppm. Fish were fed twice daily, and uneaten feed and faeces were removed after each feeding via siphoning to maintain optimal water quality.

1. Testing fish for the presence of NNV-specific antibodies

Before the preliminary trial, seven fish were randomly selected for screening of NNV-specific antibodies. Blood was collected via caudal venipuncture using 1 mL insulin syringes and transferred into an Eppendorf tube without anticoagulant to allow clot formation. Samples were stored overnight at 4ºC and then centrifuged at 3,000 × g for 7 minutes using a refrigerated centrifuge (Eppendorf, Germany) to obtain serum.

1.1. Semi-quantification of NNV-specific IgM by indirect ELISA

The presence of NNV-specific IgM antibodies in fish serum was evaluated using an indirect enzyme linked immunosorbent assay (ELISA). First, 96-well plates were coated with 100 µL of virus suspension ($10^{7.25}$ $TCID_{50}$/mL of RGNNV 378/03 P6-D4 propagated in SNN-1 cell line) and incubated for 24 hours at 25ºC (Binder GmbH, Germany). The plates were then washed three times with 300 µL of washing buffer (T-TBS, Tris Buffered Saline with 0.1% Tween 20). Next, each well received 300 µL of blocking buffer (5% skimmed milk powder in T-TBS) and was incubated for 24 hours at room

temperature. The plates were washed again with T-TBS, then 100 µl of fish serum diluted 1:100 in 50mM Na_2CO_3 was added in triplicate. Negative controls contained 100 µl of buffer (Na_2CO_3). Plates were incubated at 25 °C for 24 hours. After incubation, the serum was removed, and 300 µl of blocking buffer was added to each well and incubated for 1 h at 25 °C, followed by three consecutive washes with 300 µl T-TBS. After the washes, 100 µl of the primary monoclonal IgG anti-Sea Bass IgM antibody (Aquatic Diagnostics, UK), diluted 1:100 in blocking buffer, was added to each well and incubated at 25 °C for 1 hour. After washing, 100 µl of the secondary mouse anti-IgG-HRP antibody diluted 1:1000 in blocking buffer was added and incubated for 1 hour at 25 °C. After a final series of three washes, 100 µl of TMB substrate (BioLegend Ref.: 421101) was added to each well and incubated for 5 minutes. The enzymatic reaction was stopped with 100 µl of 2 M sulphuric acid. Absorbance was read at 450 nm using an Epoch 2 microplate reader (BioTek, USA).

All fish tested negative for NNV-specific IgM antibodies before the trial.

2. Preliminary trial - LD_{50} determination

2.1. Experimental design and sample collection

Two 30-litre tanks, each containing six juvenile European sea bass (mean weight 5 g), were used to determine the median lethal dose (LD_{50}) of NNV in the preliminary trial. Each tank was connected to a separate RAS system equipped with mechanical and biological filters. The water temperature was maintained at approximately 25°C, with a 12:12 (light: dark) photoperiod. Two concentrations of NNV (genotype RGNNV, strain DP 965.08) were tested, i.e. Tank 1: 10^6 $TCID_{50}$/fish; Tank 2:10^5 $TCID_{50}$/fish. Before infection, fish were anaesthetised with 0.5 mL/L 2-phenoxyethanol (VWR Chemicals, Portugal). After anaesthesia, each fish was placed on a tray with a damp cloth to maintain hydration and was injected intramuscularly with 0.1 mL of the virus solution containing the respective $TCID_{50}$ concentration. Injections were performed using a 1 mL syringe fitted with a 26Gx1.25mmx45/100 hypodermic needle (FINE-JECT). Following infection, fish were placed in recovery buckets with aerated tank water until fully recovered from anaesthesia and then returned to their respective tanks. During the post-infection period, fish were fed twice daily with commercial feed at 3.9% of their biomass, according to the feed producer´s table for the respective size class and water temperature (proprietary information). After each feeding, tanks were siphoned to remove uneaten feed and faeces. Water quality parameters were monitored and recorded daily. Oxygen levels and temperature were measured with an oximeter (Oxyguard, Denmark) and salinity measures using a seawater refractometer H196822 (Hana Instruments, Portugal). The ammonia content was measured using a colorimetric test kit (Tropic Marine Test Kit). Nitrite was measured if ammonia exceeded 1 ppm, using a colorimetric test kit (Red Sea Test Kit). In addition, fish behaviour was observed multiple times daily. The

Humane Endpoints Table developed for laboratory zebrafish (Figure 3) (Martins *et al.*, 2016) was used for this purpose. Observations included: physical appearance, feeding behaviour, breathing behaviour, swimming behaviour, activity level and social behaviour. Each category was scored from 0 to 3. A total score greater than 9 indicates distress, and the affected fish were euthanised. Additionally, if any category other than behaviour scored 3, fish were immediately euthanised by overdose of anaesthetic (1 mL/L of 2-phenoxyethanol). Post-mortem, brain samples were collected by carefully opening the neurocranium and stored in 1 mL of RNAlater solution for viral detection by RT-PCR.

Proposal of a pain and distress score sheet for laboratory zebrafish.	
	Score
Physical Appearance*	
Normal	0
Missing operculum, and missing fins repeatedly, indicating possible antagonist behaviours; darkening /inflammation of fin	1
Mild scoliosis/ lordosis, tegument lesions, mucus production, over or under conditioned (obese or thin), abrupt colour change, especially blanching	2
General emaciation (low body to head ratio), general body deformities, missing or protuberant scales	3
Food consumption behaviour	
Normal	0
Unresponsive to food during the first meal	1
Unresponsive to food during 5 days, not even live food	2
Unresponsive to food during 1 week not even live food as artemia or rotifers	3
Respiratory behaviour	
Normal	0
Piping or extremely low rate, almost no opercular movement	3
Swimming behaviour	
Swimming through the water column	0
Difficulties to control buoyancy and/or to maintain equilibrium	2
Systematic swimming on the surface or in the bottom of the tank	3
Activity	
Normal	0
Hyperactive (erratic swimming) or hypoactive	2
Letargic, no reaction to external stimuli	3
Social behaviour	
Normal. Shoaling.	0
Individual often isolated when group-housed	1
Individual always chasing or being chased by conspecifics	2
Individual does not respond to conspecific behaviours towards him	3
TOTAL	
Judgement: 0-1 Normal; 2-8 Monitor carefully. Consider veterinary treatment including analgesics and consider also to analyse water quality; 9-12 Suffering, provide relief, consult the specialized veterinarian, consider termination. 13-18 severe status, euthanasia, rethink experimental procedure. Euthanasia may be considered if a score of 3 is observed in any of the categories, except for behaviour-related categories, in which scores of 3 suggests repeated and close observation for a final decision regarding euthanasia. *Take in consideration organisms' age.	

Figure 3 - Humane Endpoints Table for laboratory zebrafish.

3. Passive immunization using Ccombodies-enriched feed

3.1. Feed supplementation with Ccombodies

Ccombodies were produced and shipped to Portugal by Earwyn Bio. Commercial feed pellets were supplemented with Ccombodies at three final concentrations: i.e. low dose (1 mg Ccombody/100 g of diet), medium dose (10 mg Ccombody/100 g of diet) and high dose (100 mg Ccombody/100 g of diet). The required amount of Ccombodies for each tank was calculated using the following formula:

$$Amount\ of\ Ccombodies\ (mg) = \frac{Target\ concentration\ (mg/100\ g\ feed) \times Biomass\ \times Feeding\ rate}{100}$$

The calculated amount of Ccombodies was diluted in phosphate-buffered saline (PBS), and the resulting mixture was used to coat the feed pellets. The coated feed was air-dried at room temperature. Weekly batches were prepared in advance and stored at 4°C until use. Control group feed pellets were coated with PBS only, following the same procedure.

3.2. Experimental design

A total of 120 juvenile European sea bass were used in this experiment. Prior to the trial, fish were transferred from the aquaculture room (maintained at ~15°C) to the contention room for acclimatization. Each fish was individually weighed, and the initial biomass per tank was determined (mean ± SD, 7.46 ± 1.45 g) to accurately calculate daily feed and antibody supplementation. Fish were randomly assigned to four treatment groups, each in triplicate (three tanks per treatment group, 10 fish per tank). The experimental groups are outlined in Table 1.

Table I - Treatments used in the trial.

Treatment	Virus	Ccombody concentration in feed
A (negative)	Yes	0 mg/100g of feed
B (low)	Yes	1mg/100g of feed
C (medium)	Yes	10 mg/100g of feed
D (high)	Yes	100mg/100g of feed

3.3. Feeding trial and viral challenge

Before the Ccombody feeding trial, the sea bass underwent a nineteen-day acclimatisation period at a seawater temperature of approximately 25°C. During this period, the photoperiod was gradually adjusted to a 10:14 (light: dark) cycle by reducing the light phase by 15 minutes per day. Fish were fed twice daily with commercial feed at 3.9% of their biomass throughout the acclimatisation period. Following acclimatisation, the fish were fed for seven consecutive days with their assigned diets containing different concentrations

of Ccombodies as indicated in Table 1. Fish in treatment group A received the control diet, which consisted of unmodified feed coated with PBS only.

On day 7 of Ccombody feeding, fish were anaesthetised as described above, and challenged with NNV (genotype RGNNV, strain DP 965.08) via intramuscular injection at a concentration of 10^5 $TCID_{50}$/fish, based on the LD_{50} determination. After infection, the fish were placed in the recovery buckets with aerated tank water and subsequently returned to their respective tanks. Post-infection, fish were continuously fed the Ccombody-enriched or control diet for an additional 13 days. Feeding occurred twice daily, and the feeding rate was adjusted weekly according to observed clinical signs and fish appetite. After each feeding, tanks were syphoned to remove uneaten feed and faeces. Water quality parameters – including temperature, dissolved oxygen, salinity, and ammonia - were monitored and recorded daily as previously described. Fish behaviour was observed several times per day. And the Humane Endpoints Scoring System was applied to assess distress and determine the need for euthanasia. During the trial, brain and head kidney samples were collected from moribund fish (N=3 per tank) for RNA extraction. At the end of the trial, all remaining fish were euthanised.

3.4. RNA extraction

RNA extraction was carried out at the facilities of CIIMAR (University of Porto, Matosinhos) and total RNA was extracted from brain and head kidney tissues using the Maxwell®RSC platform and the Mawell®RSC simplyRNA Tissue Kit (Promega), following the manufacturer´s protocol. First, the Thioglycerol/Homogenization solution was prepared by adding 20 µL of 1-Thioglycerol per milliliter of Homogenization Solution. For each tissue sample, 200 µL of the freshly prepared solution was used. The DNase I solution was prepared by reconstituting the lyophilized DNase I with 275 µL of Nuclease-Free Water, followed by gentle inversion and mixing. Subsequently, 5 µL of Blue Dye was added to the DNAse I solution. Each tissue sample (approximately 10 mg) was placed into a centrifuge tube containing a homogenization bead and 200 µL of the Thioglycerol/Homogenization solution. Samples were homogenized using the Precellys homogenizer at 6000 rpm in two cycles of 20 seconds, with a 15 second interval between cycles. After homogenization, 200 µL of lysis buffer were added, and samples were vortexed for 15 seconds. Next, the cartridges were prepared (Figure 4), as follows: well #1 400 µL of the prepared tubes from each sample, well #4 10 µL of the prepared DNase I solution, well #8 insertion of pipette tips, in front of each cartridge a 1.5 ml Eppendorf tube containing 50 µL of Nuclease-Free Water to collect the eluted RNA. After automated extraction, RNA quantity and purity were assessed using the Nanodrop 2000 spectrophotometer (ThermoFisher Scientific) by measuring absorbance ratios at 260/280 nm and

260/230 nm. The integrity of total RNA was verified by electrophoresis on a 2% agarose gel stained with GreenSafe Premium (NZYTech, Portugal).

Figure 4 – A) Setup and configuration of deck tray; B) Maxwell® RSC Cartridge (Source: Promega Corporation (2022).

3.5. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics software (version 29.0, IBM Corp., 2022). Mortality was expressed as percentages per treatment over time. To verify the normality of the data, the Shapiro-Wilk test was used.

As the assumptions of normality were not met, the non-parametric Kruskal-Wallis test was used to compare the mortality percentages between the different treatments. The significance level adopted was 5% ($p < 0.05$).

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Results

1. Preliminary trial - LD_{50} determination

This trial lasted 6 days, and by the fifth day, a 50% mortality rate had already been reached in both viral concentration groups, as illustrated in Figure 5.

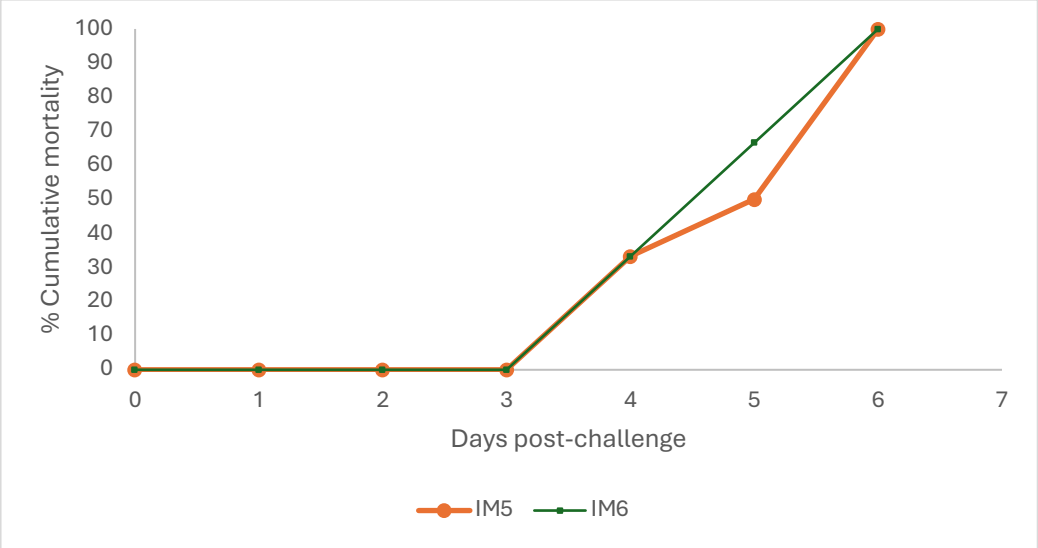

Figure 5. - Graph of cumulative mortality in the days after infection at both concentrations. (IM5 - intramuscular injection 10^5 $TCID_{50}$/fish; IM6 - intramuscular injection 10^6 $TCID_{50}$/fish). There are no statistically significant differences ($p > 0.05$).

During this preliminary trial, clinical symptoms were observed in both viral concentration groups. In the IM5 group (intramuscular injection 10^5 $TCID_{50}$/fish) the first symptoms appeared on the third day post-infection, when a fish was observed spinning (upside down). On the fourth day, a fish was observed swimming on the surface (belly up), another swimming uncontrollably and another swimming with its head down. On the fifth day, a fish was observed swimming vertically, and on the sixth day, a fish was observed spinning (upside down). In the IM6 group (intramuscular injection 10^6 $TCID_{50}$/fish), the first symptoms were also observed on the third day post-infection, with one fish spinning (upside down) and another swimming against the tank walls (accelerated). On the fourth day, the fish showed a loss of appetite, and a dark fish was observed swimming against the tank walls (accelerated), a fish swimming at the surface (belly up) and a fish spinning, on

the fifth day, a fish was observed swimming vertically against the wall, and the fish showed again a loss of appetite, and on the sixth day, a fish was observed swimming against the wall.

On the fifth day post-infection, one dead fish was observed in each of the two concentration groups. In the IM5 group (intramuscular injection 10^5 $TCID_{50}$/fish), an additional mortality was recorded on the sixth day. Throughout the experiment, symptomatic fish were euthanised in accordance with the humane endpoints table. Brain samples were collected from all euthanised fish and were consistently found to be congested.

2. Passive immunization using Ccombodies-enriched feed

Throughout this study, mortality was recorded daily in the various treatments and, as can be seen in the graph in Figure 6, the treatment in which 10mg Ccombodies/100g feed (C) was used was the one with the highest mortality (53.33%). This was followed by the 1mg Ccombodies/100g feed (B) treatment (46.66%) and the 100mg Ccombodies/100g feed (D) treatment (43.33%). On the other hand, the negative control (A), a treatment in which no antibody was used, was the one with the lowest mortality percentage (36.66%). Between the various treatments, no statistically significant differences were observed.

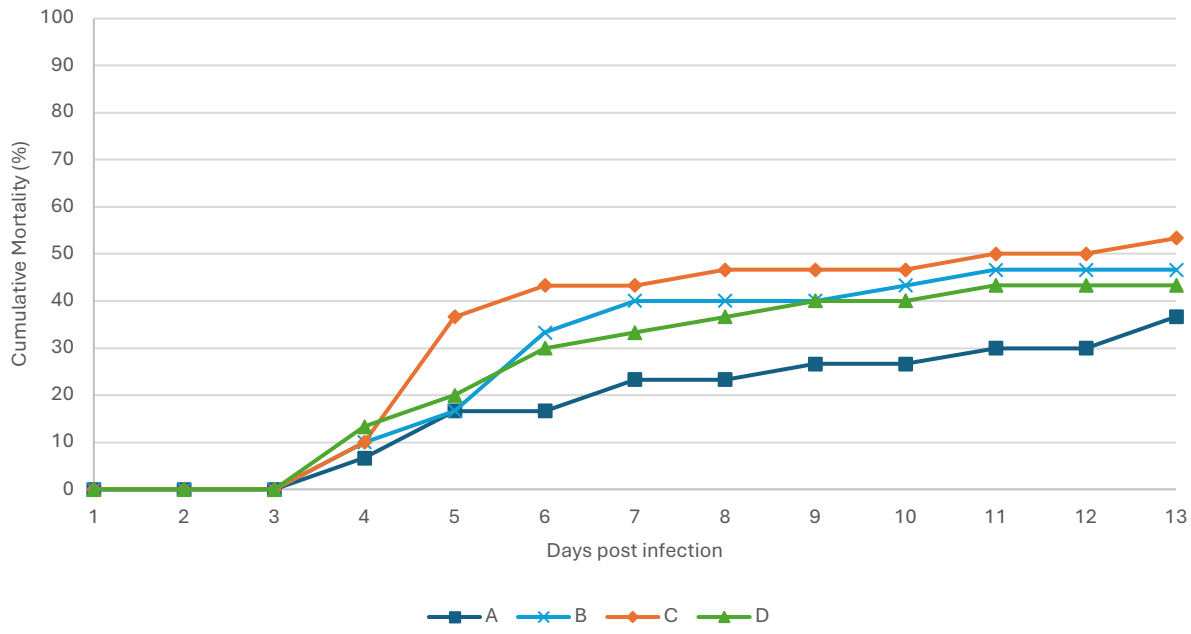

Figure 6 – Mortality kinetics for passive immunization trial. (A - negative control without antibodies; B – 1mg/100g feed; C – 10mg/100g feed; D – 100mg/100g feed). No statistically significant differences were observed ($p > 0.05$)

In this trial, clinical signs in fish were also observed in the various treatments. The first clinical signs were observed on the fourth day post-infection (dpi), in all treatments, with less agitation during feeding and a reduction in appetite, which continued to be observed throughout the trial.

The main clinical signs observed, in all treatments, during the trial included abnormal swimming (vertical, spiral, at the surface or at the bottom of the tank, often with little reaction), darkened body coloration, hyperactivity, lethargy and isolation. In treatment A (without antibody), darkened fish, vertical swimming, loss of balance and hyperactivity were predominant throughout the days, in treatment B (1mg/100g feed), darkened fish, hyperactivity, abnormal swimming (surface, bottom, vertical and spiral) and increased agitation in the tanks were observed, in treatment C (10mg/100g feed), episodes of spiral swimming, hyperactivity, darkened fish and reduced reaction at the bottom were recorded and in treatment D (100mg/100g feed), darkened fish, hyperactivity, isolation, vertical and spiral swimming, lethargy and crowding at the bottom of the tanks were observed.

As the clinical signs were recorded, the Humane Endpoints table (Figure 3) was used to understand if the clinical signs presented by the fish were indicative that they were suffering, so they should be euthanized. Whenever the fish were euthanized, the brain and head kidney were removed, which were stored in RNAlater to later proceed to RNA extraction.

3. RNA extraction

Forty-eight of the seventy-six samples obtained during the trial were used for RNA extraction, and seven brains and five head kidneys from each treatment were used.

After RNA extraction, the concentration was obtained, varying between treatments (Appendix table I). In treatment A, the values obtained were between 133.372 ng/μL and 362.544 ng/μL, in treatment B between 131.367 ng/μL and 533.244 ng/μL, in treatment C between 186.490 ng/μL and 486.627 ng/μL and in treatment D between 111.499 ng/μL and 466.261 ng/μL.

To know the purity of RNA, the 260/280 ratio was used, since it indicates the absence of proteins, phenol and other contaminants that absorb close to 280 nm. For this ratio, the ideal value is 2.0, and values between 1.9 and 2.4 are acceptable, the samples used were between these values. The 260/230 ratio was also performed, which served as a secondary measure of purity, indicating the presence of organic contaminants that absorb below 230 nm. It's ideal value is 2.2 and values between 2.0 and 2.3 are acceptable. After extraction, three samples were below 2.0, two in treatment A and one in treatment D (Appendix table I).

RNA quality and integrity were obtained by electrophoresis in 2% agarose gel (Figure 7).

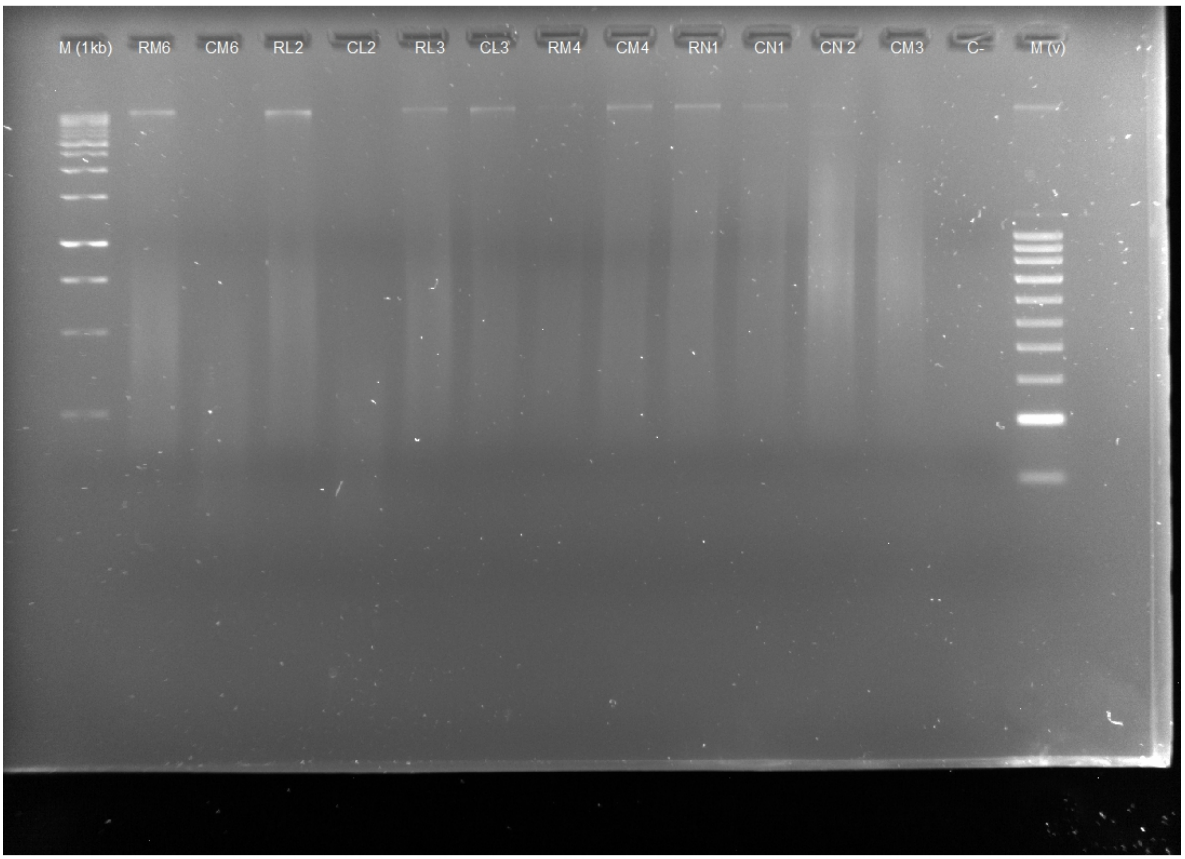

Figure 7 - Electrophoresis of the RNA extraction. Samples of treatment C. Agarose gel 2%: M(v) - NZYTech Ladder V marker; RM6 – head kidney, tank M, sample 6; CM6 – brain, tank M, sample 6; RL2 – head kidney, tank L, sample 2; CL2 – brain, tank L, sample 2; RL3 – head kidney, tank L, sample 3; CL3 – brain, tank L, sample 3; RM4 – head kidney, tank M, sample 4; CM4 – brain, tank M, sample 4; RN1 - head kidney, tank N, sample 1; CN1 – brain, tank N, sample 1; CN2 – brain, tank N, sample 2; CM3 – brain, tank M, sample 3; C- – negative control.

After observing the gel, it was found that the results obtained were not as expected. Thus, the RNA concentration was obtained again, as well as its purity, through the ratios 260/280 and 260/230 to verify if there had been any change in the samples (Appendix table II). In treatment A, the values obtained were between 136.4 ng/µL and 349.2 ng/µL, in treatment B between 123.8 ng/µL and 563.2 ng/µL, in treatment C between 171.6 ng/µL and 463.8 ng/µL and in treatment D between 112.7 ng/µL and 508.4 ng/µL. Regarding purity, all samples were between the acceptable values for both ratios.

The results obtained were identical to those obtained in the first instance, so it was decided to proceed with a new electrophoresis, this time in a 1.5% agarose gel, to obtain the quality and integrity of the RNA (Figure 8).

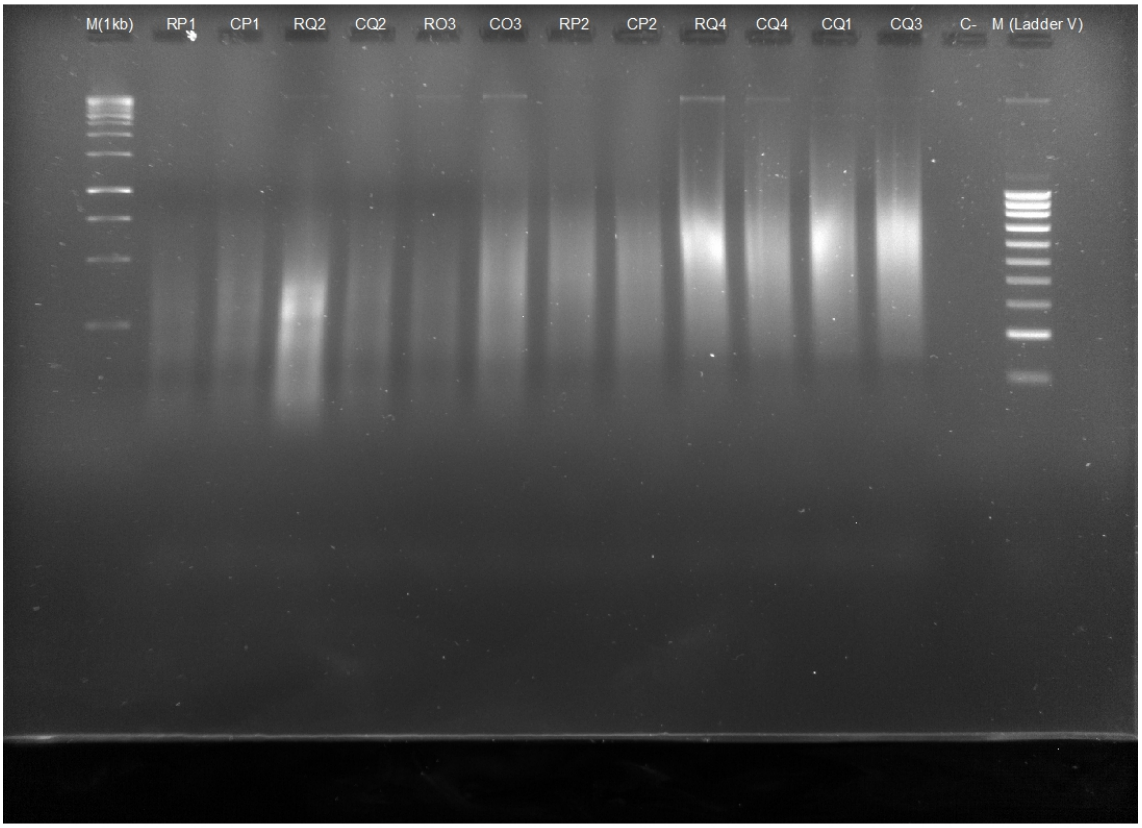

Figure 8 - Electrophoresis of the RNA extraction. Samples of treatment D. Agarose gel 1,5%: M(v) - NZYTech Ladder V marker; RP1 – head kidney, tank P, sample 1; CP1 – brain, tank P, sample 1; RQ2 – head kidney, tank Q, sample 2; CQ2 – brain, tank Q, sample 2; RO3 – head kidney, tank O, sample 3; CO3 – brain, tank O, sample 3; RP2 – head kidney, tank P, sample 2; CP2 – brain, tank P, sample 2; RQ4 - head kidney, tank Q, sample 4; CQ4 – brain, tank Q, sample 4; CQ1 – brain, tank Q, sample 1; CQ3 – brain, tank Q, sample 3; C- – negative control.

Through the gel obtained, it was again verified that the results obtained were not as expected, indicating that the RNA was degraded. Due to this, it was not possible to proceed with the remaining analyses.

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Discussion

In aquaculture, one of the most significant diseases is viral nervous necrosis (VNN), mainly affecting larvae and juveniles of several species of fish such as the European sea bass. This disease is associated with high mortality and even fish that manage to survive tend to perform poorly after recovery, which also leads to high economic losses (Munday, *et al*., 2002; Bandín & Souto, 2020). As this disease affects the host at a time when it is not yet fully immunocompetent, it is difficult to formulate a vaccine or an effective method to control the disease (Costa & Thompson, 2016). This study aimed to assess how the administration of Ccombodies affects survival of juvenile European sea bass (*Dicentrarchus labrax*) infected with nervous necrosis virus (NNV).

The preliminary LD_{50} assay is essential to determine the dose of virus that will be used throughout the assay. The results obtained in the preliminary trial demonstrated a high virulence of the virus strain used, reflected by a mortality rate of 50% reached on the fifth day post-infection in both experimental groups (IM5 and IM6). This pattern of mortality indicates a rapid progression of infection regardless of the administered viral concentration (10^5 $TCID_{50}$/fish or 10^6 $TCID_{50}$/fish), suggesting that even the lowest dose tested is sufficient to cause mortality in a short period. In both groups, clinical signs were observed, starting on the third day post-infection, including spiral swimming, loss of orientation (upside down or vertical swimming), swimming against the walls of the tank and loss of appetite, signs commonly seen in fish affected by VNN (Bandín & Souto, 2020), indicating neurological changes caused by the virus, and according to Munday *et al*. (2002) this type of symptoms are related to brain lesions, which was verified in this trial due to the presence of cerebral congestion in the euthanized fish. Although two distinct viral concentrations were tested, the pattern of symptom onset and associated mortality was quite similar, which may indicate that the dose range tested was not wide enough to produce differences between the groups. Thus, in future trials, it would be interesting to include groups with lower viral concentrations to obtain an accurate calculation of LD_{50}. Analysing the results obtained and considering that both groups reached the 50% mortality required in an LD_{50} assay, it was decided to use the lowest concentration (10^5 $TCID_{50}$/fish) in the next assay.

The trial related to passive immunization using Ccombodies-enriched feed served to test the use of recombinant antibodies to mitigate NNV infection, since fish in their early stages of life do not have a fully developed immune system so the administration of exogenous antibodies will address a possible shortage of endogenous antibodies, providing temporary protection or therapeutic effects against the pathogen (Hedegaard & Heegaard, 2016; Rajan *et al*., 2017). This approach has been successfully applied in various aquaculture species, highlighting its potential as both a prophylactic and therapeutic measure. For example, Lazarte *et al*. (2021) demonstrated that recombinant variable lymphocyte receptor B (VLRB) antibodies targeting *Vibrio parahaemolyticus* toxins could be incorporated into shrimp feed, significantly improving survival of *Litopenaeus vannamei*. Similarly, Jung *et al*. (2020) characterised VLRB antibodies from hagfish (*Eptatretus burgeri*), showing their capacity to neutralise nervous necrosis virus, thereby underscoring the versatility of alternative adaptive immune receptors as immunotherapeutics in aquatic species. Engineered VLRBs have also shown efficacy against avian influenza virus H9N2 (Im *et al*., 2018), providing a proof of concept for their application across a broad range of aquaculture pathogens. In finfish, several studies have demonstrated the feasibility of antibody-based immunoprophylaxis. Early work by Lorenzen *et al*. (2000) showed that injection of mouse antibody genes into fish resulted in transient protection, while more recent strategies have explored dietary supplementation with antibody-enriched feeds. Rajan *et al*. (2017) highlighted that passive immunisation could enhance the antiviral state and support immune development in species such as European seabass. Likewise, the authors verified that passive immunisation reduced mortality and improved survival outcomes, key parameters for the sustainability of aquaculture systems. One of the results obtained in this trial was the accumulated mortality, registering higher values in the treatment of 10 mg of antibody per 100 g of feed (53.33%), followed by the treatment with 1 mg of antibody per 100 g of feed (46.66%) and the treatment of 100 mg of antibody per 100 g of feed (43.33%). Treatment without Ccombody was the one with the lowest accumulated mortality value (36.66%), which agrees with several studies such as that of Gonzalez-Silvera *et al*. (2019) in which European sea bass weighing between 10-12g were used and after infection by NNV (10^6 $TCID_{50}$/fish, RGNNV genotype) a mortality rate of around 33% was obtained in groups in which protection against the virus was not conferred. In the study by Barsøe *et al*. (2021b) a mortality of about 30% was obtained after an infection by NNV (10^4 $TCID_{50}$/fish, RGNNV genotype) in juvenile European sea bass with average weights of 5g and in the study of Biasini *et al*. (2022) in which juvenile European sea bass with average weights of 5g were also used, a mortality of about 34.5% was obtained after an infection with NNV (10^5TCID_{50}/fish, RGNNV genotype). However, when looking at studies such as the one by Thiery *et al*. (2006), where European sea bass with 22g were used and infected with NNV (10^5TCID_{50}/fish, RGNNV genotype) and the study by Barsøe *et al*. (2021c) in which were used European

sea bass with average weights of 14g and infected with NNV (10^5TCID$_{50}$/fish, RGNNV genotype), it was found that the groups in which treatments were used to combat the pathogen had lower cumulative mortality values than the groups with no protection against the virus, which was not obtained in this study. In this trial, the clinical signs of fish in the various treatments were also observed and recorded. As in the LD_{50} assay, it was possible to observe a set of clinical signs compatible with Betanodavirus infection, such as changes in behavior, uncoordinated swimming (rotation, vertical or upside-down swimming), darkening of the body, hyperactivity and decreased appetite. These signs, described in the literature as characteristic of infection by the nerve necrosis virus (NNV) (Bandín & Souto, 2020), were recorded in all treatments, with small variations in frequency and intensity. In the various treatments, the first clinical signs (non-related to food) were recorded on the fifth day post-infection (dpi), which is in line with the results obtained by Barsøe *et al*. (2021a) and Biasini *et al*. (2022), and clinical signs continued to be recorded on the remaining days of the trial. Regarding treatment A (without antibody), these results were expected since, after being infected by intramuscular injection, they did not receive any type of immunological treatment. In treatment B (1 mg Ccombody/100 g feed), the clinical signs observed were, in general, like those of treatment A, which may indicate that the dose of antibody used may not have been sufficient to promote a relevant protective response. Treatment C (10 mg Ccombody/100 g feed) had the highest number of mortalities. A possible explanation for the results obtained, both for the cumulative mortality and for the clinical signs observed, could be related to the fact that the antibodies were delivered externally to the body and were not protected against enzymatic degradation in the digestive tract, which may have significantly reduced their efficacy (Lee *et al*., 2000). The way in which the antibodies were incorporated into the diet, through their dilution in PBS and subsequent coating of the feed pellets, could also be a possible explanation, since this procedure may have favored the dispersion of the antibodies in the water before ingestion by the fish, compromising the effective dose administered as well as the degradation or loss of the functional activity of the antibodies (Akbarian & Chen, 2022). In future studies, it would be interesting to incorporate the antibodies into a feed production plant, which would reduce possible problems related to the dispersion of antibodies in water. When comparing the feed formulation method used in this study with that described by Zhang *et al*. (2024), it is evident that in the latter study the pellets were left to dry at ambient temperature for a longer period (72 h), which may enhance antibody absorption and reduce its dispersion in the water. Therefore, in future studies, it would be worthwhile to increase the drying time of the pellets. Additionally, incorporating a substance during feed formulation that allows for antibody encapsulation could help protect it from degradation in the intestinal tract, as demonstrated by Gao *et al*., (2016), who used β-cyclodextrin for this purpose. The use of microencapsulation, as performed in the studies of Sotomayor-Gerding *et al*. (2020) and Zhang *et al*. (2020), is an excellent strategy for the oral administration of polyclonal antibodies, as it provides

protection against degradation by gastric enzymes and allows a more controlled release into the intestine, where absorption is potentially more effective (Wu *et al*., 2011; Encina *et al*., 2016; Hashim *et al*., 2021). It would also be interesting in future studies to add a treatment in which the fish were not infected, and a higher dose of antibody was provided, to understand if a higher dose negatively affects individuals. Also, could be interesting try different feed strategies, such as feeding fish with Ccombodies for a longer period before infection. However, after the antibodies were tested *in vitro* by other researchers from the Moredun Institute, it was found that they were not effective against the nervous necrosis virus (NNV), which may explain the absence of statistically significant differences in the mortality results obtained between the antibody and non-antibody treatments, as well as the similarity of clinical signs observed in the different treatments. In future studies, it will be essential to obtain *in vitro* results beforehand to assess whether the antibody is effective against the virus. This data will make it possible to decide whether the antibody can be used *in vivo* assays and whether adjustments need to be made to its development.

In the *in vivo* assay, as the fish were euthanized, the brain and head kidney were removed and stored in RNAlater, and then the RNA was extracted. This method of RNA storage was used as it minimizes the need for immediate processing of the samples, allowing them to be stored for more than a week at lower temperatures (Passow *et al*., 2019), which was verified in this study, since the samples were kept at -80ºC until the RNA was extracted.

The extraction of RNA from the brain and head kidney samples was carried out at the facilities of CIIMAR (University of Porto, Matosinhos) in an automated way through the platform Maxwell®RSC, ensuring greater effectiveness and lower risk of contamination. Soon after RNA extraction, the concentration and purity of the RNA of the various samples were obtained, verifying that for both parameters the values were acceptable, however, the RNA integrity evaluated by agarose gel electrophoresis, already at the Cetemares facilities, did not reveal well-defined 28S and 18S bands, indicating RNA degradation (Imbeaud *et al*., 2005; Fleige & Pfaffl, 2006; Schroeder *et al*., 2006). This result shows that acceptable concentrations and purity indices are not necessarily indicative of intact RNA, since degraded samples can maintain these parameters. Degradation does not appear to be associated with gel formulation or possible gel contaminations since two markers were used in the gels and both presented a correct and well-defined separation (Agarose Gel Electrophoresis of RNA | Thermo Fisher Scientific - DE, n.d.). The storage form (-80ºC) also does not seem to be associated with degradation, since low temperatures preserve RNA for long periods (Vehniäinen *et al*., 2019). It is therefore plausible that loss of integrity occurred after extraction, during transport between laboratories, due to possible thermal variations. To prevent this issue, analyses could be performed at the extraction site, or RNA could be converted into cDNA immediately after extraction, as cDNA is more stable (Exploring cDNA, 2025). It was planned to quantify the viral load by RT-qPCR, however, due to RNA degradation, unfortunately, it was

not possible to proceed with the analyses. Although it was not possible to quantify the viral load in the brain and cranial kidney, the identical results obtained regarding mortality rates and clinical signs allow us to deduce that the viral quantification values would not present significant differences between the different treatments either.

Although no favourable results were obtained in this study, it should be noted that the use of recombinant antibodies allows positive results against NNV infections, as demonstrated in the study carried out by Jung *et al*. (2020). It is also important to note that passive immunization is essential to combat diseases in aquaculture, and several studies, such as the one by Liu *et al*. (2021) and Liang *et al*. (2024), demonstrated that oral administration of antibodies reduced viral load and mortality in fish infected with NNV.

Conclusion

The results obtained in this study confirm the high virulence of the NNV strain used, with significant mortality in infection assays, even at the lowest viral dose. The LD_{50} assay allowed the identification of an adequate concentration of virus for the following tests, demonstrating the rapid progression of infection in juvenile European sea bass, with typical clinical signs consistent with those described in the literature for infections with NNV.

In the passive immunization trial with *Ccombody* diets, it was found that oral administration of recombinant antibodies did not result in effective protection against NNV infection. The absence of statistically significant differences in cumulative mortality levels and clinical expression between treatments suggests that the antibodies used did not show relevant antiviral activity *in vivo*. This conclusion is supported by *in vitro* assays previously carried out by other researchers under the Moredun, which demonstrated the ineffectiveness of the antibodies tested against NNV. In addition, factors such as the enzymatic degradation of antibodies in the digestive tract, their possible dispersal in water before ingestion and the structural instability of antibodies may have compromised the effectiveness of oral treatment.

Regarding the molecular analysis, the integrity of the extracted RNA was compromised, despite acceptable concentrations and purity indexes. The degradation observed after electrophoresis indicates that care with the transport of samples between the extraction and analysis sites should be reviewed. RNA instability is a critical factor in gene expression and viral load quantification studies, and its inadequate handling significantly compromises the results obtained.

In future studies, it is recommended to explore strategies to protect recombinant antibodies, namely through microencapsulation, ensuring their stability and controlled release in the intestine. In addition, the formulation of foods with antibodies should be reviewed and adapted to industrial processes that minimize losses during administration. In parallel, it will be crucial to ensure that the antibodies used have proven antiviral efficacy, ideally validated through robust *in vitro* assays prior to their *in vivo* application. It is also suggested that future molecular analyses, namely quantification of viral load by RT-qPCR, be carried out immediately after RNA extraction, or that RNA be converted into cDNA quickly, to ensure the integrity of the samples and the reliability of the results. These methodological improvements will allow a more accurate assessment of the potential of passive immunization with recombinant antibodies in aquaculture, contributing to the development of innovative and effective strategies to combat viral diseases in fish.

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Appendix

Table I - Quantity and quality of RNA (CIIMAR).

Treatment	Sample	Concentration of nucleic acids (ng/µL)	260/280 (nm)	260/230 (nm)
A (without antibody)	RG1	156.244	2.115	1.990
	CG1	228.735	2.121	2.102
	RG2	143.180	2.025	1.982
	CG2	181.947	2.146	2.172
	RF2	177.252	2.127	2.112
	CF2	133.372	2.166	2.147
	RF3	270.975	2.091	2.062
	CF3	266.771	2.115	2.160
	RF4	282.396	2.106	2.120
	CF4	362.544	2.138	2.169
	CE1	253.195	2.139	2.215
	CF1	352.530	2.123	2.223
B (1mg/100g feed)	RI3	131.367	2.104	2.032
	CI3	143.264	2.109	2.027
	RH3	153.750	2.116	2.071
	CH3	246.495	2.147	2.170
	RJ1	369.940	2.093	2.112
	CJ1	366.771	2.118	2.178
	RI4	400.070	2.120	2.158
	CI4	366.598	2.114	2.183
	RH2	201.603	2.122	2.164
	CH2	533.244	2.134	2.181
	CH1	341.185	2.147	2.226
	CI1	277.630	2.111	2.187
C (10mg/100g feed)	RM6	333.849	2.136	2.157
	CM6	223.136	2.136	2.114
	RL2	303.696	2.133	2.160
	CL2	297.801	2.149	2.178
	RL3	486.627	2.126	2.217
	CL3	239.138	2.113	2.168
	RN4	188.467	2.129	2.143
	CN4	380.761	2.144	2.185
	RN1	364.928	2.132	2.169
	CN1	213.948	2.140	2.208
	CN2	417.055	2.135	2.212
	CM3	186.490	2.148	2.228
D (10mg/100g feed)	RP1	111.499	2.055	1.951
	CP1	146.096	2.118	2.158
	RQ2	466.261	2.148	2.154
	CQ2	166.791	2.119	2.107
	RO3	125.472	2.141	2.102
	CO3	266.864	2.153	2.210
	RP2	176.111	2.139	2.172
	CP2	369.157	2.149	2.212
	RQ4	395.992	2.142	2.203
	CQ4	316.991	2.144	2.215
	CQ1	286.227	2.140	2.227
	CQ3	304.597	2.127	2.210

Table II - Quantity and quality of RNA (CETEMARES).

Treatment	Sample	Concentration of nucleic acids (ng/μL)	260/280 (nm)	260/230 (nm)
A (without antibody)	RG1	148.3	2.05	2.06
	CG1	230.9	2.09	2.20
	RG2	138.5	1.98	2.13
	CG2	178.4	2.10	2.31
	RF2	194.1	2.10	2.21
	CF2	136.4	2.07	2.24
	RF3	248.2	2.08	2.11
	CF3	257.4	2.10	2.30
	RF4	264.3	2.07	2.21
	CF4	349.2	2.10	2.22
	CE1	250.7	2.10	2.31
	CF1	339.2	2.11	2.31
B (1mg/100g feed)	RI3	123.8	2.05	2.12
	CI3	137.8	2.07	2.23
	RH3	148.7	2.06	2.18
	CH3	246.3	2.09	2.28
	RJ1	345.1	2.09	2.26
	CJ1	355.8	2.10	2.28
	RI4	384.7	2.09	2.22
	CI4	357.8	2.11	2.29
	RH2	204.8	2.07	2.22
	CH2	563.2	2.11	2.34
	CH1	337.0	2.11	2.32
	CI1	260.0	2.09	2.26
C (10mg/100g feed)	RM6	340.8	2.10	2.26
	CM6	219.1	2.09	2.25
	RL2	296.4	2.09	2.23
	CL2	274.5	2.08	2.14
	RL3	463.8	2.09	2.31
	CL3	267.2	2.09	2.23
	RN4	171.6	2.05	2.14
	CN4	359.3	2.10	2.24
	RN1	348.1	2.09	2.28
	CN1	188.3	2.05	2.00
	CN2	408.3	2.10	2.30
	CM3	179.0	2.09	2.34
D (10mg/100g feed)	RP1	112.7	2.01	2.15
	CP1	158.3	2.10	2.32
	RQ2	508.4	2.06	2.29
	CQ2	159.8	2.07	2.22
	RO3	115.0	2.05	2.22
	CO3	259.8	2.09	2.31
	RP2	175.0	2.08	2.29
	CP2	373.0	2.11	2.31
	RQ4	379.2	2.11	2.31
	CQ4	307.0	2.11	2.31
	CQ1	274.6	2.11	2.34
	CQ3	293.6	2.10	2.33