Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biology

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Impact of diet formulation on gill health status of Atlantic Salmon (*Salmo salar*) infected by Amoebic Gill Disease (AGD)

Master's thesis in Health Management in Aquaculture (AquaH)
Supervisor: Rolf Erik Olsen
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Preface

First, I would like to thank Elisabeth Aasum, and Simon Leigh Wadsworth for granting me the opportunity to write my master thesis in collaboration with Biomar AS. I would also like to thank Torunn Forberg for allowing me to use one of her feeding trials for my project and providing full guidance during the whole process. Torunn, thank you for always being available to help, for all your kindness and for accepting the role of my external supervisor in this thesis. I would also like to thank all of the staff at ILAB for helping me during the samplings and, especially, to the trial coordinator, Susanne Håvardstun Eide, for providing me with a detailed description of the trial.

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Finally, I would like to thank my wife, Livia, for joining me on this adventure across the world, my parents, Denise and Cassiano, and my brother, Dante, for all the love and support that they always provide me.

Abstract

Aquaculture is the fastest growing food sector in the world and is also one of the biggest promises of a sustainable food source capable of supporting the ever-increasing world population. However, from a health management point of view, the fast growth of this sector is of big concern. The intensification of aquaculture production results in systems with higher densities, creating the perfect environment for diseases to spread. One current example is the increasing incidence of the Amoebic Gill Disease (AGD) in Atlantic salmon's (*Salmo salar*) aquaculture industry and its negative impacts, such as, increased production costs, lower growth, and higher mortality. AGD, caused by the parasite Ne*oparamoeba perurans*, has been commonly treated with hydrogen peroxide or freshwater baths, but no preventative measures are in place. Nonetheless, different studies have shown promising results in using functional feed as a preventative measure to many diseases, including AGD.

The aim of this study is to test two diet formulations (control and functional feed) and compare the overall gill health and the response of ten different genes related to immune and pro-inflammatory processes in uninfected and AGD-infected Atlantic salmon. The results obtained showed a significant difference in four of the genes, which consisted of an upregulation of cathelicidin 2, mucin 18 and tumor necrosis factor α-3, and a down regulation of interleukin 4-13a in the infected group, when compared to the uninfected group. Furthermore, the expression of cathelicidin 2 and mucin 18 showed encouraging results to support the hypothesis that the functional feed provides a protective effect against the disease, by better regulating the host's inflammatory response. However, the results obtained did not provide enough evidence in order to fully validate the functional diet as a successful preventative method against AGD. Therefore, further research is needed to provide more knowledge on the use of functional ingredients and their role in combatting AGD outbreaks in the aquaculture industry.

Key words: Amoebic Gill Disease (AGD), Atlantic salmon, Neoparamoeba perurans, aquaculture, gene expression, functional feed.

Sammendrag

Akvakultur er den raskest voksende matsektoren i verden og er også et av de største løftene om en bærekraftig matkilde som er i stand til å støtte den stadig økende verdensbefolkningen. Fra et helseledelsessynspunkt er imidlertid den raske veksten i denne sektoren av stor bekymring. Intensiveringen av akvakulturproduksjonen resulterer i systemer med høyere tettheter, og skaper det perfekte miljøet for sykdommer å spre seg. Et aktuelt eksempel er den økende forekomsten av amøbe-gjellesykdommen (AGD) i atlantisk laks (*Salmo Salar*) akvakulturnæringen og dens negative virkninger, som økte produksjonskostnader, lavere vekst og høyere dødelighet. AGD, forårsaket av parasitten *Neoparamoeba perurans*, har ofte blitt behandlet med hydrogenperoksid eller ferskvannsbad, men ingen forebyggende tiltak er på plass. Ikke desto mindre har forskjellige studier vist lovende resultater ved bruk av funksjonelt fôr som et forebyggende tiltak mot mange sykdommer, inkludert AGD.

Målet med denne studien er å teste to diettformuleringer (kontroll og funksjonelt fôr) og sammenligne den generelle gjellehelsen og responsen til ti ulike gener relatert til immun- og pro-inflammatoriske prosesser i uinfisert og AGD-infisert atlantisk laks. Resultatene som ble oppnådd viste en signifikant forskjell i fire av genene, som besto av en oppregulering av cathelicidin 2, mucin 18 og tumornekrosefaktor α-3, og en nedregulering av interleukin 4-13a i den infiserte gruppen, sammenlignet med uinfisert gruppe. Videre viste ekspresjonen av cathelicidin 2 og mucin 18 oppmuntrende resultater for å støtte hypotesen om at det funksjonelle fôret gir en beskyttende effekt mot sykdommen, ved å bedre regulere vertens inflammatoriske respons. Resultatene som ble oppnådd ga imidlertid ikke nok bevis til å fullt ut validere det funksjonelle kostholdet som en vellykket forebyggende metode mot AGD. Det er derfor behov for ytterligere forskning for å gi mer kunnskap om bruken av funksjonelle ingredienser og deres rolle i å bekjempe AGD-utbrudd i havbruksnæringen.

Stikkord: Amoebic Gill Disease (AGD), atlantisk laks, Neoparamoeba perurans, akvakultur, genuttrykk, funksjonelt fôr.

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List of Abbreviations

AGD	Amoebic Gill Disease
AMP	Antimicrobial peptide
Cq	Cycle threshold
DHA	Docosahexaenoic
DPC	Days post challenge
DS	Double stranded
EOs	Essential oils
EPA	Eicosapentaenoic
FAs	Fatty Acids
FW	Forward
FOTS	Forsøksdyrforvaltningens tilsyns- og søknadssystem
ILAB	Aquatic and Industrial Laboratory
IPNV	Infectious pancreatic necrosis
IQR	Interquartile range
ISAV	Infectious salmon anemia virus
LC-PUFA	Long-chain polyunsaturated fatty acids
MHC	Major Histocompatibility Complex
MIQE	Minimum information for publication of quantitative real-time PCR experiments
MOS	Mannanoligosaccharides
NF	Normalisation factor
NRQ	Normalised relative quantities
NTNU	The Norwegian University of Science and Technology
PAMP	Pathogen associated molecular pattern
PL	Phospholipid
PMCV	Piscine myocarditis virus
PRV	Piscine orthoreovirus
RE	Reverse
RQ	Relative quantities
RT-qPCR	Real time-quantitative polymerase chain reaction
SAV	Salmonid alphavirus
SGR	Specific growth rate
SS	Single stranded
TAG	Triacylglycerides
TCR	T-Cell receptor
TM	Melting temperature

1. Introduction

Fish gills are commonly known for their respiratory role. Nonetheless, other essential processes such as, osmoregulation, calcium homeostasis, excretion of nitrogenous waste (ammonia), acid-base regulation, and hormone production, also occur on fish gills. In order to optimize the gaseous exchange, fish gills are in close contact to the marine environment, protected only by the operculum. This arrangement leaves the gills, which are very delicate, exposed to many external disturbances (e.g. toxins, pathogens, organisms, and particulate matter), making them highly vulnerable to irritation, physical damage, or even, infection (Herrero et al., 2018).

Many pathogens, such as viruses, parasites, and bacteria, have been found to target the fish gills and endanger the performance of its key mechanisms. In the industry of farmed Atlantic salmon (*Salmo salar*), gill diseases are considered to be one of the main health challenges today, due to the significant economic losses as a result of mortality, inadequate growth, and cost of treatments (Herrero et al., 2018). In particular, the amoebic gill disease (AGD), which has been first documented thirty years ago, and is of increasing concern to the global aquaculture industry (Oldham et al., 2016).

It is often hard to provide a complete definition on what gill health is. On many occasions, it has been interpreted as the presence or absence of a detectable change, when compared to the expected normal reference. However, it should also be based on a combination of behaviour, clinical signs, gross pathology, histopathology, and other laboratory findings (Foyle et al., 2020, Mitchell et al., 2012).

1.1 Amoebic Gill Disease (AGD)

Amoebic Gill Disease (AGD) was first described in Tasmania, Australia, in the 1980s, leading to increased production costs, due to lower growth, and increased mortality and treatment expenses ever since (Oldham et al., 2016). AGD is caused by the marine, free-living, amphizoic amoeba *Neoparamoeba perurans* and behaves as a facultative ectoparasite. *Neoparamoeba spp.* belongs to the family of naked, lobose amoebae and lacks well-organized surface structures. These amoebae can be found in high concentrations in a wide variety of habitats, ranging from coastal regions to the open sea. The natural distribution and reservoirs of *N. perurans,* outside of fish farms, have yet to be identified. However, while results from previous studies have ruled out wild fish as a significant reservoir of the parasite. Other studies indicate possible involvement of cleaner fish during AGD outbreaks (Oldham et al., 2016).

Treatments against AGD include hydrogen peroxide or freshwater baths, but no preventative measures are in place (Marcos-López et al., 2018). Some experimental vaccines have been tested but none showed successful immunization against *N. perurans* infection (Valdenegro-Vega et al., 2015; McGrath et al., 2022). However, different studies have shown promising results in the manipulation of feed composition as a preventative measure against many diseases (Martinez-rubio et al., 2014; Dawood et al., 2018; Mullins et al., 2020).

N. perurans has been observed in fourteen countries, distributed across six continents and in fifteen species of finfish (Oldham et al., 2016). Due to the expansion of the disease's geographic distribution and host range, the impact caused by AGD has increased dramatically and is of major concern to the global marine aquaculture industry (Marcos-López et al., 2018). AGD's ubiquitous distribution also means that outbreaks

have been reported in temperatures ranging as low as 7 °C to as high as 20 °C. Besides indicating that the parasite has a wide temperature range of infection, the different reports have shown that first appearances of the disease have been preceded by abnormally high temperatures (Oldham et al., 2016). The specific changes in gill physiology and in pathogenic virulence caused by climate change are still uncertain, however, it is estimated that higher temperatures could be beneficial for the attachment and growth of the *N. perurans* amoeba, influencing the development of AGD (Foyle et al., 2020). That being said, the continuous intensification of aquaculture and the effects of climate change will most likely increase the risks of AGD outbreaks. To prevent this situation, there is a strong need for the development of novel strategies for treatment and management of the disease.

1.1.1 Disease expression

The amoebic gill disease induces a highly localized response in the fish gills, which is microscopically characterized by epithelial hyperplasia, fusion of the lamellar epithelium, presence of interlamellar vesicles, and increased number of goblet cells (Figure 1). Clinically, the disease leads to anorexia, respiratory distress, lethargy, and aggregation at the water surface. Due to increased production of gill mucus, which is the organism's first physical and chemical barrier against waterborne pathogens, white mucoid patches can also be macroscopically seen on the gill filaments, depending on the severity of the disease (Figure 2). This is used for the initial diagnosis of infected fish (Oldham et al., 2016; Marcos-López et al., 2018; Mullins et al., 2020).

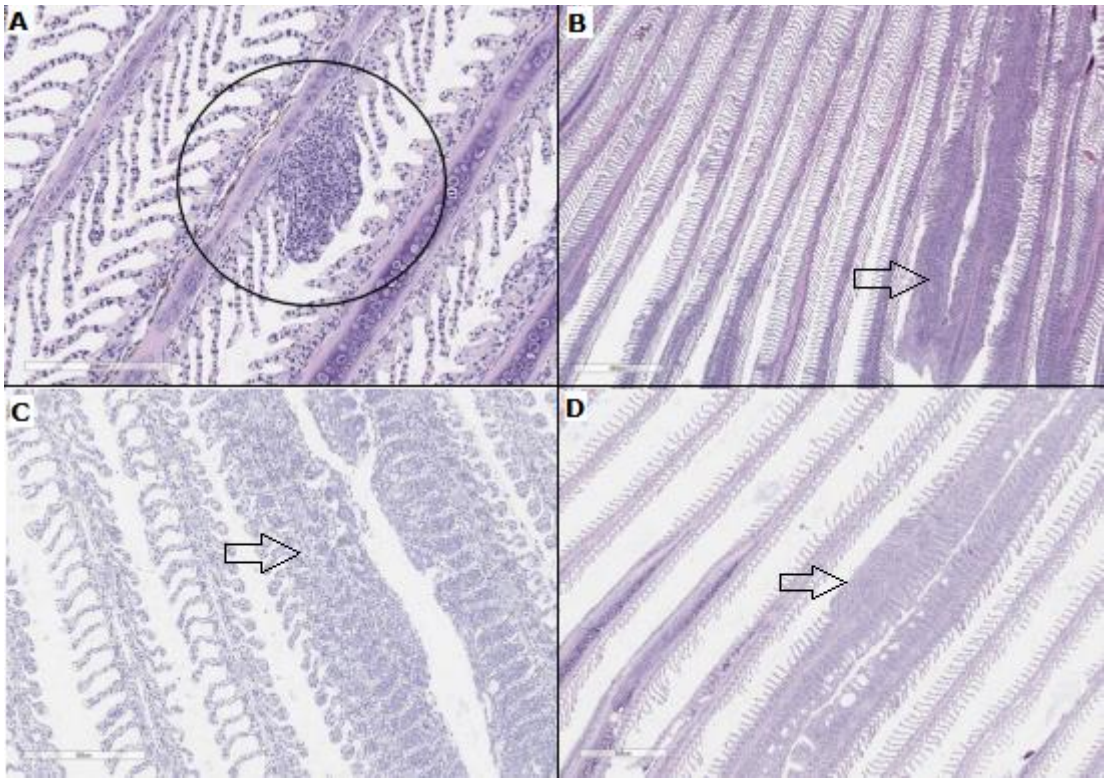


Figure 1. Histopathology assessment of AGD infection (H&E staining) performed by PHARMAQ. (A) Example of focal hyperplasia. (B-D) Examples of epithelial hyperplasia and fusion of the lamellar epithelium, indicated by the arrows.

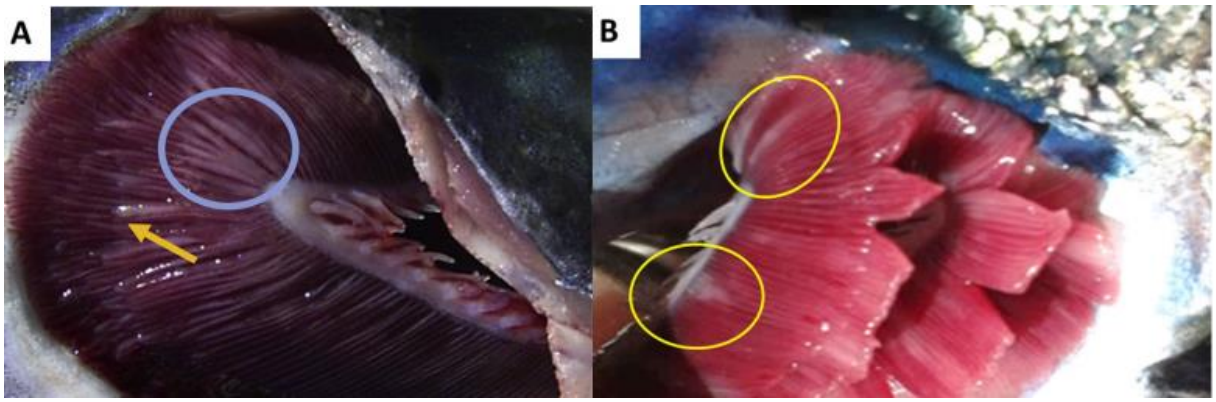


Figure 2. Macroscopic AGD lesions (A) Grossly thickened gill filaments (yellow arrow) and mucoid patches (blue circle) (modified from Herrero et al., 2018). (B) Mucoid patches (yellow circles) in experimentally AGD infected fish (from Marcos-López et al., 2018).

In the aquaculture industry, AGD infections will have major impacts during the seawater grow-out phase. In best-case scenarios, the disease will cause mild infections, reduced performance, and impaired fish welfare. However, if left untreated, in worst-case scenarios, it can result in up to 80% mortality (Oldham et al., 2016).

1.1.2 Fish Immune Response and AGD

The fish immune system is responsible for many layers of defence mechanisms that are in place to protect the organism against infection. These mechanisms are usually divided into the innate immune response and the adaptive immune response.

The innate immune response is a rapid and non-specific mechanism and is the organism's first line of defence against pathogens. It consists of constitutive physical and chemical factors, that are always present, such as epithelial surfaces and mucus layers that protect the body against the attachment and/or penetration of microorganisms, and inducible factors, that can be upregulated if the primary barriers have been breached (Watts et al., 2001).

The mucus layers are usually rich with antimicrobial peptides (AMPs) and antimicrobial enzymes. The AMPs are small peptides that can be divided into membrane disruptive, inducing membrane permeabilization, and non-membrane disruptive, where they are internalized and will act on intracellular targets. In fish, AMPs are divided into five main classes: β-defensins, cathelicidins, hepcidins, histone-derived peptides, and piscidins (Dalmo & Bøgwald, 2022). The presence of microorganisms or tissue damage can elicit a local inflammatory response and will result in the upregulation of the inducible factors. This response mobilises the activation of different cell signalling pathways and induce the production of acute phase proteins, complement factors and inflammatory mediators, such as chemokines and cytokines, which will lead to the mobilisation of a variety of immune cells to the site of infection, including phagocytes and macrophages (Watts et al., 2001).

The adaptive immune response is activated by the innate response and is comprised of specific mechanisms designed to improve pathogen recognition through the development of an immunological memory. This response includes lymphocytes, immunoglobulins, T cell receptors (TCR), products of the major histocompatibility complex (MHC), and the production of antibodies (Rauta et al., 2012). MHC class II are antigen presenting molecules to T cell receptors (TCR) on CD4+ helper T cells, activated in the presence of

exogenous antigens, usually originated from a bacterial or parasitic infection (Yamaguchi & Dijkstra, 2019; Abós et al., 2022).

Cytokines act as modulators of both innate and adaptive immune responses. Once a wound or an infection is recognized by the body, the innate response initiates an influx of macrophages and granulocytes, guided by chemokines, to the damaged site. These molecules are then able to initiate many antimicrobial processes and give way to a cascade of pro-inflammatory cytokines, such as interleukin-1β (IL-1 β) and tumor necrosis factor-α (TNF-α), which helps sustain the inflammatory response and fight the infection (Secombes, 2022). The two mentioned cytokines are commonly used immune-regulatory genes and are well characterized in fish (Watts et al., 2001, Rauta et al., 2012).

Gene expression is easily altered by the presence of a pathogen. During an infection, different pathways from both innate and adaptive immune responses are activated and result in different up or down regulation of the expressed genes. The difference in gene expression will be specific to the pathogen and how it attacks the body.

During an AGD infection, it is still unclear whether the host's first response is initiated due to the direct contact of the amoeba on the gill epithelium, if it is prompted by the attachment of the parasite to local mucins or whether it is a response to a secreted substance. Amoebae have been observed to secrete proteases capable of degrading mucins and host tissue, however, histopathological analysis of AGD infected fish have not shown any evidence that *N. perurans* secretes cell damaging enzymes (Nowak et al., 2014; Marcos-López et al., 2017a).

In the early days of infection, an upregulation of C type lectin has been observed and could indicate the recognition of glycan epitopes on the amoeba. At the same time, a down regulation of the mannose-binding protein C (MBP-C) was detected, indicating a possible mechanism used by *N. perurans* to evade the host's immune response (Nowak et al., 2014). The recognition of the parasite elicits a typical inflammatory response, displaying evidence of cellular infiltration of key pro-inflammatory cytokines, such as IL-1β and TNFα, especially in the regions with the most severe AGD lesions. The increased expression of these two cytokines takes place mainly due to resident immune cells that are already present in the gills and epithelial cells (Pennacchi et al., 2014). The AGD characteristic hyperplastic response of the epithelial and mucus cells also explains the increase in IL-1β levels, as epithelial cells and fibroblasts are a source of this cytokine. It has also been noted that AGD infected gills show an increased expression of immune cell's cellular markers, such as professional antigen presenting cells (MHC II), B cells (IgM, IgT, MHC II) and T cells (TCR, CD4, CD8), which indicates the presence of an immune cellular response and a possible antibody response, due to the presence of the B cells (Pennacchi et al., 2014).

Marcos-López et al. (2017a) showed that there is greater enzymatic variations in the gill mucus when compared to the serum, indicating their ability to elicit a local response to the infection. Furthermore, it was noted that the levels of IgM and most enzymes decreased as the infection advanced and became more severe. This indicates that the humoral immune response becomes compromised as the exposure time to the infection increases. That being said, many studies have already shown that the use of immunostimulants and the activation of the innate immunity have the ability to reduce AGD related mortalities (Marcos-López et al., 2017a).

1.1.3 Immunonutrition

Immunonutrition is a term commonly used to express the concept of using specific nutrients or dietary compounds to modulate and improve the immune response of an organism. This is a topic of increasing interest to the aquaculture sector as a preventative method for disease in cultured fish.

Proper nutrition is essential for adequate growth and health maintenance of all animals. While nutrient deficiency is known to negatively impact the immune response, exceeding the minimum nutrient requirement levels for optimal growth has been shown to significantly improve fish immunocompetence and disease resistance (Pohlenz & Gatlin, 2014). During an infection, the need for specific nutrients increases considerably so it is important to fully understand the role that each compound play during the immune challenge.

Besides their crucial role in the energy production of cells, fatty acids are also vital for contributing to the membrane's physical and functional properties, acting as modifiers of protein structure, regulating gene expression, and, even, in modulating the immune response and affecting inflammatory mediators. The use of marine oils in feed is essential for providing high levels of long-chain n-3 polyunsaturated fatty acids (LC-PUFA), especially eicosapentaenoic (EPA) and docosahexaenoic (DHA), due to their good health impacts in fish (Trichet, 2010). These fatty acids are commonly provided in feed through the addition of fish meal (FM). However, due to increasing prices and lack of availability, new sources have been sought and a promising option is the addition of krill meal (KM). While the FAs in FM are available in triacylglycerides (TAG) form, in the KM, they are available in phospholipid (PL) form. Higher levels of EPA and DHA are concentrated in the PL compared to TAG and, due to their molecular structure, are more readily and easily absorbed by the organism (Linder et al., 2010). It is important to note that, once in the fish's organism, omega 3 fatty acids are prone to peroxidation, thus, proper levels of antioxidant nutrients are essential to maintain their stability (Trichet, 2010).

Two of the most important cellular antioxidants are vitamins C and E, which are well known for their ability to boost the immune response. Vitamin E is present in cell membranes, protecting lipids from peroxidation, and helping in the functioning of all membrane receptors. Vitamin C is in the cell's cytoplasm, protecting it from oxidation, and, together with vitamin E, has been shown to stimulate cytotoxic activity, increase lymphocyte counts and to modulate phagocyte function by increasing phagocytosis and pathogen killing capacity (Trichet, 2010; Kiron, 2012). Furthermore, the combination of these two vitamins has also shown to increase antibody production and immunological memory (Pohlenz & Gatlin, 2014).

In addition, the use of non-nutritive compounds has been studied and shows promising results in enhancing the immune response of many aquatic animals. Mannanoligosaccharides (MOS) are complex carbohydrate fibers, derived from the cell wall of live yeast (*Saccharomyces cerevisiae*). Fibres like MOS cannot be degraded by fish but can be utilized by certain intestinal bacteria. The use of MOS as a prebiotic has been proven not only to improve the gut's microbiota and affect the intestinal mucus layer, but also to enhance the gill and skin mucosal barriers (Kiron, 2012; Leclercq et al., 2020). Furthermore, β-glucans, plant and algae extracts have been shown to act as pathogen associated molecular pattern (PAMP) molecules and help in the detection and recognition of pathogens (Pohlenz & Gatlin, 2014).

Essential oils (EOs) are purified plant extracts obtained by distillation and represent a major group of phytogenic feed additives (Trichet, 2010). In the past, EOs were mainly used for their antistress and antioxidant properties, as well as their role as gastrointestinal health promoters. Recently, these phytogenic extracts have shown to enhance the immunocompetence and disease resistance, prevent outbreaks, promote fish growth, stimulate appetite, and to have antibacterial and antiparasitic activities (Sutili et al., 2018).

1.2 Research question

The aim of this study is to validate whether functional diets could be used as an efficient preventative method for AGD outbreaks in Atlantic salmon aquaculture. Therefore, the immune response of both uninfected and infected fish, fed with two different diets (control and functional feed), was evaluated by comparing the differences in gene expression. Furthermore, histopathology analysis and visual AGD scoring were used to assess disease severity in between the different dietary groups.

For a functional feed to be accepted as a valid preventative method against AGD, it is necessary that the comparison between the control feed can show its ability to prevent or weaken the severity of the infection and, also, to enhance the fish's immune response. Thus, better preparing the fish to fight off diseases and increase their robustness.

The main hypothesis of this study is that the functional feed will be able to lower the overall severity of an AGD infection when compared to the control diet. In addition, it is also expected that the functional diet will:

- Lower the inflammatory response and oxidative cellular stress in the infected fish.
- Increase the immune competence and robustness of challenged fish.
- Result in increased levels of mucus production due to the addition of MOS.

In order for the above statements to be verified, it is also expected that the infection of the fish during challenge will be enough to elicit a typical disease response, will increase the immune and inflammatory processes, and that the severity of the disease will be significantly different between the control and the functional feed.

2. Materials and Methods

2.1 Feed Trial

The experiment was approved by the Norwegian Food Safety Authority (Mattilsynet), under FOTS ID number: 29669. The experiment was set up by Biomar AS at the Aquatic and Industrial Laboratory (ILAB), in Bergen, Norway, and started in August and ended in October of 2022.

2.1.1 Fish Origin

All fish used in the experiment originated from Stofnfiskur, Egghouse Vogavik, Iceland, and a total of 27,000 Atlantic salmon was delivered as eyed roe to ILAB in September 2021, under the batch label of ILAB_21_502. Prior to delivery, the parental fish of the gonadal products were screened for different pathogens (ISAV, SAV, IPNV, PMCV and PRV) and none were detected. All eggs were hatched in October and the fish were start fed from December 2021. Once they reached 15 grams, 30 individuals had their gills, kidneys and heart examined by RT-qPCR for the presence of IPNV, ISAV, PRV, and PMCV,

and an additional 30 individuals had their hearts examined for histological changes. No pathogen or abnormal changes were detected in any of the analysis. Furthermore, all fish were kept unvaccinated and, to ensure that they remained uninfected, they were submitted to monthly inspections.

2.1.2 Study Design

This trial was designed by Biomar AS with the main goal of testing the differences between four different feed formulations on Atlantic salmon submitted to an AGD challenge and later, to a hydrogen peroxide treatment. The trial can be roughly divided in to three mains parts: the pre-feeding phase, the AGD challenge, and the hydrogen peroxide treatment, as shown in Figure 3. The current study will only focus on data obtained in the first two samplings of the experiment and from two, out of the four, tested diets: the control and the functional feed.

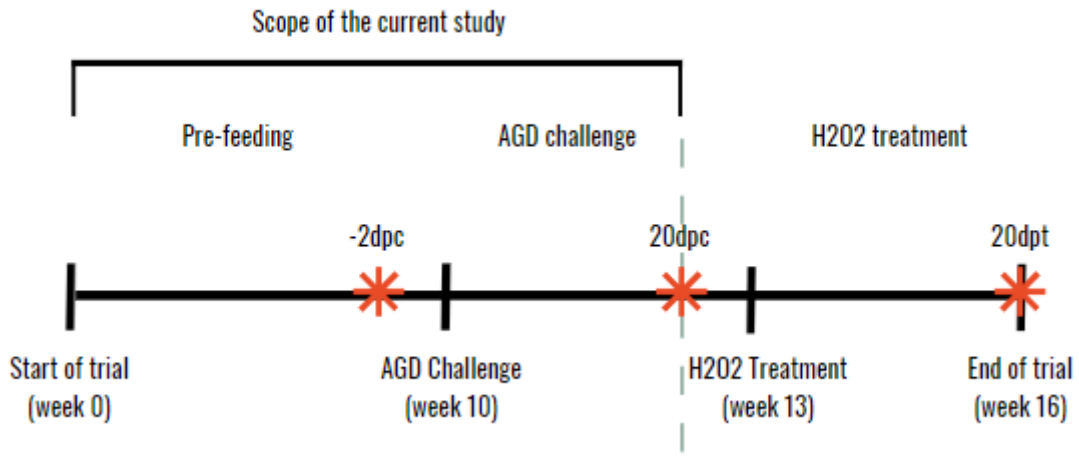


Figure 3. Timeline of the complete trial designed by Biomar AS. The samplings are indicated by the red asterisk and the grey dashed line delimitates the scope of the present study. Dpc: days post challenge; Dpt: days post treatment.

For this study, a total of 330 Atlantic salmon smolts, with a start weight of approximately 57 g, were distributed into 6 different conical shaped tanks of 450 L, established in a flow-through system. The trial started with a salinity of 25 ppt and the acclimatisation to full sea water (>33 ppt) was done during the initial five days of the experiment. The water temperature was kept at 12 °C until the AGD challenge, where it was increased to 14 °C and maintained stable until the end of the experiment. The tanks were kept in a controlled laboratory and checked daily. The tanks had a water flow of 16-20 L/min and were kept in a 12:12 light regime. Triplicate tanks were randomly assigned to each of the two diets. The fish were fed the diets for 10 weeks after which, they were subjected to the AGD challenge. As shown in Figure 3, the first sampling took place two days before the challenge (-2dpc) and the second sampling happened twenty days post challenge (20dpc). Figure 4 displays an example of a fish collected during the second sampling.

Figure 4. Example of one of the sampled fish (20dpc).

2.1.3 Feed formulation

The control and the functional feed diet used in the experiment were both produced by extrusion in Biomar's Technology Center, Brande, Denmark. However, since the detailed formulation of both diets is a commercial property of Biomar AS, it cannot be fully disclosed in this study. A simplified version of both formulations is provided in Table 1.

Table 1. Formulation of the control and the functional feed.

	Control	Functional Feed
EPA+DHA	High*	High*
Krill meal	-	X
Fish meal	X	X
Vitamin E	[redacted]	[redacted]
Vitamin C	[redacted]	[redacted]
MOS	-	X
Phytogenic extracts	-	X

MOS: Mannanoligosaccharides; * Same level in both diets, both higher than the industry standard (6-7.5% of fatty acids).

It is important to note that, even though, the levels of EPA and DHA are the same in both diets, their origin is not. In the control diet, the levels of fatty acids come primarily from fish meal, whereas, in the functional diet, krill meal is added as an extra ingredient.

2.1.4 Amoeba challenge

The *N. perurans* isolate used in this study originated from AGD infected Atlantic salmon, from commercial fish farms, sampled in Autumn 2013 in Hordaland County, Western Norway (University of Bergen's (UoB) identifier: H02/13Pp C2 – isolate #2). The fish had their infected gill mucus and tissue scraped onto malt yeast agar (MYA) plates with autoclaved seawater and incubated at 15 °C. After confirmation of the amoeba, with an inverted microscope, a small piece of agar was transferred to a new MYA plate with a layer of autoclaved seawater and the amoeba isolate was kept in continuous culture in malt yeast broth (MYB) at 15 °C at the ILAB facilities (Blindheim et al., 2023; Tröße et al., 2020).

In order to prepare the inoculum for the experiment, the number of viable amoebae was determined with a CASY Model TT Cell counter (Innovatis, Roche Diagnostics) and each tank was infected with approximately 8400 amoebas per liter. During the infection, the inlet water was closed, pressured oxygen was added to each tank through diffusors, and the water volume was reduced from 450 to 150 L. The exposure lasted for one hour.

2.1.5 Sampling regime

As mentioned, in this study, data were collected at two different samplings, one at -2dpc and the other at 20dpc. During both samplings, five fish were removed from each tank and euthanized by an overdose of Finquel Vet (1,5 gram per 10 liter of sea water). All fish were weighted, length measured, and scored based on the guidelines for standardization of AGD-gill scoring described by the Norwegian Veterinary Institute's report (Hytterød et al., 2018). The gill scoring table used by ILAB's staff is shown in Table 2.

Then, the whole right-sided third gill arch was removed for histopathology analysis, and a small sample from the left-sided third gill arch was collected for RT-qPCR analysis, placed in a tube with RNALater, refrigerated overnight, and stored at -80 °C until use. During the last sampling, additional swab samples from the gill mucus were taken for positive identification of the parasite by qualitative PCR.

Table 2. Macroscopic gill scoring system to determine AGD degree (modified from Taylor et al. 2009)

Infection Level	Gill Score	Gross Description
Clear	0	No sign of infection and healthy red colour
Very light	1	1 white spot
Light	2	Accumulations of several small spots or large spots (< 20% of gill area)
Moderate	3	Established thickened mucus patch or spot grouping equal or greater than 20% of gill area
Advanced	4	Established lesions covering 50% of gill area
Heavy	5	Established lesions covering most of the gill surface

2.2 Total RNA extraction and cDNA synthesis

The gill samples were removed from the tubes containing RNALater. Total RNA was extracted using RNeasy Mini Plus Kit (Qiagen, Germany) according to the manufacturer's instructions. The gill tissue was dried against tissue paper, weighted, and, a maximum of 35 mg, was homogenized in 600 µl of RLT Plus Buffer, using 5mm stainless steel beads (Qiagen, Germany), in the Qiagen TissueLyzer II for 8 to 10 minutes at 20 Hz. The optional DNase I (Qiagen, Germany) step was included to ensure complete elimination of genomic DNA. The final RNA was eluted in 30 µl of nuclease-free water and stored at -80 °C. Quantification of RNA was obtained with NanoDrop ND-1000 spectrometer (Thermo Fisher Scientific) and RNA quality and integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent 2100 Bioanalyzer (Thermo Fisher Scientific). All extracted samples showed a RIN value of 10. The cDNA synthesis was performed with the QuantiTect Reverse Transcription Kit (Qiagen, Germany) and final samples were stored at -20 °C.

2.3 Gene and Primer selection

The selection of genes analysed in the present study was based on previous publications of most relevant pathways to be affected following an AGD infection in Atlantic salmon. These include representatives of pathways involved in mucus production, immune-regulation and -markers, cellular oxidative metabolism, and inflammatory processes (Table 3). Two potential housekeeping genes were also included. All primer sequences were selected from previous studies and checked with BLAST to rule out possible

amplification of similar undesired products. The final selection of genes, and their corresponding primers and references, are shown in Table 3.

Table 3. Genes selected for the qPCR and their corresponding forward (FW) and reverse (RE) primers and references.

Gene	FW and RE Primers	Reference
1. Mucin 5 (Muc 5)	FW: CCGTGCTGGGAGACATTGTGAAGT RE: TGCTGGAGAGGGATAGGGTAAC	Marcos López et al., 2018
2. Mucin 18 (Muc 18)	FW: AAGAGCAGCGAGGTGGTG RE: TCCGTTGACTTGGCAGATGA	Marcos López et al., 2018
3. Th2 interleukin: IL4-13a	FW: GCATCGTTGTGAAGAGCCAAGA RE: GAAGTCTCCTCAGCTCCACCT	Marcos López et al., 2018
4. TNF – alpha 3 (TNFα3)	FW: GTGTATGTGGGAGCAGTGTT RE: GAAGCCTGTTCTCTGTGACT	Marcos López et al., 2018
5. Heat shock 70 kDa protein (HSP70)	FW: CCTGCCTACTTCAACGATTCACAGAGACA RE: CCAGCGATCACTCCAGCGTCCTTA	Marcos López et al., 2018
6. β-defensins – 3 (β def-3)	FW: GTCATTGCTTGTGGAATACAAGAG RE: GAAGCAAGGCACAAACGAAG	McGrath et al., 2022
7. β-defensins – 4 (β def-4)	FW: CACATGTGATGTAAATGAGGCA RE: TGGTAGTTCTGCTGACAGAC	McGrath et al., 2022
8. Cathelicidin 2 (Cath 2)	FW: AAGCCCAGCGGAGGCTCTAGG RE: GCCAAACCCAGGACGAGAGCC	McGrath et al., 2022
9. Interleukin 1β (IL1β)	FW: GCTGGAGAGTGTGTGGAAGA RE: TGCTTCCCTCCTGCTCGTAG	Wynne et al., 2008
10. MHClass IIβ (MHC II β)	FW: ACCCGTCCCTGCCTGAG RE: TGTAGTAGATGAGTCCTGCCAAG	Young et al., 2008
Housekeeping genes:		
11. β-actin (β-act)	FW: CCATCCAGGCAGTGTTGT RE: CGGAGTCCATGACGATACC	Marcos López et al., 2018
12. Elongation factor 1-α (ELF1α)	FW: TGCCCCTCCAGGATGTCTAC RE: CACGGCCCACAGGTACTG	Wynne et al., 2008

All primers were ordered from Invitrogen by Thermo Fisher Scientific in a hydrophilized state. Based on the nanomole quantity, provided for each primer by the company, primer stock solutions of 100 µM concentration were created by the addition of nuclease free water. All primers were further diluted to a concentration of 10 µM.

2.4 Primer testing

To ensure optimal performance of each primer pair used, sensitivity and efficiency tests were performed prior to the RT-qPCR runs.

2.4.1 Sensitivity test

The sensitivity test is essential to determine the optimal temperature of each gene's primer pair (FW and RE) during the qPCR run. All primers were tested in duplicates, with

and without the addition of cDNA, at different temperatures. The reactions prepared without cDNA are necessary for assessing the formation of primer-dimers and other unwanted products. For this analysis, the primers were tested against a cDNA pool that contained small amounts of all the samples used in this study. To create the pool, a 1:5 dilution was made by the addition of 80 µl of nuclease free water to each of the wells containing the cDNA samples. Then, for the final cDNA pool, 10 µl of each sample was added to the same tube and homogenized.

The RT-qPCR program used was set up in Roche's LightCycler-96 instrument using the "Gradient" function, during the three-step amplification phase, and the thermal profile set up, shown in Figure 5.

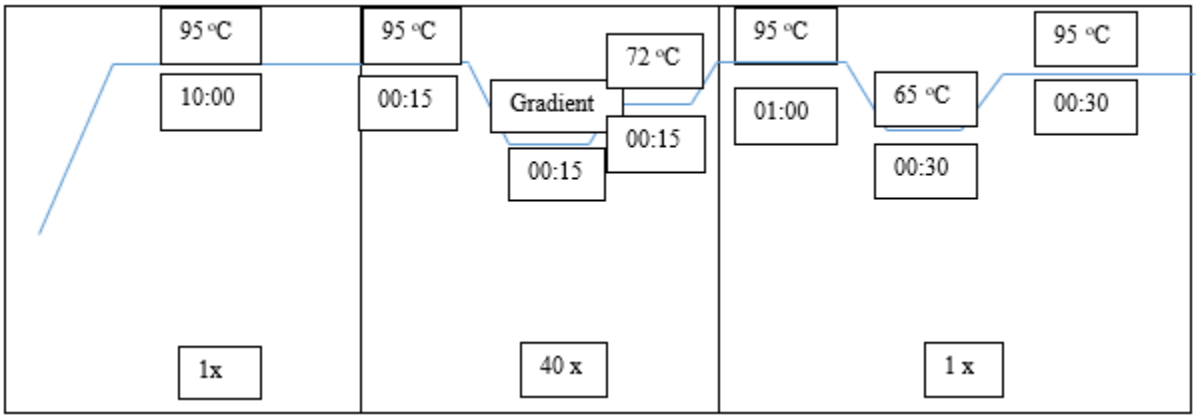


Figure 5. qPCR thermal profile set up for the primer sensitivity test

The eight different temperatures tested were: 51, 52.9, 55.3, 57.9, 60.7, 63.4, 67.9 and 70 °C. Each reaction was prepared following Table 4 and each qPCR plate was arranged as shown in Appendix 1.

Table 4. Master Mix preparation for the sensitivity test.

	With cDNA	Without cDNA
Number of reactions	1	1
Light Cycler SYBR (2x)	10 µl	10 µl
Primer FW (10 µM)	1 µl	1 µl
Primer RE (10 µM)	1 µl	1 µl
Nuclease free water	3 µl	8 µl
cDNA pool (1:5)	5 µl	-
Total	20 µl	20 µl

After the qPCR run, the optimal temperature for each gene was obtained through the analysis of the amplification curves and the melting peaks of each of the tested temperatures. The amplification curve shows how much of the fluorescence signal is detected in each one of the qPCR cycles. Thus, it is possible to observe and identify which temperatures yielded faster product amplification and better curve profiles. The melting peak allows for the identification of the temperature in which half of the sample's DNA is single stranded (ss) and the other half is double stranded (ds), indicating the size of the product obtained during the amplification. Additionally, the melting curve profile can indicate whether different products are being amplified by the same primer pair or not. As an example, the amplification curve and the melting peak for the Mucin 5 gene is shown in Figures 6 and 7 below, respectively.

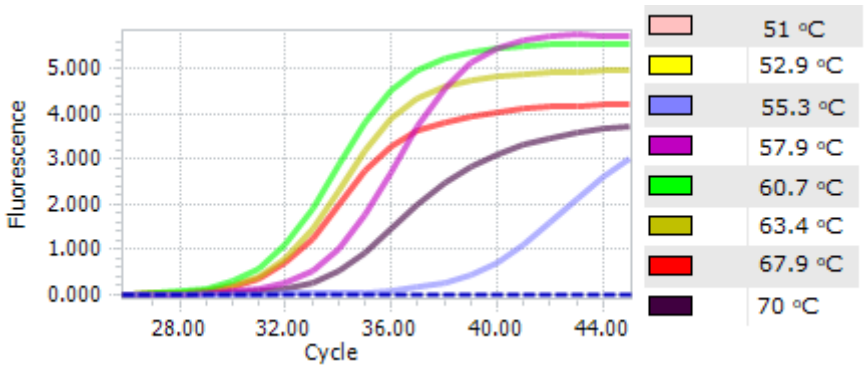


Figure 6. Amplification curves for Mucin 5

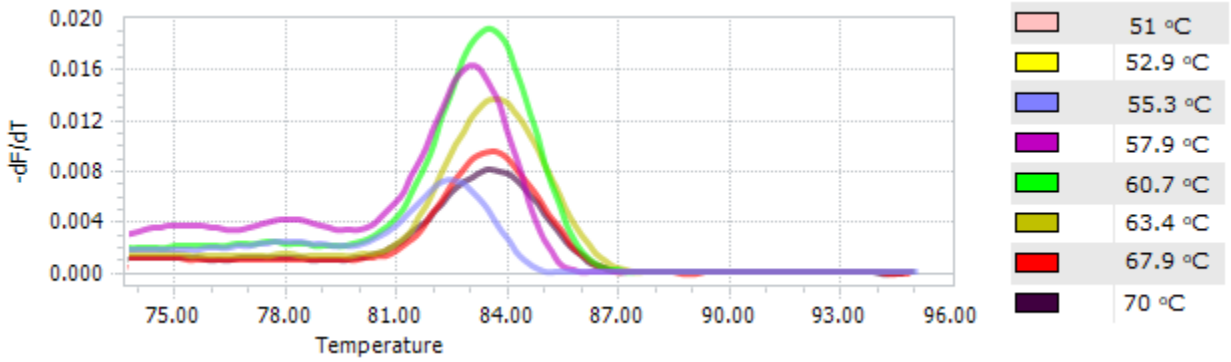


Figure 7. Melting peaks for Mucin 5.

The analysis of the amplification curve (Figure 6) shows that the best amplification temperature for Mucin 5 is 60.7 °C. This temperature yielded an earlier amplification when compared to the other temperatures (Cq = 29.64) and the final curve follows the expected profile for qPCR amplification. Furthermore, through Figure 7, it is possible to observe that no undesired products were amplified, and that the melting temperature (Tm = 83.40 °C) obtained yielded a good-sized product.

The analysis of the data from all twelve genes resulted in the establishment of two optimal temperature groups, one for the temperature of 58 °C and one for 60 °C. The corresponding temperatures and genes used in the final qPCR assays are shown in Table 5 below and the corresponding amplification curves and melting peaks for all genes analysed are in Appendix 2.

Table 5. Optimal temperatures for each of the studied genes.

Temperature	**58 °C**	**60 °C**
Gene name	Mucin 18	Mucin 5
	ILF4-13a	TNF-α3
	Cathelicidin 2	β defensins-3
	IL1 β	β defensins-4
	MHC II β	HSP70
	β-actin	
	Elongation Factor 1α	

2.4.2 Efficiency test

The primer amplification efficiency of each one of the tested genes was calculated through the LinRegPCR software. For that, the data from the amplification curves of the

final RT-qPCR plates, from each one of the genes, were exported from LightCycler 96's and imported into LinRegPCR's software. Then, after analysing the efficiency of each one of the obtained curves, the programme calculated the mean amplification efficiency for each gene, which is shown in Table 6.

Table 6. Primer amplification efficiency.

Gene	Amplification Efficiency
Mucin 5	1.934
Mucin 18	1.928
ILF4-13a	1.956
TNF – alpha 3	1.920
Heat shock 70 kDa protein	1.940
β-defensins – 3	1.927
β-defensins – 4	1.921
Cathelicidin 2	1.932
Interleukin 1β	1.934
MHClass IIβ	1.933
β-actin (β-act)	1.923
Elongation factor 1-α	1.952

2.5 RT-qPCR

To determine the optimal dilution of the cDNA, the original 1:5 cDNA pool used in the sensitivity test, in section 2.4.1, was prepared into 1:5, 1:10, 1:20, 1:25, 1:30 and 1:40 dilutions. The dilutions were then run together with each of the primer pairs in two different plates, one at 58 and one at 60 ^{o}C, according to each gene's optimal temperature shown in Table 5. After analysing the Cq values for each of the dilutions, the genes were also divided into optimal cDNA dilutions, either 1:5 and 1:10, shown in Table 7.

Table 7. Optimal RT-qPCR dilution for each of the studied genes

Dilution	**1:5**	**1:10**
Gene name	Mucin 18	Cathelicidin 2
	Mucin 5	MHC II β
	TNF-α3	β-actin
	IL1 β	Elongation Factor 1α
	HSP70	
	β defensins-3	
	β defensins-4	
	ILF4-13a	

All quantitative real-time PCR (RT-qPCR) reactions were performed with the SYBR green method using the Light Cycler kit and equipment from Roche, and a 96-well PCR array layout. In each well, 15 µl of master mix and 5 µl cDNA (1:x) were added. The master mix was prepared as described in Table 8 and the thermal profile of the qPCR run is displayed in Figure 8. In order to avoid interplate bias, all samples belonging to the same gene were run on the same plate. Thus, in total, twelve plates were analysed, each corresponding to one gene of interest. The template for the plate set-up used for all

genes is shown in Appendix 4. In all assays, in order to identify any deviation in the qPCR runs, triplicate wells containing the same sample were run as calibrators, however, no differences were identified between them. Furthermore, two controls were added in all assays, a No-RT (reverse transcriptase) control, to assess whether there was DNA contamination during the RNA extraction, and a No-RNA control, to investigate the formation of primer dimers and the presence of external contamination of nucleic acid. In all assays, the amplification curves for both of the controls did not express significant outcomes.

Table 8. Mastermix preparation for RT-qPCR.

Mastermix	1X
Light Cycler SYBR (2x)	10 µl
Primer Fw (10 µM)	1 µl
Primer Re (10 µM)	1 µl
RNase/DNase free water	3 µl
cDNA (1:x)	5 µl
Total	20 µl

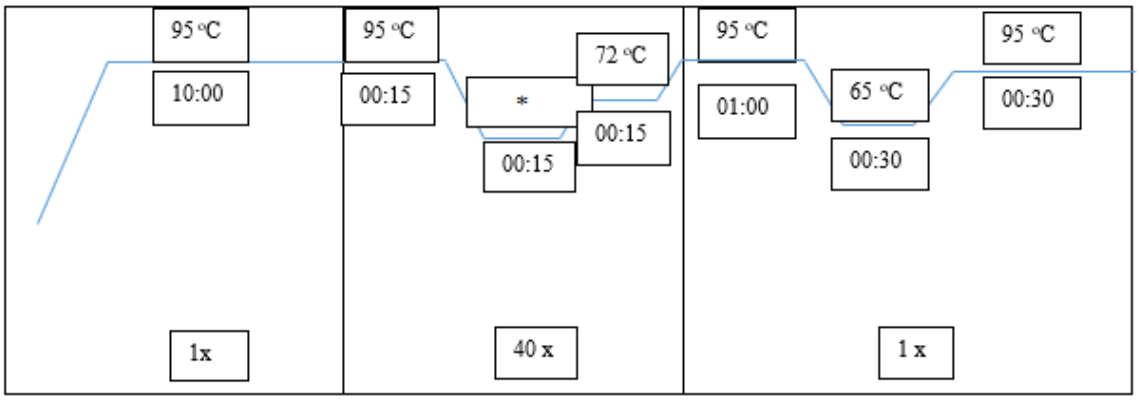


Figure 8. Thermal profile set up for qPCR.

2.6 Data Analysis

After running all twelve plates, the LightCycler 96 software provided the Cq values for each one of the samples. The Cq value is the MIQE standard name for Ct (cycle threshold) and represents the cycle in which the sample reached the detection threshold of the equipment. Therefore, a low Cq value belongs to samples with higher genetic material, reaching the threshold faster than samples with a high Cq value and, consequently, a lower load of genetic material.

Cq values for all samples and genes were extracted from the LightCycler 96 program and the data analysis was done using the qbase+ software, version 3.4 (Biogazelle, Zwijnaarde, Belgium - www.qbaseplus.com). This software uses a generalized model of

the delta-delta-Ct approach, allowing for the normalisation with multiple reference genes and gene specific amplification efficiencies (qBase+ manual, 2017). In this case, two reference genes were used to normalise the results (βactin and Elongation factor 1-α) and specific PCR efficiencies were used for each one of the genes. The efficiencies were calculated with the LinRegPCR software (as shown in Table 6) and manually imported into qBase+.

All calculations performed by the qBase+ software are described in Hellemans et al., 2007. In summary, Cq values are transformed into relative quantities (RQs) using the specific PCR efficiencies. Then, the RQ values are normalised through the calculation of a normalization factor (NF) based on the two reference genes. The normalised RQs (NRQs) are obtained by dividing the RQ by the sample specific NF. The NRQ value represents the relative quantity (gene expression or copy number) between different samples for a given gene. Final NRQ results were scaled to the uninfected group, which means that the expression levels of that group were set to one, making all results relative to that sample group and facilitating the interpretation of the results. It is important to note that rescaling the data does not affect the fold changes between samples, it only re-adjusts the scale (qBase+ manual, 2017). To prepare the final NRQ results for parametric tests, they were log transformed during export from the qBase+ software and imported into R for further statistical analysis.

A two-way ANOVA was performed to check for the interaction between the factors (diet and infection) for all genes. When the interaction effect was not significant ($p>0,05$), the null hypothesis of no interaction was accepted, and the interaction factor was removed from the model. The assumptions of equal variances were checked by analysing the boxplot of each gene and by performing Levene's test for homogeneity of variance. Given that the p-value was not significant ($p>0,05$) for all samples, equal variance was accepted for all analysed genes. Furthermore, the assumption of normality was investigated by analysing the QQ-plot of the residuals of the final ANOVA model and their distribution. All genes showed a normal distribution, and all residuals QQ-plots are present in Appendix 3. Finally, post-hoc Tukey's Honestly-Significant-Difference (TukeyHSD) test was used on the genes that showed a significant difference to correctly identify which pairwise differences were statistically different from each other.

For better visualisation, boxplots of the NRQ values were created for each gene with the GraphPad Prism version 9.5.1.733 for Windows. The whiskers and outliers of the boxplots were established by Tukey's method. Meaning that the interquartile range (IQR) was calculated from the difference between the 25^{th} and the 75^{th} percentiles. The length of the upper whisker corresponds to the value in the data set that is equal to or less than the 75^{th} percentile plus 1.5 times the IQR. Any data larger than that was plotted as an outlier. The same pattern was used for the lower whisker, where its limit was drawn to the value equal to or lower than the 25^{th} percentile minus 1.5xIQR (GraphPad Prism User's Guide).

3. Results

3.1 Qualitative PCR

Even though, all fish in the experiment were challenged with the AGD pathogen, that does not mean that all fish became infected. In order to get a positive confirmation of the infection, swab samples were used in a qualitative PCR to identify the presence or absence of the *N. perurans* amoeba. Based on the Cq values fish were divided into three categories (not detected, low infection and moderate infection). This analysis was performed by the PHARMAQ Analytiq company, and the results are displayed in Figure 9.

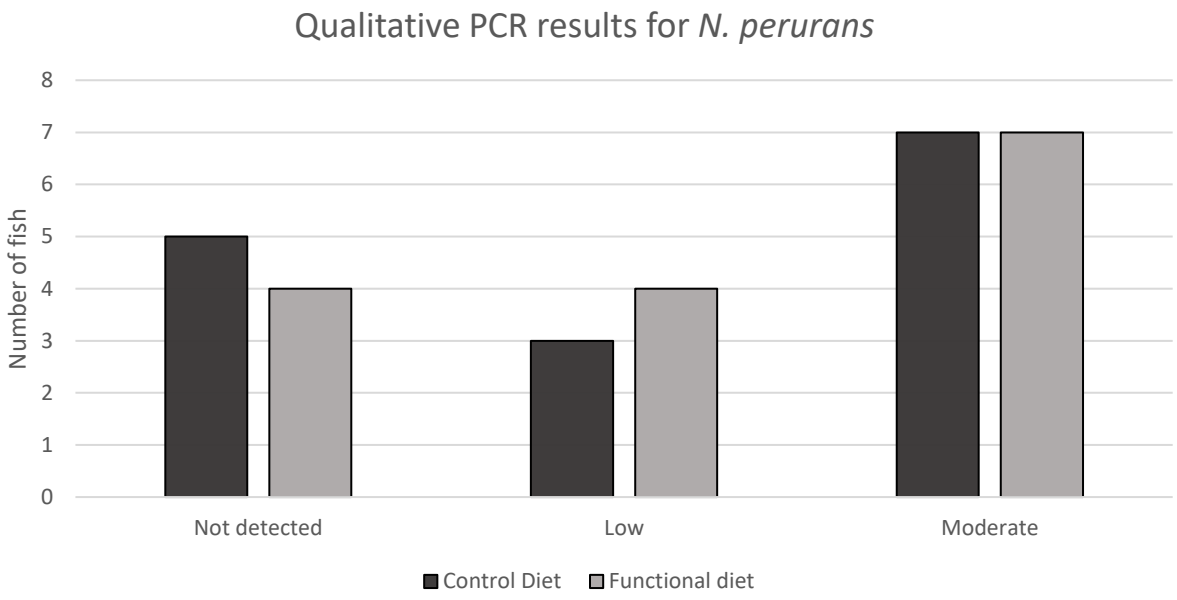


Figure 9. Results from the qualitative PCR analysis, showing the number of fish with moderate, low or no presence of *N. perurans*, 20 days after challenge, for both diets (control and functional feed). Data are combined from 3 tanks of fish fed with the control and 3 tanks of fish fed with the functional diet.

The results (Figure 9), confirm the presence of the pathogen in only 21 out of 30 sampled fish 20 days after challenge. This means that the 9 fish that received a negative result did not contain sufficient genetic material from the pathogen to be detected by the analysis' threshold. Both data sets, one with all 30 infected fish and one with only the 21 PCR-positive, were compared, but no significant differences were observed between them. Therefore, the nine PCR-negative fish were excluded from the data analysis shown in this study, to ensure that the comparison between uninfected and infected fish was only based on fish that showed a detectable infection. From the nine fish that received a negative PCR result, 4 belonged to the functional diet group and 5 to the control diet. Thus, the final sample number for the present study is uninfected (n=30) and infected (n=21), where functional diet has n=11 and control has n=10.

From the results, it should also be noted that from the 21 PCR-positive fish, 14 were classified with a moderate infection (7 functional feed and 7 control feed) and 7 were classified with a low infection (4 functional feed and 3 control feed). The gene expression between low and moderate infection also did not result in significant differences.

3.2 Overall Gill Health

The overall gill health assessment of uninfected and infected fish relied on data from both the AGD gill scoring, performed by the ILAB staff, and the histopathology analysis performed by the PHARMAQ Analytiq company.

3.2.1 Visual AGD score

The AGD scoring technique is a fast tool to rank the organism's disease severity relying solely on visual observations, as described in Table 2. The score was taken for all four gill arches from a total of 51 fish (30 before the AGD challenge and 21 afterwards). The differences between the arches are divided in the two dietary groups and can be visualised in Figure 10.

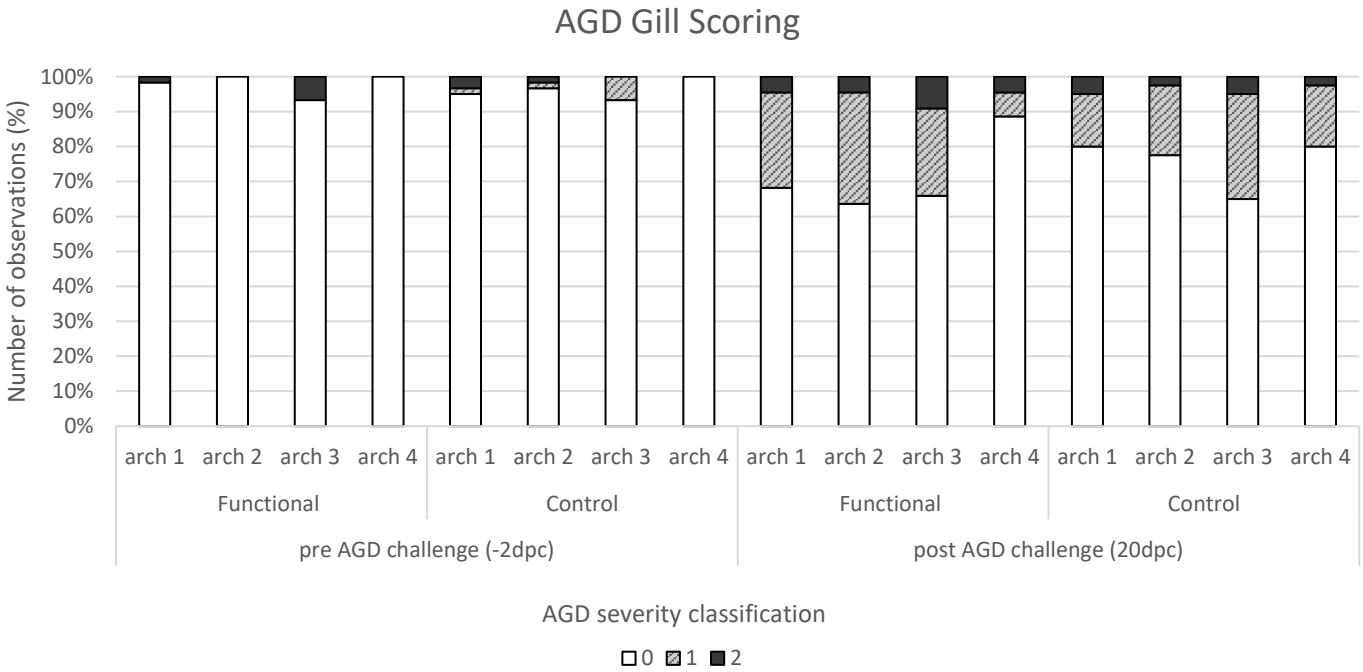


Figure 10. Gill scoring results for all four gill arches before (n=30) and after (n=21) the AGD challenge. The infection level is given by 0 = clear (no visible infection), 1 = very light, and 2 = light (modified from Taylor et al. 2009). Data are combined from all 6 tanks used in the experiment.

From the results above (Figure 10), it is possible to observe that, even prior to the challenge with the pathogen, some fish already showed signs of increased mucus production. Furthermore, it is clear that, after the infection, the number of fish showing overexpression of mucus is much higher than before the challenge. However, the highest classification obtained was only a 2, which corresponds to a light infection. The comparison of the AGD score in between dietary groups does not provide any substantial differences.

3.2.2 Histopathology analysis

Visual AGD scoring is a macroscopic technique frequently used to identify the disease's severity. However, to obtain a more complete picture of the overall disease status, additional methods like histopathology are needed.

After careful analysis of the sampled third gill arch, PHARMAQ Analytiq could not find any evidence of necrosis, epithelial apoptosis, filament inflammation, presence of AGD,

amoeba, intracellular or filamentous bacteria, fungi, Costia, Trichodina, or intracellular brown pigment and subepithelial inflammation in any of the analysed samples. On the other hand, some abnormal tissue, vascular lesions, and lamellar hyperplasia and inflammation were found in some of the samples, as shown in Figure 11. These changes were given grades from 0 to 4, following PHARMAQ's scoring system:

- 0- No lesions present;
- 1- Minimal pathology;
- 2- Mild: lesion distribution: < 10% of the respiratory surface of the lamellae;
- 3- Moderate: lesion distribution: between 10 – 50 % of the respiratory surface of the lamellae;
- 4- Severe: lesion distribution: >50% of the respiratory surface of the lamellae.

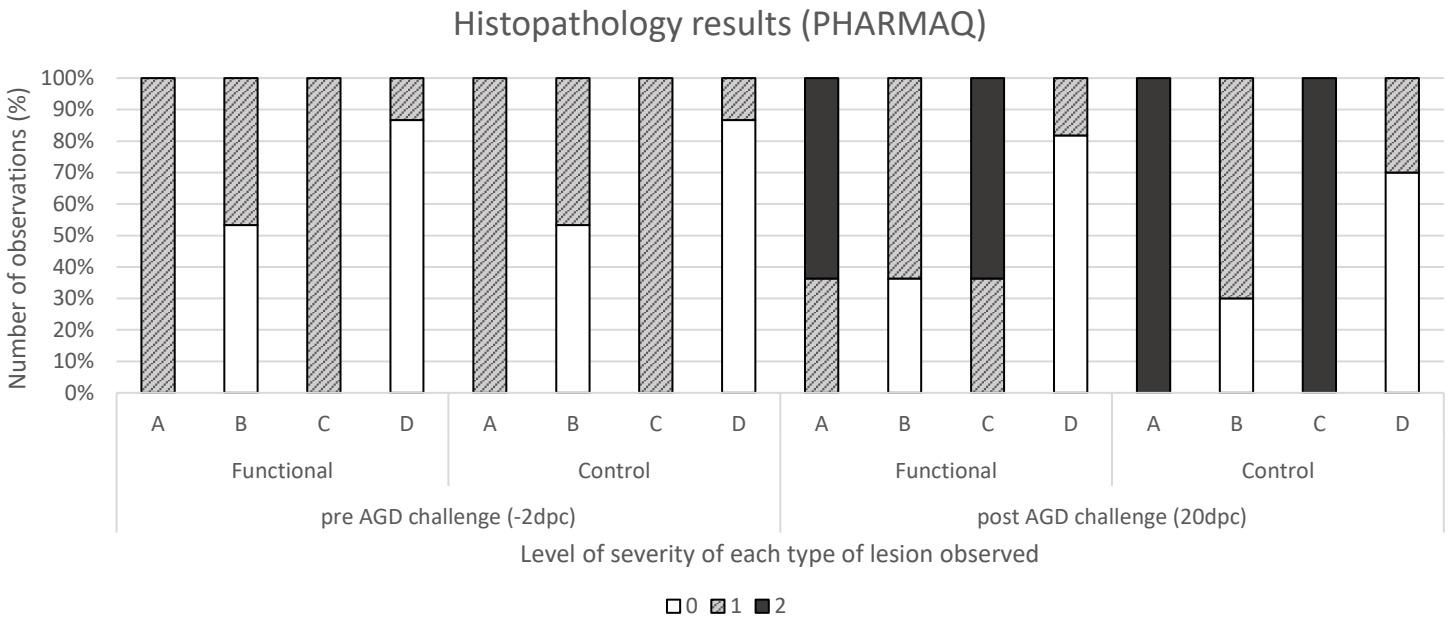


Figure 11. Histopathological results obtained by PHARMAQ Analytiq for uninfected (-2dpc) and infected (20dpc) samples ranked from 0 to 4, according to severity. Type of lesions observed are as follows: (A) Total amount of abnormal tissue; (B) Hyperplasia of the lamellar epithelium; (C) Lamellar inflammation; (D) Vascular lesions (total). Data are a combination of the 3 tanks fed with the control diet and the 3 tanks fed with the functional diet.

The results in Figure 11 indicate that, even before challenge, many fish already showed signs of minimal pathology (grade 1) for most of the lesion types assessed. For types (A) and (C), for example, there were signs in 100% of the sampled fish. This result is important for understanding that even uninfected fish can show signs of irritation in the gills that are unrelated to an AGD infection. However, it is clear that, after the infection, the frequency of mild lesions (grade 2) increased. Again, for lesions (A) and (C), the increase was of 100% in the control group and of around 70% in the functional group. There were, however, no observations of moderate (3) or severe (4) pathologies indicating only a mild AGD infection. Additionally, no dietary effects could be identified from the results obtained.

3.3 Specific Growth Rate

The specific growth rate is a useful measure to report the percentage body weight increase of fish per day and is calculated through Equation 1 below, where "wt" is the

weight at time t, "wi" is the initial weight and "Δt" is the number of days passed between weighting (Millot, et al., 2008).

$$SGR = \frac{(Ln(wt) - Ln(wi))}{\Delta t} x\ 100 \quad (1)$$

The body weight of the fish was measured at the start of the trial, at the first sampling (-2dpc) and at the last one (20dpc). The SGR1 was calculated between the start weight and the first sampling (-2dpc), the SGR2 was calculated between the first (-2dpc) and the second (20dpc) sampling and the SGRTotal was calculated between the start weight and the 20dpc sampling for both diets (control and functional). The differences between the two diets were compared through a Welch two sample t-test but no significant differences were found in the growth rate between fish of the different dietary groups in any of the tested time points, as shown in Table 9. The normality and homogeneity of variances assumptions were tested through the Shapiro-Wilk and the Levene's test, respectively, and no changes were detected.

Table 9. Mean ± SD specific growth rate (SGR) for each diet's triplicate tanks, sampling points and the significance of a Welch two sample t-test results between the two diets.

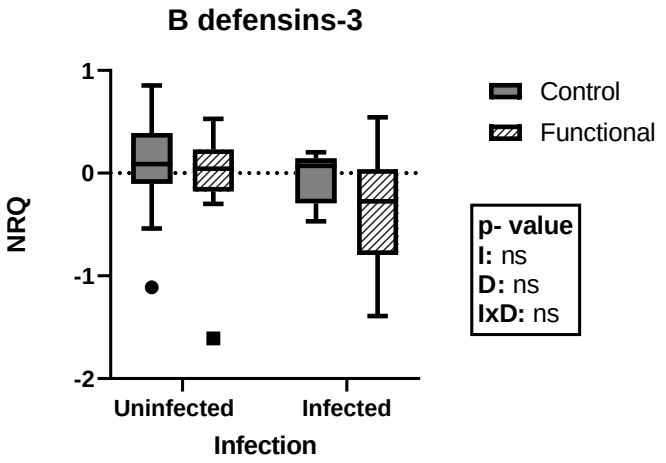
	SGR1	SGR2	SGRTotal
Control Diet	2.12 ± 0.05	1.23 ± 0.45	1.92 ± 0.15
Functional Diet	2.06 ± 0.09	0.86 ± 0.48	1.78 ± 0.08
Statistics	NS	NS	NS

NS: non-significant (p-value: ≥ 0.05)

3.4 RT-PCR

3.4.1 Gene expression results

As described in section 2.6, the normalised relative quantities (NRQ) values of each gene, obtained in the qBASE+ software, were used for interpreting the results in the RStudio software. The boxplots of all genes are shown in Figure 12 and show the p-value results for all factors analysed in the Two-way ANOVA. Additionally, the letters show the results obtained in the post-hoc Tukey HSD test performed to identify exactly where the significant differences lied within the groups. The plots containing no letters indicate that the post-hoc test was not able to identify significant difference within the groups. Furthermore, the summary table containing the mean NRQ ± SE of all analysed genes and their corresponding results for the Two-way ANOVA test and Levene's test (LV) for homogeneity of variances is displayed in Appendix 5 and the box plot for the two housekeeping genes used, β-actin and Elongation factor 1-α, are in Appendix 6.


B defensins-3
NRQ
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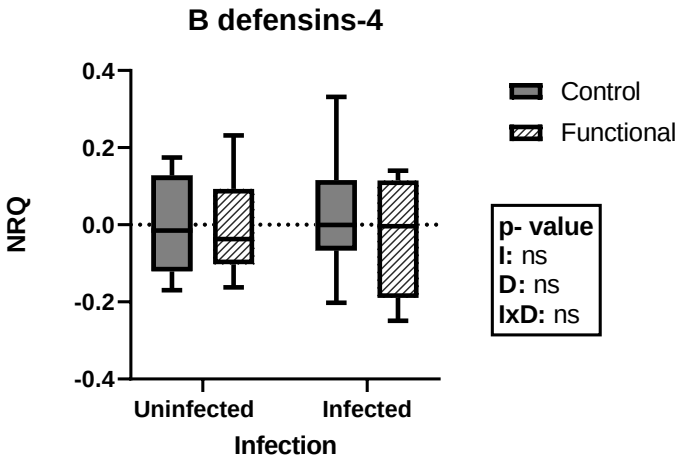
0
-1
-2
Uninfected
Infected
Infection
Control
Functional
p- value
I: ns
D: ns
IxD: ns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NRQ
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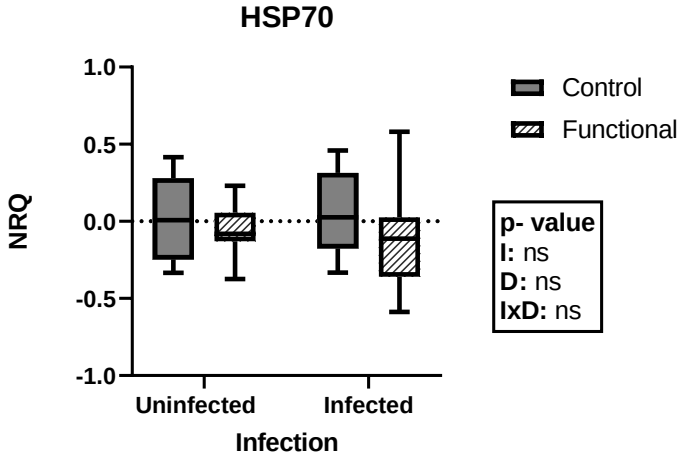
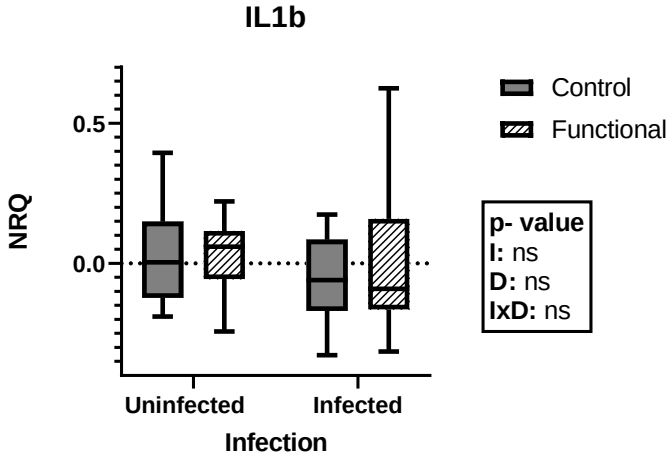
0.2
0.0
-0.2
-0.4
Uninfected
Infected
Infection
Control
Functional
p- value
I: ns
D: ns
IxD: ns


HSP70
NRQ
1.0
0.5
0.0
-0.5
-1.0
Uninfected
Infected
Infection
Control
Functional
p- value
I: ns
D: ns
IxD: ns


IL1b
NRQ
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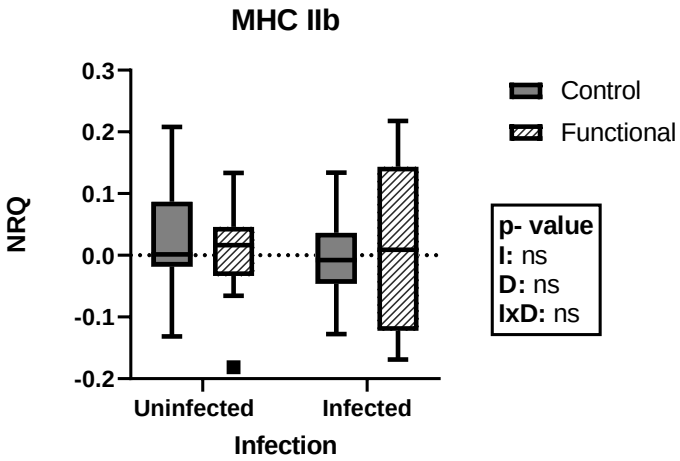
0.0
Uninfected
Infected
Infection
Control
Functional
p- value
I: ns
D: ns
IxD: ns


MHC IIb
NRQ
0.3
0.2
0.1
0.0
-0.1
-0.2
Uninfected
Infected
Infection
Control
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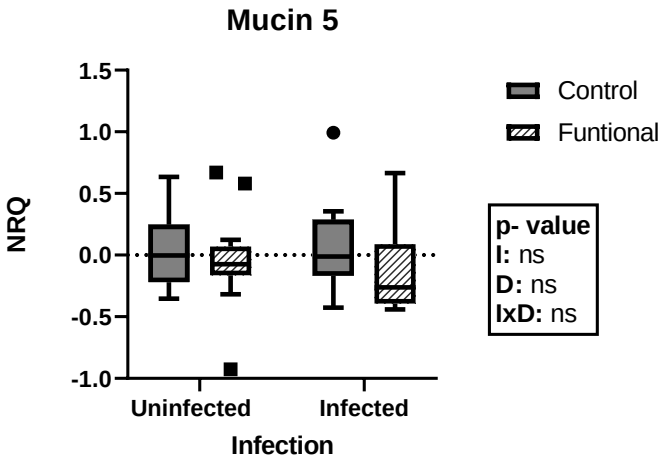
p- value
I: ns
D: ns
IxD: ns


Mucin 5
NRQ
1.5
1.0
0.5
0.0
-0.5
-1.0
Uninfected
Infected
Infection
Control
Funtional
p- value
I: ns
D: ns
IxD: ns

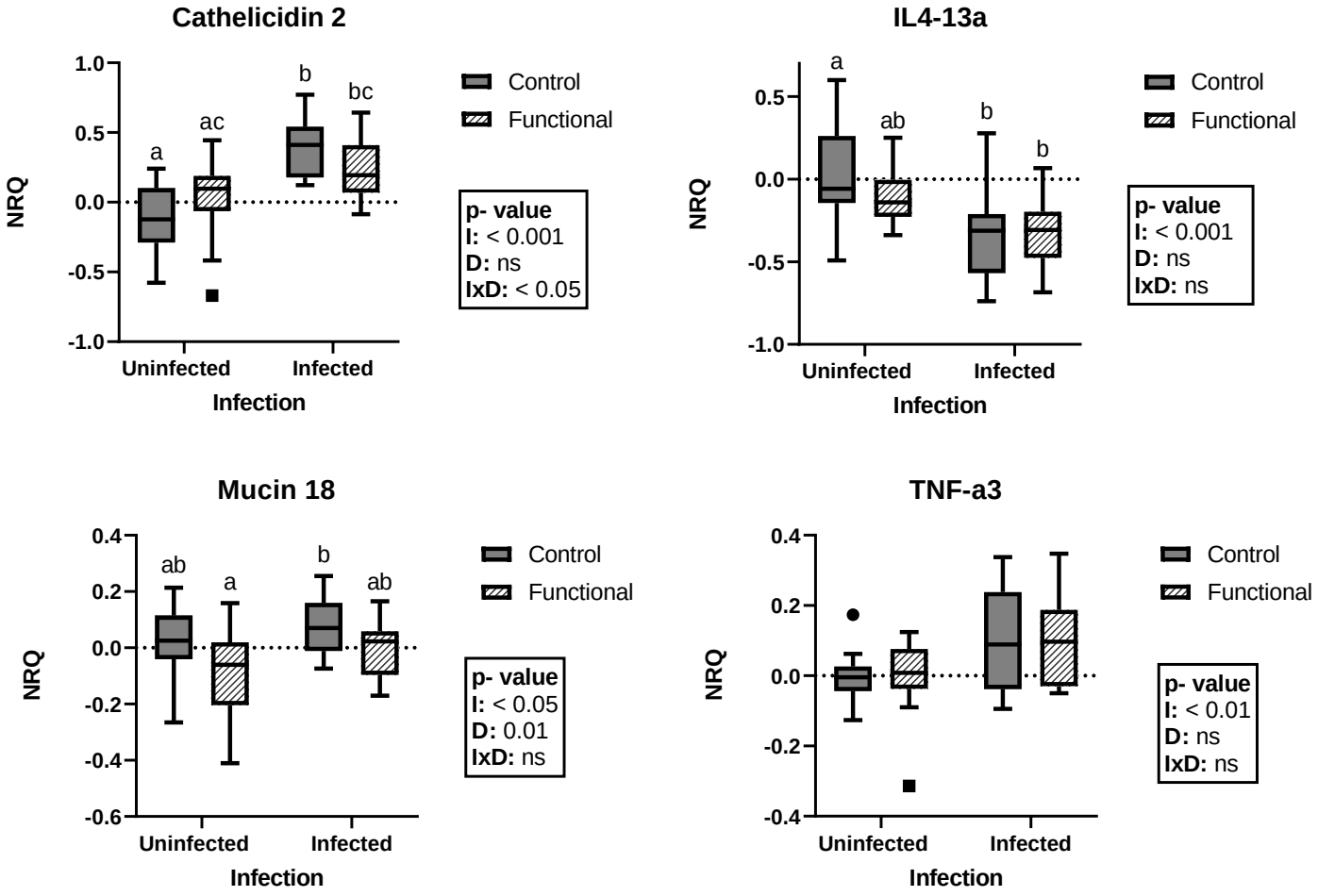


Figure 12. Box plot for all ten genes with the corresponding p-value result obtained in the Two-way ANOVA. Data are normalised relative quantities ± SD. Different letters represent significant differences obtained in the post-hoc Tukey HSD test (p-value < 0.05). I: infection; D: diet; IxD: interaction factor between infection and diet; ns: non-significant (p-value ≥ 0.05).

From Figure 12 it is possible to observe that significant differences within the infection, feed or the interaction factor were present in 4 out of the 10 genes analysed. The significant differences within the infection group indicated that, overall, IL4-13a was down regulated, whereas cathelicidin 2, mucin 18 and TNF-α3 were upregulated during the infection. Additionally, mucin 18's significant dietary effect indicated that there was an upregulation in the infected control group, when compared to the uninfected functional group. Finally, cathelicidin 2 expressed a significant value for the interaction factor between diet and infection, which resulted in the upregulation of both infected groups, when compared to the uninfected control, and the upregulation of the infected control, when compared to the uninfected functional diet. The other six analysed genes did not show any significant differences.

4. Discussion

Many studies have aimed to understand the role that functional feeds can have as a preventative measure against diseases in aquaculture. Martinez-Rubio et al. (2014) concluded that altering the composition of feed can have a positive impact in controlling inflammatory diseases in Atlantic salmon. Dietary lipid content and fatty acid composition were found to have important immune-modulatory roles in response to viral infections, such as cardiomyopathy syndrome (CMS) and heart and skeletal muscle inflammation (HSMI). Dawood et al. (2018) compiled studies on the use of different feed additives and their useful applications for the aquaculture industry. Inactivated natural microbes or microbial products, such as, β-glucans, lipopolysaccharides, lactoferrin (LF), chitin, and peptidoglycans have shown important functional properties with good potential to improve fish health, immune function, and, therefore, increase the hosts' resistance towards pathogenic diseases. Likewise, plant extracts were found to have several types of active components that can also act as immunostimulants and improve the fish's response to viral, bacterial, and parasitic infections.

Specifically, for AGD, Mullins et al. (2020) studied the effect that different concentrations and combinations of functional ingredients can have on the survival and immune response of Atlantic salmon. The results suggested a positive effect, especially attributed to the addition of arginine, micro additives, and vitamins C and E.

In this study, the presence of higher concentrations of vitamins C and E and the addition of mannanoligosaccharides (MOS), phytogenic extracts and krill meal as functional ingredients was intended to provide fish with better protection and enhanced immune responses when challenged with *N. perurans*, the causative agent of AGD.

The addition of krill meal, on top of fish meal, is nutritionally interesting as the omega-3 fatty acids (n-3 FAs) are more readily available. This is in part explained by the fact that most n-3 PUFAs are bound to phospholipids (PLs), whereas in fish meal (FM), a larger portion is bound to triglycerides (TGs). This difference has been associated with several health promoting aspects in many animals (humans, swine, and rodents) and is thought to behave similarly in fish. These benefits can be attributed to the higher absorption efficacy of EPA and DHA when provided in the PL form compared to the TG. The availability of n-3 PUFAs is crucial to regulate the inflammatory response, to maintain cellular and body homeostasis, and to provide a healthy neurological balance (Burri et al., 2012).

In order to evaluate the impact of the two different diet formulations on the fish, visual AGD scorings and histopathological analysis were used to assess the overall gill health and, the gene expression of ten markers, was used to assess the main changes at a molecular level. Additionally, the specific growth rate (SGR) was calculated between both sampling points and between the start and the end of the experiment. The results were compared between diets, but no significant differences in growth were observed.

Prior to the challenge with the pathogen, the comparison between the two dietary groups did not show significant differences in the gill health observations or in the expression of the tested genes. After the challenge, the visual AGD gill scoring verified that there was an increase in the frequency of grade 2 scores (light infection), indicating some effect of the infection in the mucus expression in the gills, when compared to the uninfected fish. Based on previous results, it was expected that the addition of MOS to the functional feed

would cause an overexpression of mucus (Leclercq et al., 2020), however, both visual data and expression of the analyzed mucins (muc5 and muc18) did not support this statement. In fact, the expression of mucin 5 did not result in significant differences within neither the infection nor the dietary groups.

Mucins are glycoproteins secreted by goblet cells that comprise the primary structural components of mucus and are divided into secretory and membrane bound. The first is associated with the rheological properties (viscosity and elasticity) of mucus and the latter is associated with the formation of the glycocalyx that covers epithelial cells (Roussel & Delmotte, 2004). Muc5 and muc18 belong to the secreted and the membrane-bound groups, respectively. The mucus layer of epithelial surfaces has many essential roles in lubrification, mechanical and immune defence, ion regulation, and host-pathogen interaction. In order to increase physical and chemical barriers, mucus hypersecretion is a common response to limit parasitic and bacterial infections, especially in the gills, skin, and gut (Marcos-López & Rodger, 2020).

As mentioned in the introduction, AGD typically induces an inflammatory response in the fish gills, commonly characterized by epithelial hyperplasia, fusion of the lamellar epithelium, and presence of interlamellar vesicles (Oldham et al., 2016). The presence and severity of these lesions were investigated through histopathology analyses, and they indicated an increase in severity in the infected group, when compared to the uninfected group. The comparison between feed types also showed a slightly higher frequency of more severe observations in the control group than in the functional feed. However, as no fish showed lesions with a classification higher than 2 (mild), it seems that the infection achieved was not as severe as expected in the experimental design. Furthermore, even though, the count of observations was slightly higher in the control group, there is no evidence of a significant difference when comparing the AGD score and the histopathological findings of both diets.

Once a parasite has been recognized, the organism's first response is the activation of both innate and adaptive immune responses. Heat shock proteins, such as HSP70, are markers for cellular stress and oxidation. During an infection, they are also responsible for activating antigen presenting cells, such as MHC class II, and inducing the pro-inflammatory cytokine cascade to the site of infection (Marcos-López et al., 2017b). Tumor necrosis factor alpha (TNFα) and interleukin 1β (IL-1β) have major roles in the cellular infiltration process triggered by the cytokine cascade, especially in sites of severe AGD lesions. Additionally, epithelial cells, which are typically overexpressed during an AGD infection, are a source of IL-1β, contributing to their overexpression (Pennacchi et al., 2014). Likewise, HSP70, MHC IIβ, and TNFα3 have also been observed in previous studies to be significantly altered as a result of an AGD infection.

As opposite to what is seen in literature, in the current study, no significant differences between infection or dietary groups were observed for HSP70, MHC IIβ or IL-1β. Even though, no significant dietary effect was detected in the expression of TNFα3, a significant upregulation was observed in the infected group, when compared to the uninfected group. It is worth mentioning that the infected group showed a large variation within the data, which could be an indication of individual differences in response to the infection. The study by Pennacchi et al. (2014), also identified an upregulation in the expression of TNFα3 during an AGD infection in Atlantic salmon, particularly in lesion-specific areas. However, Marcos-López et al. (2018) observed a significant down regulation of TNFα3 during the infection. The low expression of TNFα3 can indicate an

impairment of the immune system towards the disease but, considering that this gene can also help induce apoptosis and growth arrest, it could also indicate that the down regulation is aiding the high cell proliferation that takes place during an infection. Thus, the role that tumor necrosis factor-α3 has during the AGD infection in Atlantic salmon is still not fully understood and requires further studies.

The present study also identified significant differences in expression levels of three other genes. While cathelicidin 2 and mucin 18 were upregulated upon infection, IL4-13a was downregulated. The TukeyHSD test for both cathelicidin 2 and IL4-13a found significant differences between the uninfected control group and both infected groups (control and functional feed), suggesting that infection had a significant effect on gene expression regardless of the diet that fish were fed. This result provides confirmation that the AGD infection can have direct influences on the expression of antimicrobial peptides (AMPs), such as cath2, and pro-inflammatory cytokines, such as IL4-13a.

The role of the T helper 2 (Th2) cytokine, IL4-13a, has been investigated by Marcos-López et al. (2018) and the expression of cathelicidin 2, has been studied by McGrath et al. (2022). Both studies reported a significant upregulation in expression of both genes in the gill tissue of *N. perurans* challenged salmon. In this study, however, the same pattern in expression is only observed for cathelicidin 2, whereas, as mentioned, IL4-13a was downregulated. This trend is opposite to what was expected of AGD infected fish, but it can also explain the lack of mucin's 5 upregulation, as observed by Marcos-López et al. (2018). In mammals, the upregulation of Th2 cytokines, such as IL4-13a, stimulates goblet cell hyperplasia and, consequently, induces overexpression of mucin 5 (Marcos-López & Rodger, 2020). As it has been observed in previous studies, parasites have the ability to develop mechanisms to evade the host's immune response (Schmid-Hempel, 2009), thus, the suppression in expression of key cytokines, such as, IL4-13a, and, consequently, the expression of mucin 5, could be an example of these mechanisms. However, the infection's lack of significant influence on the other analysed genes, which are also involved in key processes of the host's immune response, indicates that, most likely, the pathogenic load during challenge might not have been sufficient to elicit a substantial inflammatory response. Thus, the upregulation of the IL4-13a cytokine and, consequently, mucin 5 was not necessary.

Mucin 5 is thought to be the primary component of the characteristic AGD white mucus patches in the gills (Marcos-López et al., 2018). The lack of significant differences in its expression are consistent with the visual AGD score obtained, and, together with the histopathological analysis, indicate that the fish did not show signs of a severe infection in any of the dietary groups post-challenge.

In the context of fish health, McGrath et al. (2022) showed that, particularly regarding parasitic infections, two families of antimicrobial peptides (AMPs) have a strong influence: β-defensins (βD) and cathelicidins. Based on McGrath's study, besides the upregulation of cath2, a statistically significant upregulation of β-defensins – 3 (βD-3) and – 4 (βD-4) was expected. In the present study, however, βD-3 and βD-4 did not show significant differences between uninfected and infected fish. Low levels of AMP's have shown to inhibit inflammation, while higher expression is associated with pro-inflammatory response and chemoattractant activity for different types of inflammatory cells (Das et al., 2022). The low expression of β-defensins obtained in the current study, for both dietary groups, can indicate that the fish were lowly infected and, therefore, there is little evidence of a substantial inflammatory response. A decreased expression of

β-defensins also has a role in resolving inflammation, especially once the danger has been neutralized or during the presence of low pathogenic load (Das et al., 2022), indicating that the fish's organisms might have already fought off the infection.

The analysis of cathelicidin 2 and mucin 18's results also indicated a significant difference between the uninfected functional group and the infected control group, which suggests that the functional diet may have had a protective outcome against negative effects of infection on gene expression. Mucin 18 belongs to the immunoglobulin superfamily (IgSF), which consists of several molecules expressed at the cell surface that contributes to several cellular activities, such as adhesion and immune responses. Muc18 is known to have pro-inflammatory roles and to be upregulated as a response to airway inflammation (Dermody et al., 2009; Marcos-López et al., 2018). Additionally, antimicrobial peptides, like cathelicidin 2, have essential roles as mediators of innate immune response, due to their strong antimicrobial and immunomodulatory activity, and are typically upregulated in the presence of pathogens (Katzenback, 2015). The significant higher expression of both cath2 and muc18 in the fish that received the control diet supports the hypothesis that the gills of control fed fish were slightly more susceptible towards inflammation, caused by the amoeba's presence, than the fish fed with the functional diet. Thus, also supporting the hypothesis that the functional feed provides a better outcome at containing the inflammatory process in fish, when compared to the control diet. This is a promising result towards verifying the hypothesis that functional feed can increase fish robustness and make them more prepared to fight off pathogenic invasions.

Furthermore, the expression of cath2 also showed a significant interaction factor (diet x infection), suggesting that both infection and diet have significant effects in its upregulation, and that the effect of the functional diet on gene expression may depend on whether the fish are infected or not.

Overall, the comparison in expression of all ten genes in uninfected and infected fish, fed with the control and the functional diet, provided little significant dietary effects. However, the difference in expression between the infected control and the uninfected functional feed, for mucin 18 and cathelicidin 2, provided promising results in suggesting a protective outcome of the functional feed against negative effects of infection on gene expression. Moreover, even though, a significant effect of the infection was observed in the expression levels of four of the analysed genes, the gene expression results, together with the visual AGD scoring, and the histopathology analysis, suggests that the fish were only mildly infected. The typical amoebae concentration for a challenge is 500 amoeba/L and a high dosage is considered to be 2500 amoeba/L (Nowak et al., 2014). Since, in this trial, the concentration used was 8400 amoeba/L, it is unlikely that the pathogen density was responsible for the low level of infection. Different factors, such as fish size during challenge or amoeba density and virulence, could have interfered with the experiment leading to a milder infection level than expected. Thus, the lack of immune response stimulation could explain why no significant differences, in response to the infection, were observed in the other six analysed genes.

5. Conclusion

In this study, Atlantic salmon smolts were fed two different diets, a control and a functional feed, and, after a pre-feeding phase of 10 weeks, were submitted to a pathogenic challenge with *N. perurans*, the causative agent of AGD. The aim of the study was to assess whether the functional feed would be a viable preventative method towards the disease. For that, visual AGD scorings, histopathological analysis, growth rate, and the expression levels of ten genes, related to inflammatory processes and immune responses in the host, were investigated.

The comparison amongst the different dietary groups showed that there were no changes in growth and that, predominantly, there was no evidence of a significant difference in disease expression between them. Overall, a large variation within the groups was observed, which could be explained by the presence of individual differences in response to the infection. These results imply the presence of substantial disparity in susceptibility towards the disease amongst the fish, but the motive for this variation is unknown, as it could not be explained by the differences between the dietary groups. Furthermore, since the gene expression analysis only resulted in significant differences in four, out of the ten, genes, it is fair to conclude that, overall, the functional diet did not significantly alter immune and pro-inflammatory genes, mucus production or cellular stress levels in the current experiment, as was expected from the study's hypotheses. It is possible that the pre-feeding period (10 weeks) was too short, that the size of the fish during challenge and, therefore, their disease resistance was too high, that the pathogen's virulence was impaired or that some other factor could have prevented the arousal of a substantial immune response to the infection, in both groups.

Nonetheless, one important result was observed in the expression of cathelicidin 2 and of mucin 18, which, by revealing a significant upregulation in the expression of infected control, when compared to the uninfected functional feed, suggested that the functional diet could have indeed acted towards preventing the negative effects of the disease. However, more evidence is needed in order to validate the functional diet as a preventative method for AGD.

That said, there is still a lack in knowledge on whether altering the formulation of feed can have a beneficial impact on the infection rate of AGD in Atlantic salmon or not. In order to fully assess the effects that the functional diet has on the immune response of AGD infected fish, the exposure to a more severe infection is suggested. Additionally, future studies comparing different functional feed formulations would also provide precious knowledge on the matter. Based on existing literature, it is clear that the optimization of diet formulation, through the addition of functional ingredients, shows a considerable promise towards becoming an effective preventative measure against many diseases, including AGD.

The amoebic gill disease is responsible for many of the health problems seen in today's Atlantic salmon aquaculture industry and finding an efficient solution to deal with this disease is of extreme importance. The continuous growth and increasingly intensification of the industry, together with the changing climate can contribute towards aggravating the situation in the near future. The use of functional ingredients and optimization of feed formulation shows great promise and should continue to be further studied.

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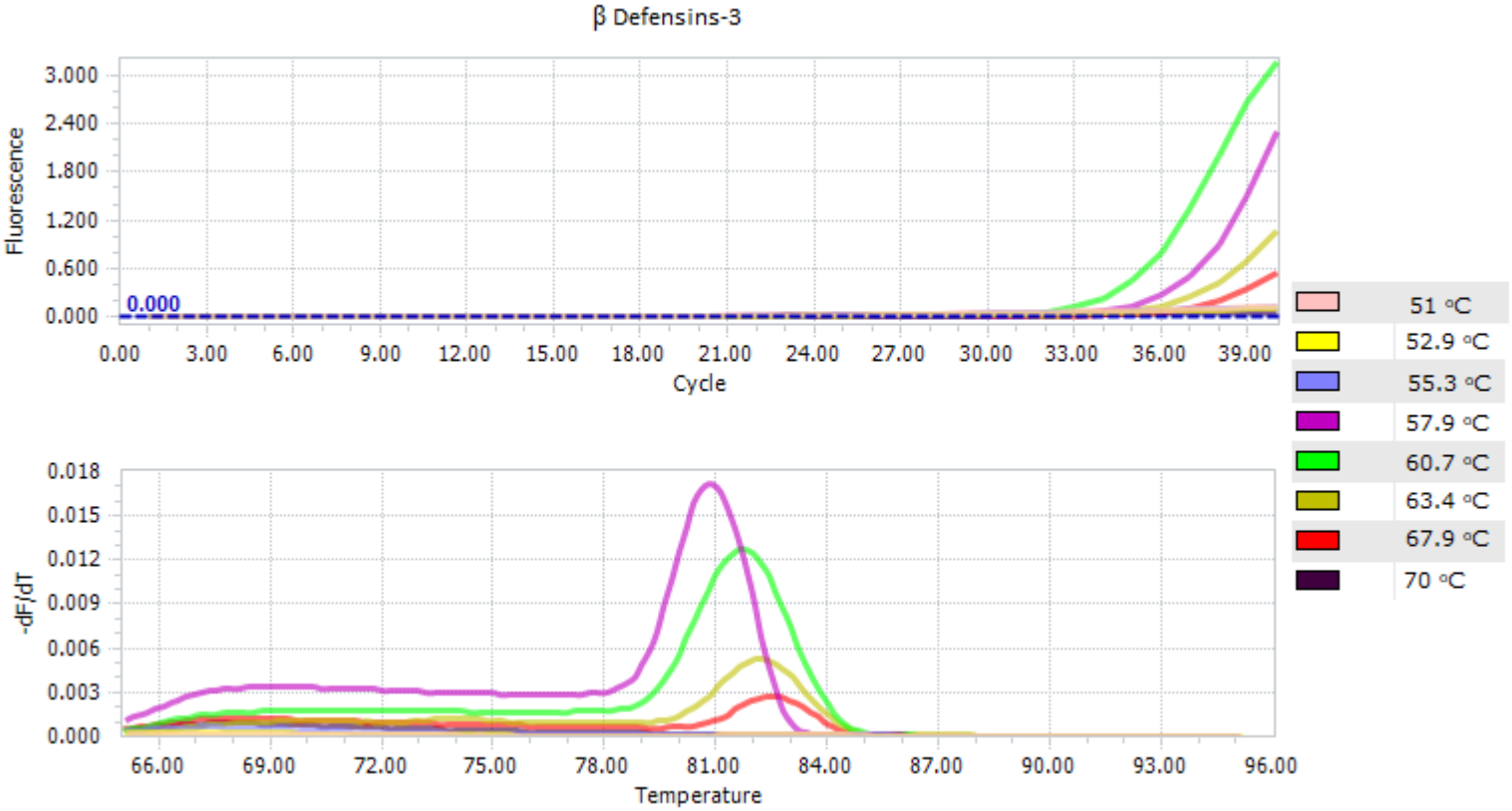
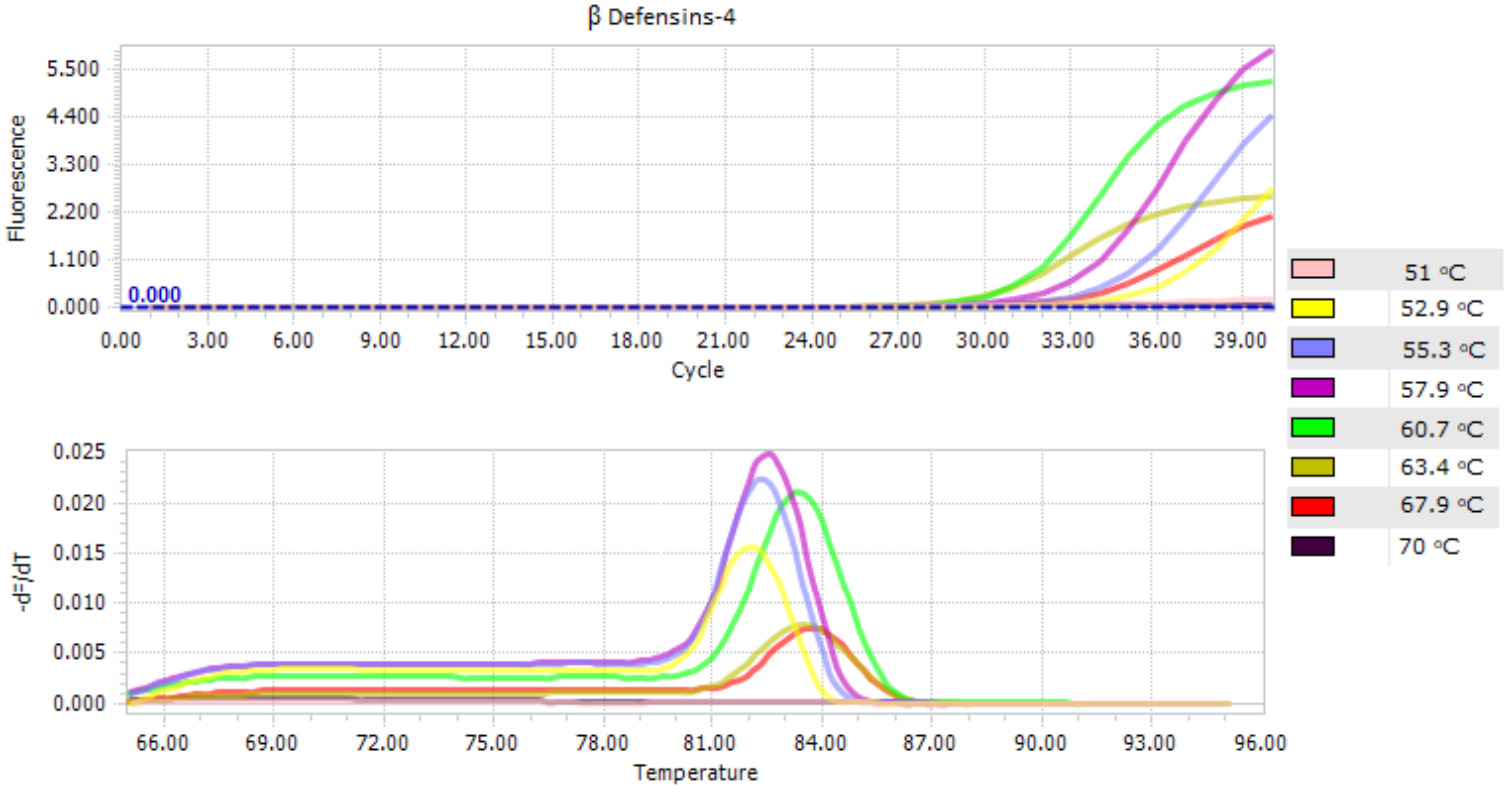
7. Appendices

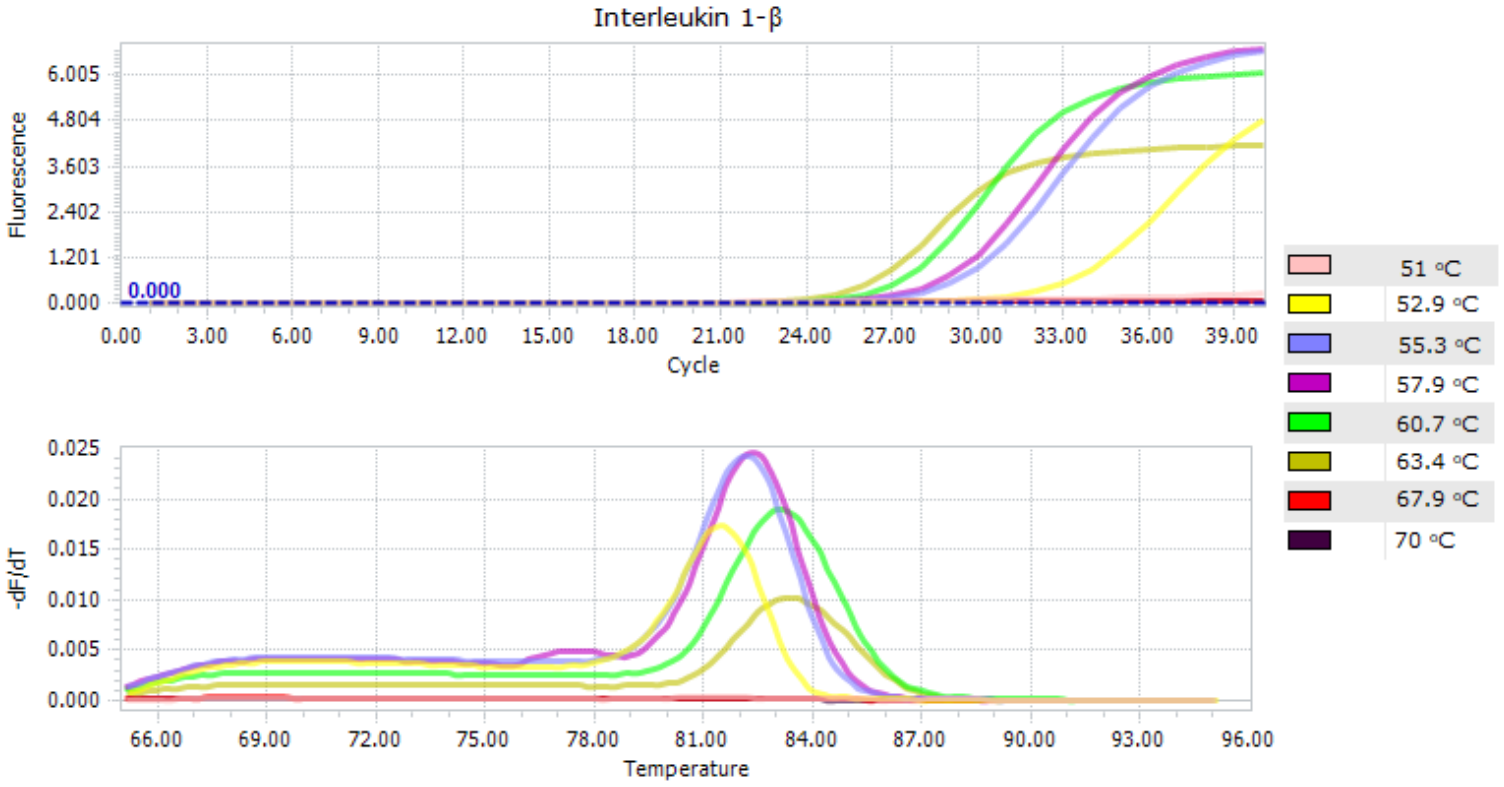
Appendix 1: qPCR plate set up for the primer sensitivity test.

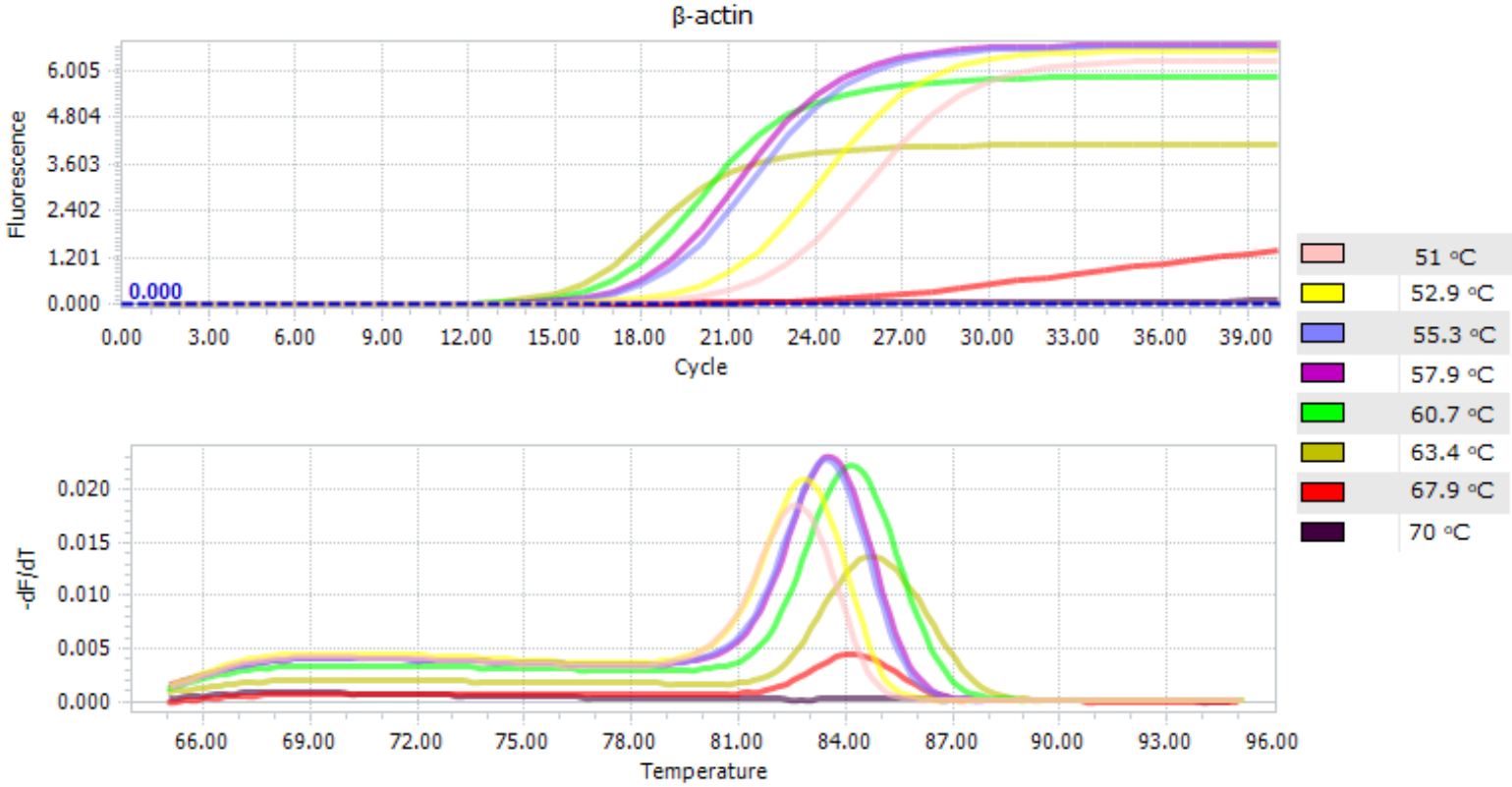
None	Gene1 T°C:51	Gene1 T°C:52.9	Gene1 T°C:55.3	Gene1 T°C:57.9	Gene1 T°C:60.7	Gene1 T°C:63.4	None	Gene1 T°C:67.9	None	None	Gene1 T°C:70
None	Gene2 T°C:51	Gene2 T°C:52.9	Gene2 T°C:55.3	Gene2 T°C:57.9	Gene2 T°C:60.7	Gene2 T°C:63.4	None	Gene2 T°C:67.9	None	None	Gene2 T°C:70
None	Gene3 T°C:51	Gene3 T°C:52.9	Gene3 T°C:55.3	Gene3 T°C:57.9	Gene3 T°C:60.7	Gene3 T°C:63.4	None	Gene3 T°C:67.9	None	None	Gene3 T°C:70
None	Gene4 T°C:51	Gene4 T°C:52.9	Gene4 T°C:55.3	Gene4 T°C:57.9	Gene4 T°C:60.7	Gene4 T°C:63.4	None	Gene4 T°C:67.9	None	None	Gene4 T°C:70
None	Gene1 No cDNA T°C:51	Gene1 No cDNA T°C:52.9	Gene1 No cDNA T°C:55.3	Gene1 No cDNA T°C:57.9	Gene1 No cDNA T°C:60.7	Gene1 No cDNA T°C:63.4	None	Gene1 No cDNA T°C:67.9	None	None	Gene1 No cDNA T°C:70
None	Gene2 No cDNA T°C:51	Gene2 No cDNA T°C:52.9	Gene2 No cDNA T°C:55.3	Gene2 No cDNA T°C:57.9	Gene2 No cDNA T°C:60.7	Gene2 No cDNA T°C:63.4	None	Gene2 No cDNA T°C:67.9	None	None	Gene2 No cDNA T°C:70
None	Gene3 No cDNA T°C:51	Gene3 No cDNA T°C:52.9	Gene3 No cDNA T°C:55.3	Gene3 No cDNA T°C:57.9	Gene3 No cDNA T°C:60.7	Gene3 No cDNA T°C:63.4	None	Gene3 No cDNA T°C:67.9	None	None	Gene3 No cDNA T°C:70
None	Gene4 No cDNA T°C:51	Gene4 No cDNA T°C:52.9	Gene4 No cDNA T°C:55.3	Gene4 No cDNA T°C:57.9	Gene4 No cDNA T°C:60.7	Gene4 No cDNA T°C:63.4	None	Gene4 No cDNA T°C:67.9	None	None	Gene4 No cDNA T°C:70

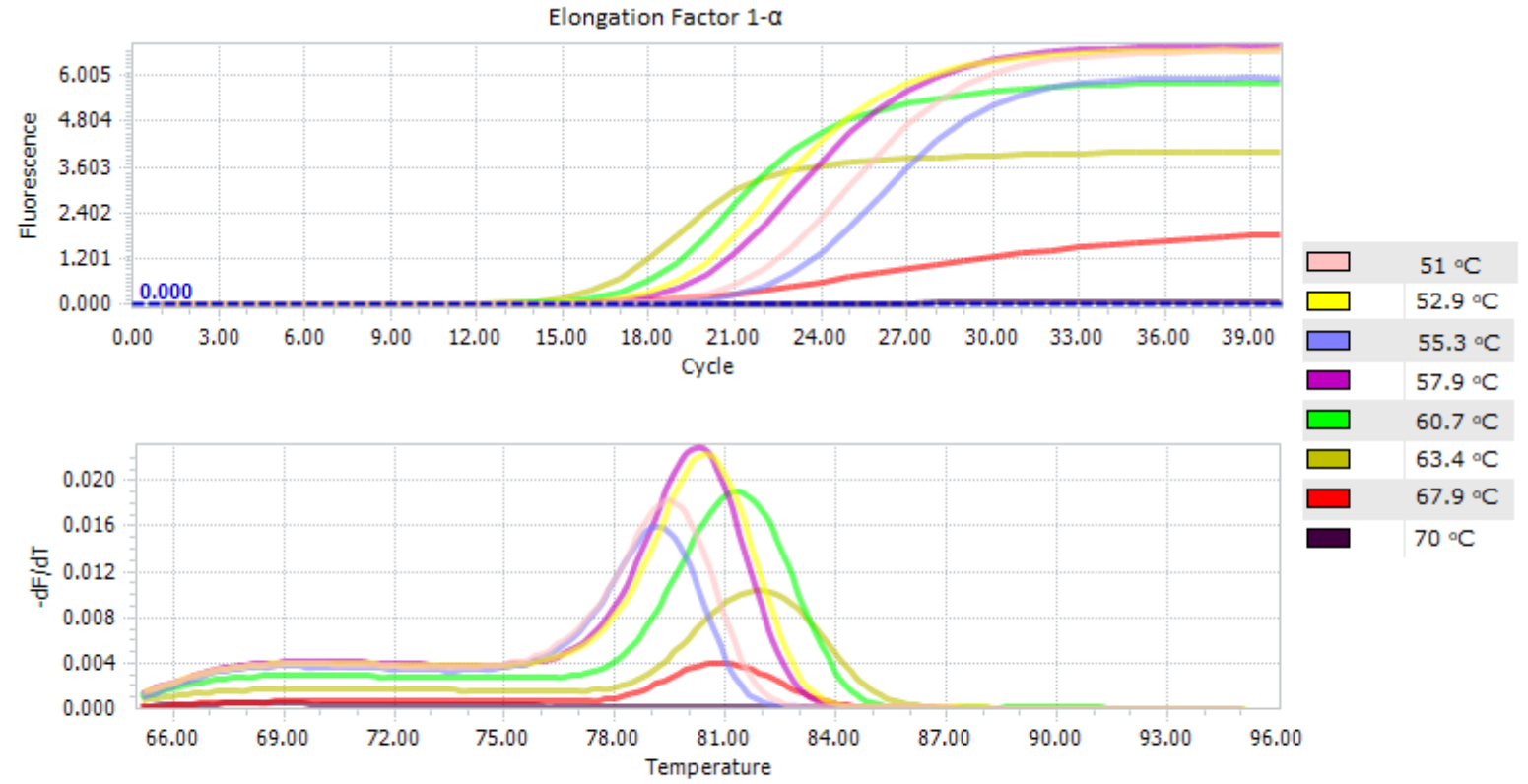
Appendix 2: Amplification curves and melting peaks obtained during the primer sensitivity test for all genes.

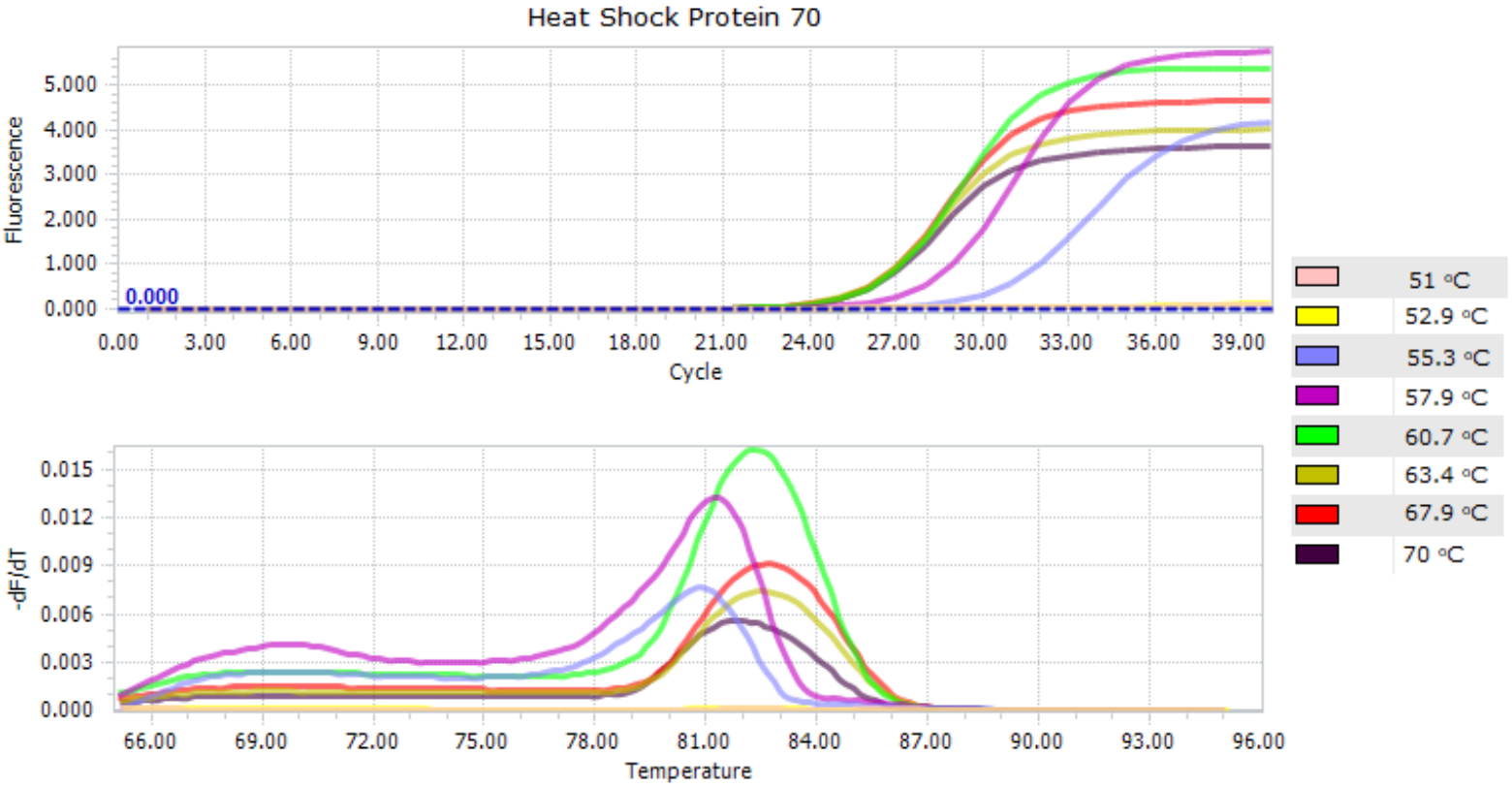


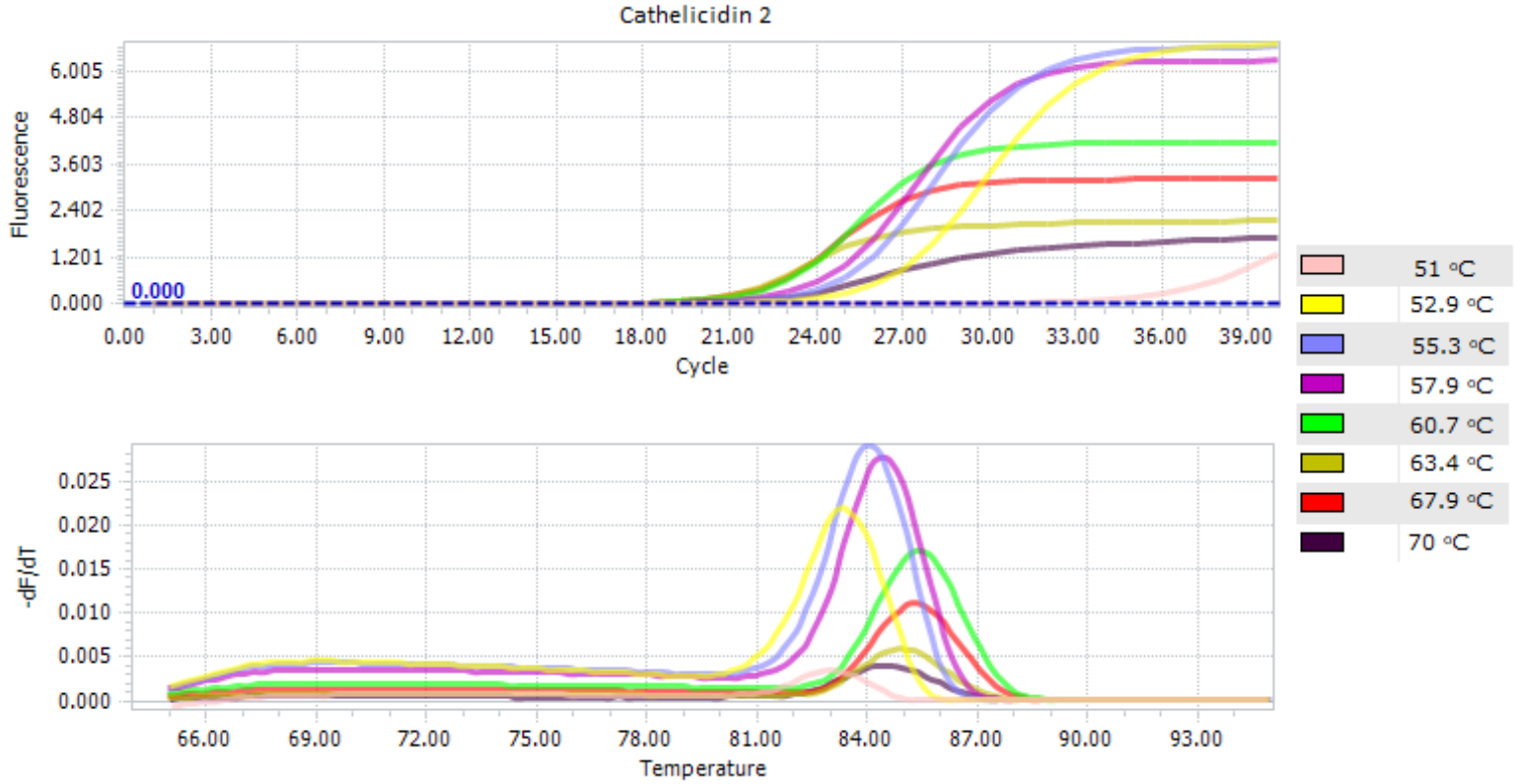


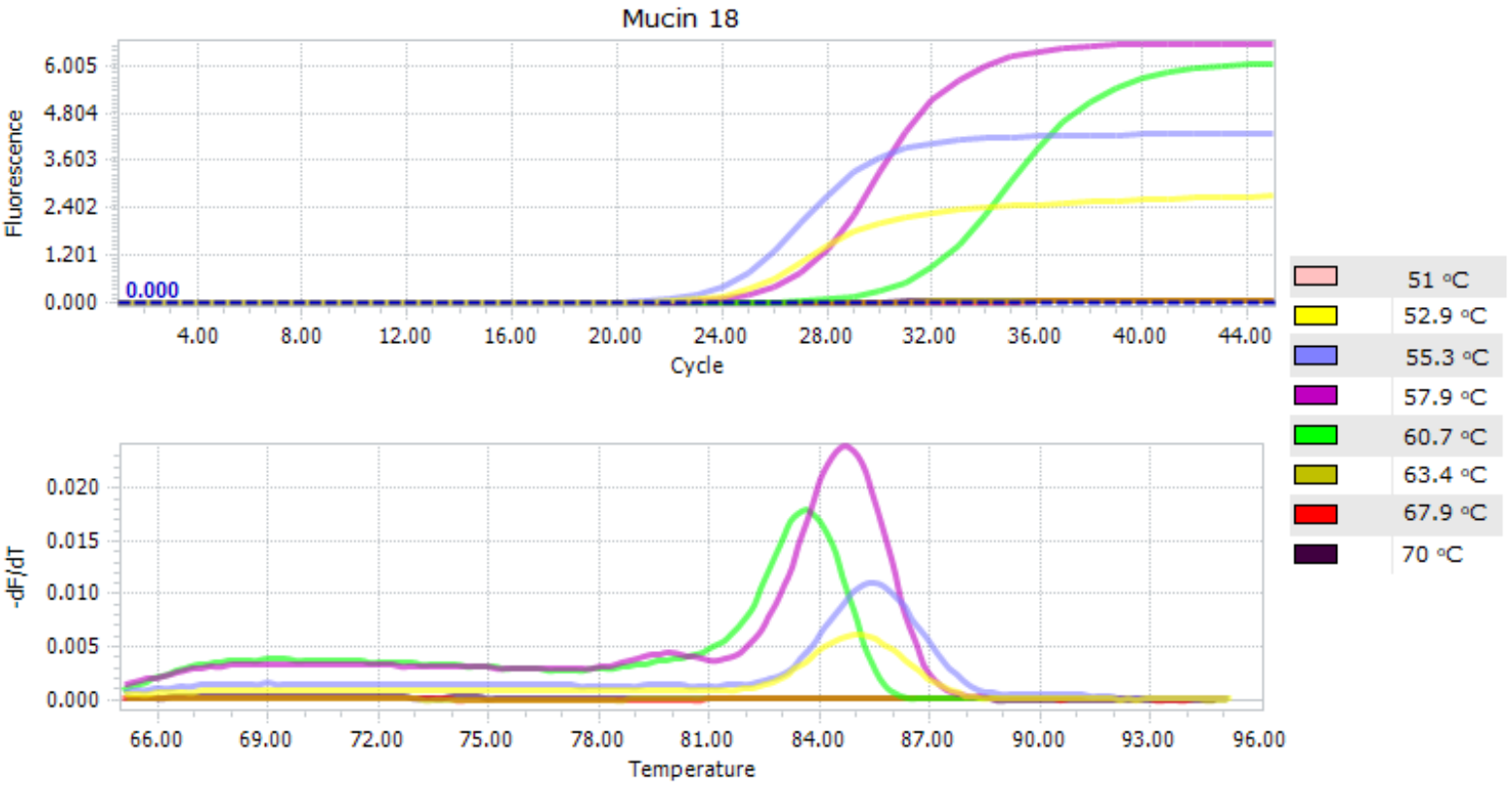

Interleukin 1-β
Fluorescence
6.005
4.804
3.603
2.402
1.201
0.000
0.000
0.00 3.00 6.00 9.00 12.00 15.00 18.00 21.00 24.00 27.00 30.00 33.00 36.00 39.00
Cycle
-dF/dT
0.025
0.020
0.015
0.010
0.005
0.000
66.00 69.00 72.00 75.00 78.00 81.00 84.00 87.00 90.00 93.00 96.00
Temperature
51 °C
52.9 °C
55.3 °C
57.9 °C
60.7 °C
63.4 °C
67.9 °C
70 °C

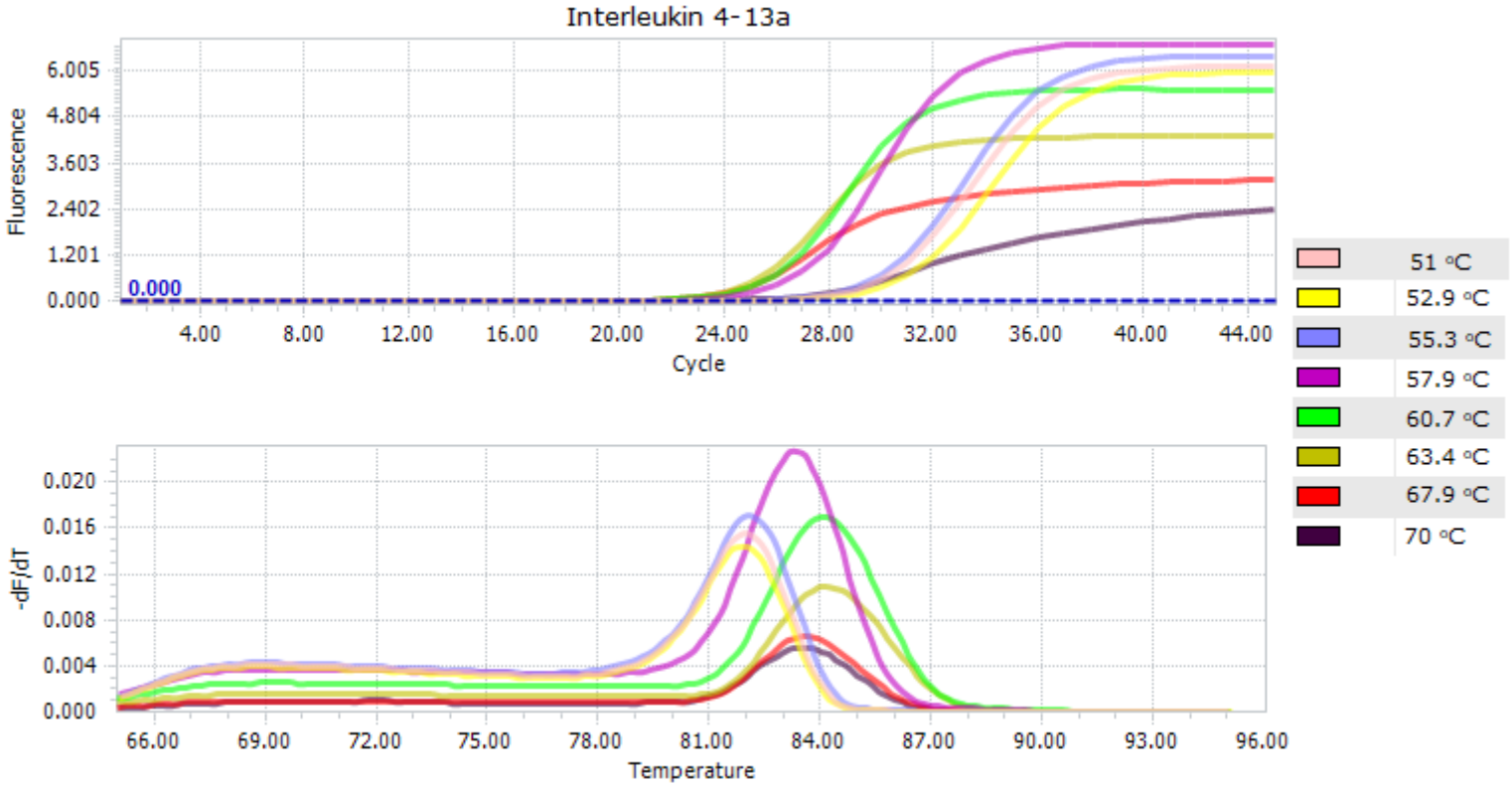

β-actin
Fluorescence
6.005
4.804
3.603
2.402
1.201
0.000
0.000
0.00 3.00 6.00 9.00 12.00 15.00 18.00 21.00 24.00 27.00 30.00 33.00 36.00 39.00
Cycle
-dF/dT
0.020
0.015
0.010
0.005
0.000
66.00 69.00 72.00 75.00 78.00 81.00 84.00 87.00 90.00 93.00 96.00
Temperature
51 °C
52.9 °C
55.3 °C
57.9 °C
60.7 °C
63.4 °C
67.9 °C
70 °C

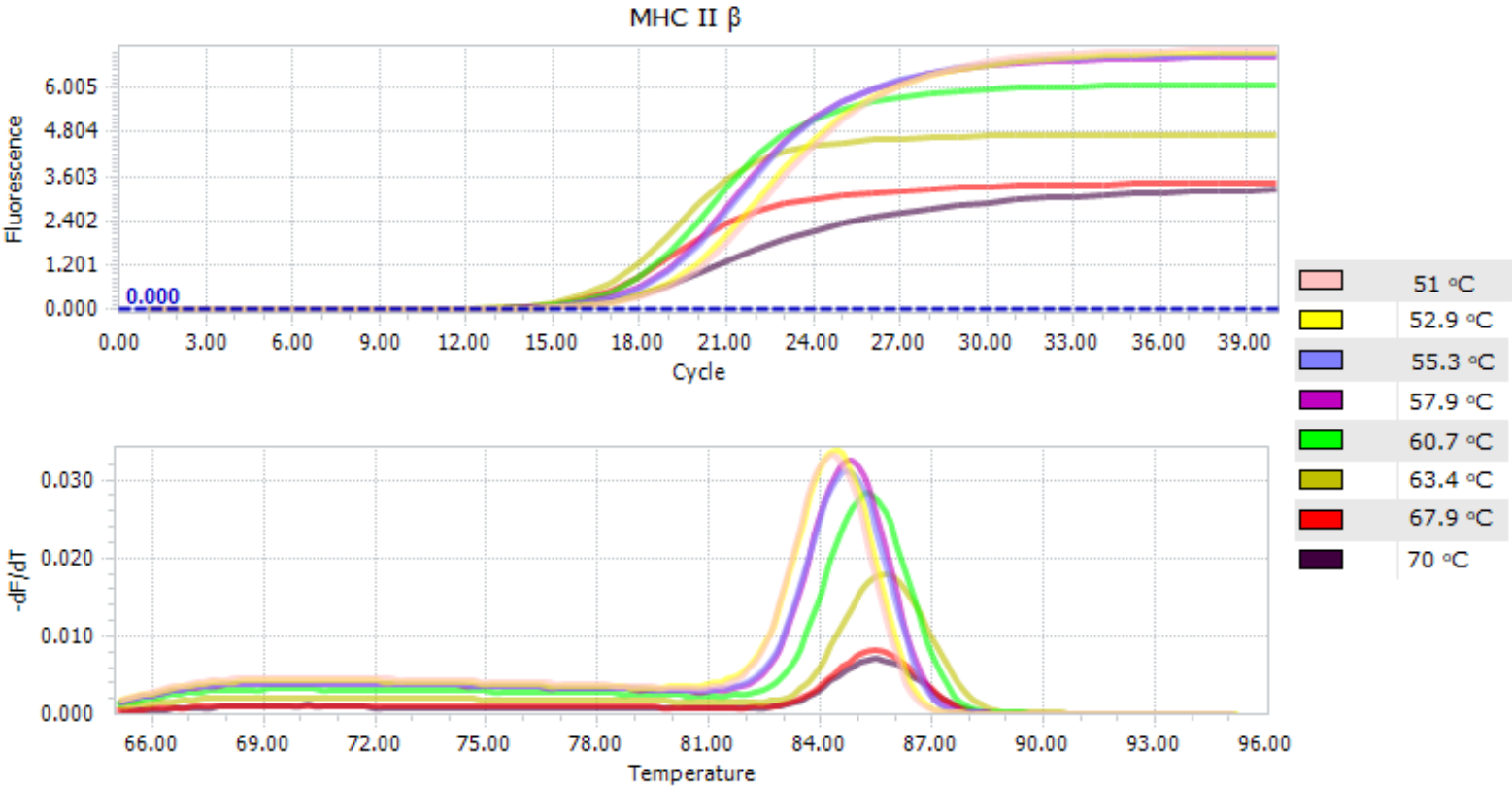

Elongation Factor 1-α
Fluorescence
6.005
4.804
3.603
2.402
1.201
0.000
0.000
0.00 3.00 6.00 9.00 12.00 15.00 18.00 21.00 24.00 27.00 30.00 33.00 36.00 39.00
Cycle
-dF/dT
0.020
0.016
0.012
0.008
0.004
0.000
66.00 69.00 72.00 75.00 78.00 81.00 84.00 87.00 90.00 93.00 96.00
Temperature
51 °C
52.9 °C
55.3 °C
57.9 °C
60.7 °C
63.4 °C
67.9 °C
70 °C

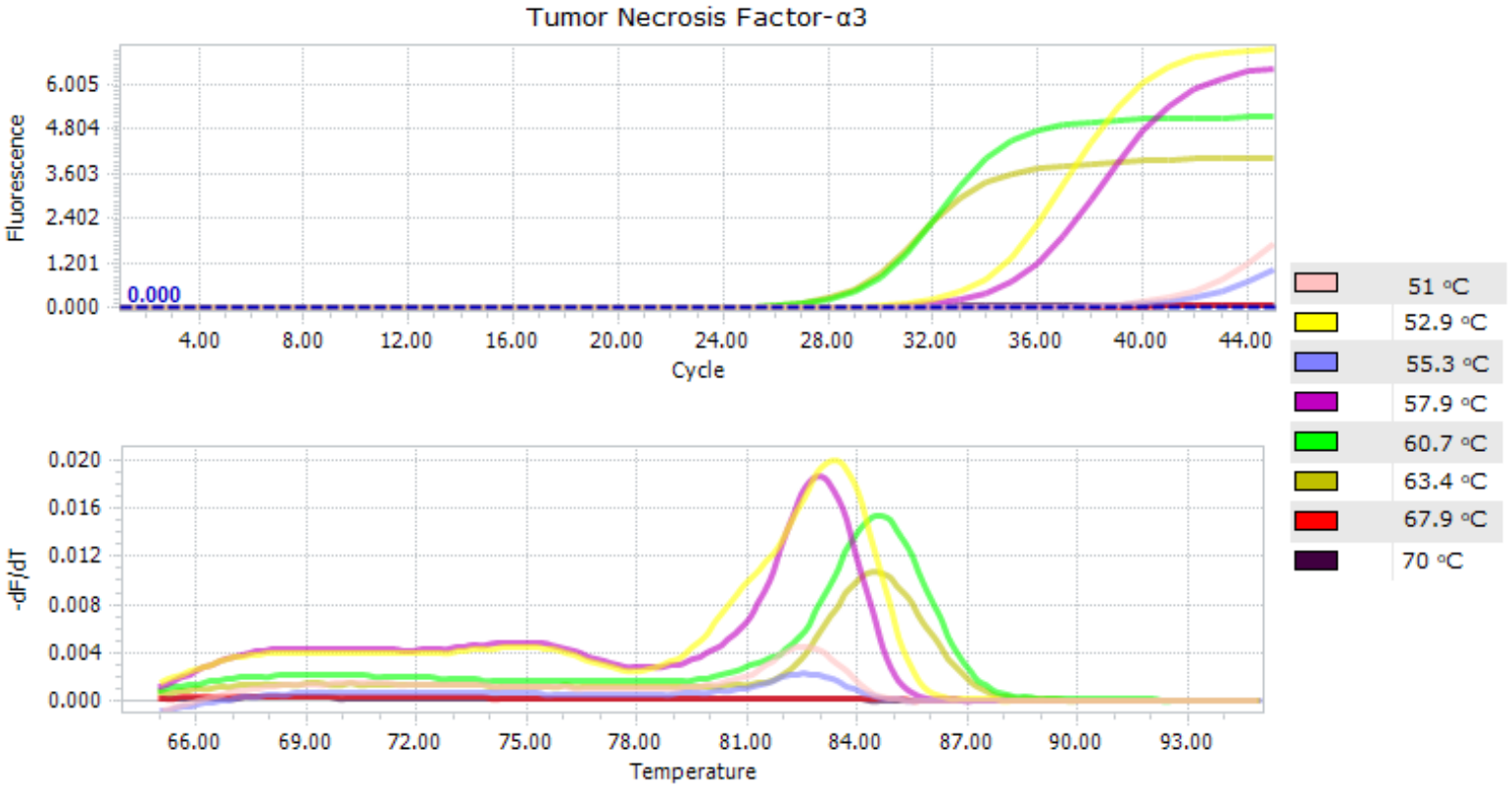

Heat Shock Protein 70
Fluorescence
5.000
4.000
3.000
2.000
1.000
0.000
0.000
0.00 3.00 6.00 9.00 12.00 15.00 18.00 21.00 24.00 27.00 30.00 33.00 36.00 39.00
Cycle
-dF/dT
0.015
0.012
0.009
0.006
0.003
0.000
66.00 69.00 72.00 75.00 78.00 81.00 84.00 87.00 90.00 93.00 96.00
Temperature
51 °C
52.9 °C
55.3 °C
57.9 °C
60.7 °C
63.4 °C
67.9 °C
70 °C


Cathelicidin 2
Fluorescence
6.005
4.804
3.603
2.402
1.201
0.000
0.000
0.00 3.00 6.00 9.00 12.00 15.00 18.00 21.00 24.00 27.00 30.00 33.00 36.00 39.00
Cycle
-dF/dT
0.025
0.020
0.015
0.010
0.005
0.000
66.00 69.00 72.00 75.00 78.00 81.00 84.00 87.00 90.00 93.00
Temperature
51 °C
52.9 °C
55.3 °C
57.9 °C
60.7 °C
63.4 °C
67.9 °C
70 °C

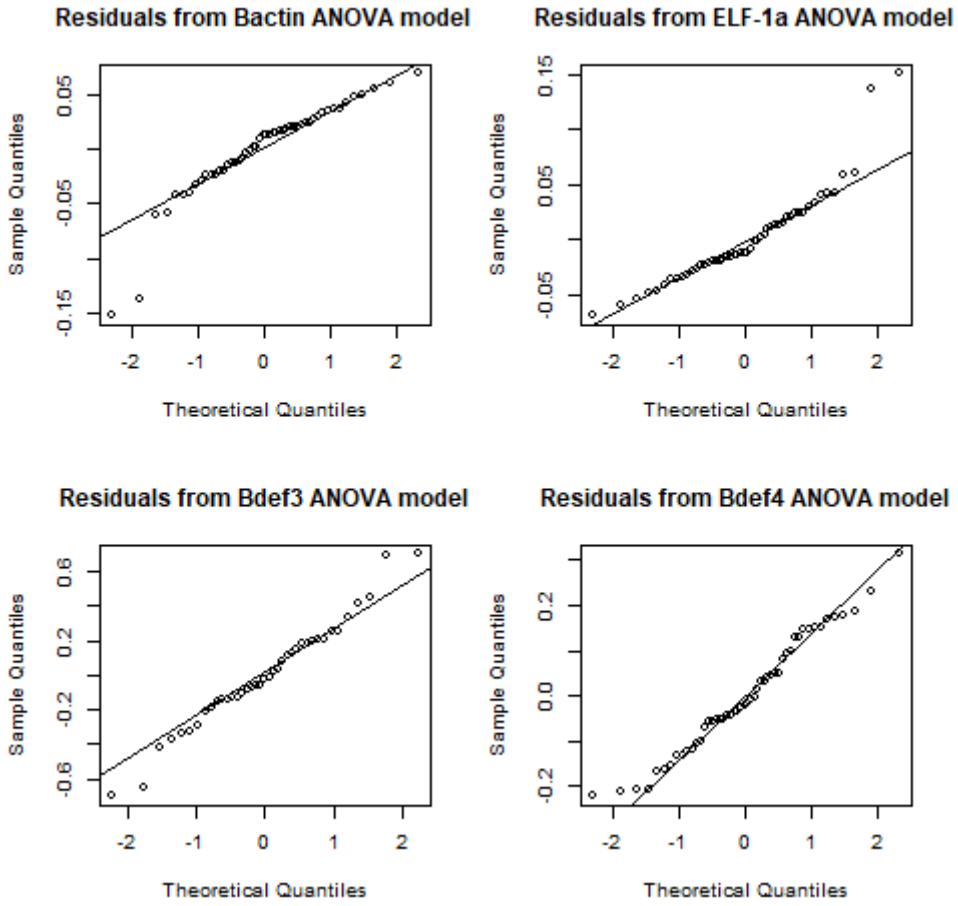

Mucin 18
Fluorescence
6.005
4.804
3.603
2.402
1.201
0.000
0.000
4.00 8.00 12.00 16.00 20.00 24.00 28.00 32.00 36.00 40.00 44.00
Cycle
-dF/dT
0.020
0.015
0.010
0.005
0.000
66.00 69.00 72.00 75.00 78.00 81.00 84.00 87.00 90.00 93.00 96.00
Temperature
51 °C
52.9 °C
55.3 °C
57.9 °C
60.7 °C
63.4 °C
67.9 °C
70 °C

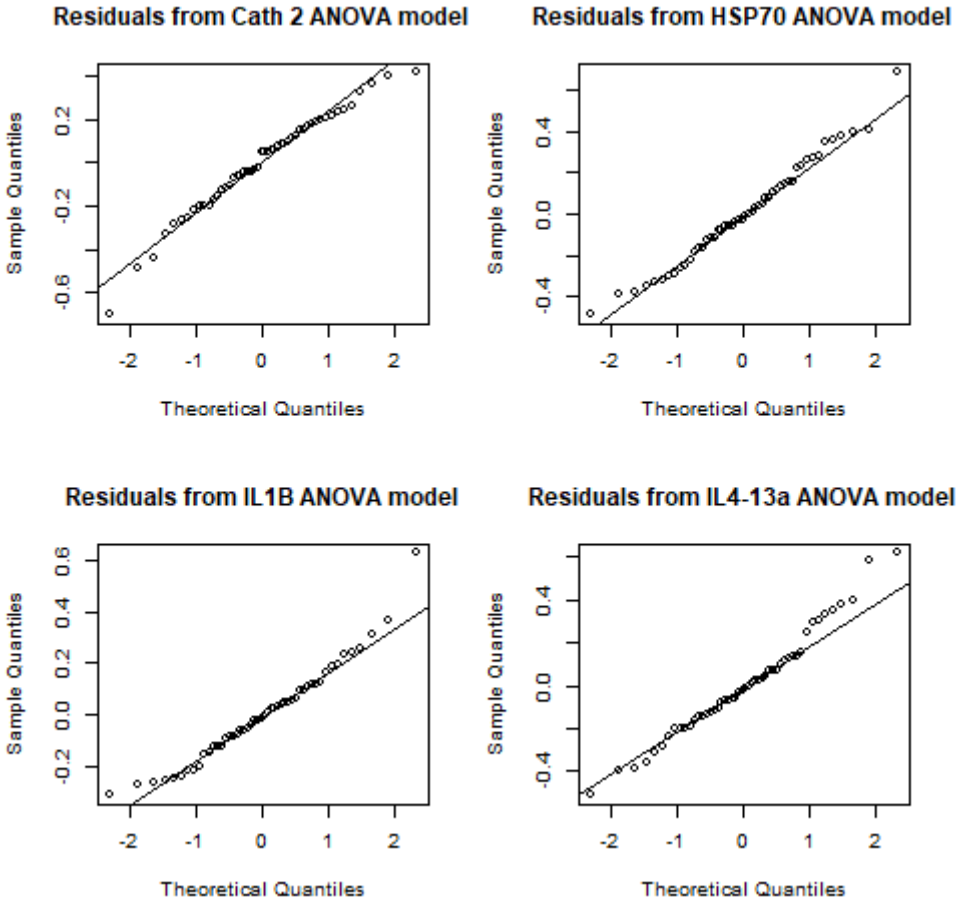

Interleukin 4-13a
Fluorescence
6.005
4.804
3.603
2.402
1.201
0.000
0.000
4.00
8.00
12.00
16.00
20.00
24.00
28.00
32.00
36.00
40.00
44.00
Cycle
-dF/dT
0.020
0.016
0.012
0.008
0.004
0.000
66.00
69.00
72.00
75.00
78.00
81.00
84.00
87.00
90.00
93.00
96.00
Temperature
51 °C
52.9 °C
55.3 °C
57.9 °C
60.7 °C
63.4 °C
67.9 °C
70 °C

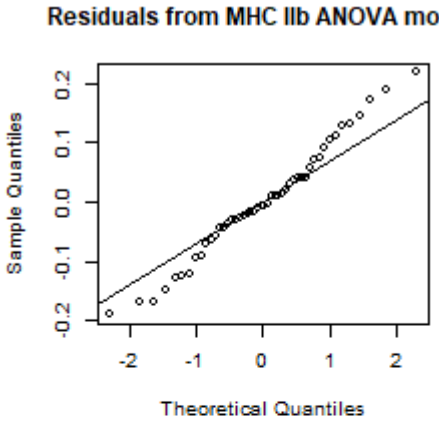

MHC II β
Fluorescence
6.005
4.804
3.603
2.402
1.201
0.000
0.000
0.00
3.00
6.00
9.00
12.00
15.00
18.00
21.00
24.00
27.00
30.00
33.00
36.00
39.00
Cycle
-dF/dT
0.030
0.020
0.010
0.000
66.00
69.00
72.00
75.00
78.00
81.00
84.00
87.00
90.00
93.00
96.00
Temperature
51 °C
52.9 °C
55.3 °C
57.9 °C
60.7 °C
63.4 °C
67.9 °C
70 °C

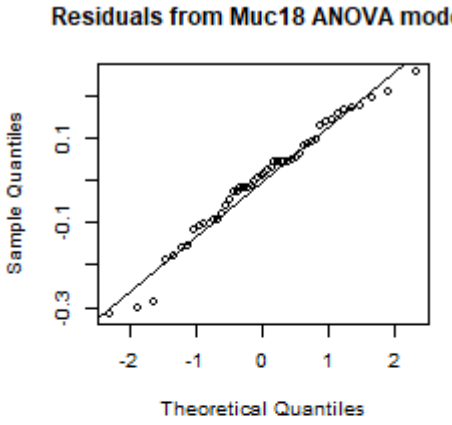

Tumor Necrosis Factor-α3
Fluorescence
6.005
4.804
3.603
2.402
1.201
0.000
0.000
4.00
8.00
12.00
16.00
20.00
24.00
28.00
32.00
36.00
40.00
44.00
Cycle
51 °C
52.9 °C
55.3 °C
57.9 °C
60.7 °C
63.4 °C
67.9 °C
70 °C
-dF/dT
0.020
0.016
0.012
0.008
0.004
0.000
66.00
69.00
72.00
75.00
78.00
81.00
84.00
87.00
90.00
93.00
Temperature

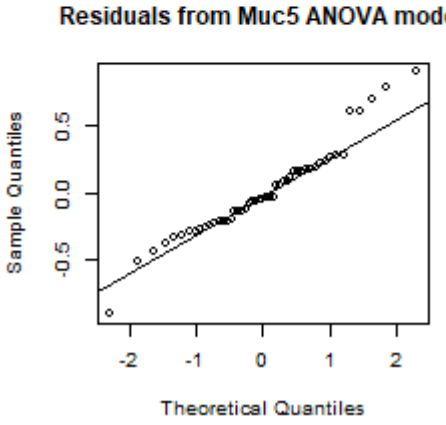
Appendix 3: Plot of residuals from ANOVA model for all genes.

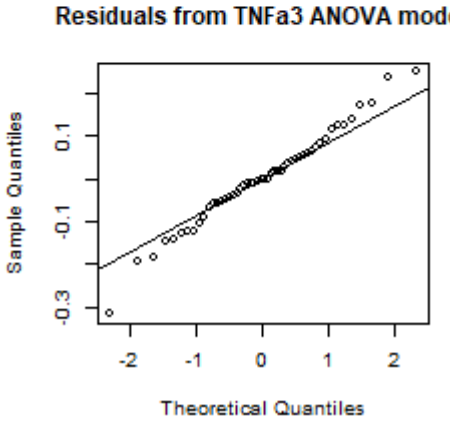



Residuals from Cath 2 ANOVA model
Sample Quantiles
Theoretical Quantiles
Residuals from HSP70 ANOVA model
Sample Quantiles
Theoretical Quantiles
Residuals from IL1B ANOVA model
Sample Quantiles
Theoretical Quantiles
Residuals from IL4-13a ANOVA model
Sample Quantiles
Theoretical Quantiles


Residuals from MHC IIb ANOVA model
Sample Quantiles
-0.2
-0.1
0.0
0.1
0.2
-2
-1
0
1
2
Theoretical Quantiles


Residuals from Muc18 ANOVA model
Sample Quantiles
-0.3
-0.1
0.1
-2
-1
0
1
2
Theoretical Quantiles


Residuals from Muc5 ANOVA model
Sample Quantiles
-0.5
0.0
0.5
-2
-1
0
1
2
Theoretical Quantiles


Residuals from TNFa3 ANOVA model
Sample Quantiles
-0.3
-0.1
0.1
-2
-1
0
1
2
Theoretical Quantiles

Appendix 4: Template plate set-up used for RT-qPCR runs of all genes

U1	U2	U3	U4	U5	U6	U7	U8	U9	U10	U11	U12
U13	U14	U15	U16	U17	U18	U19	U20	U21	U22	U23	U24
U25	U26	U27	U28	U29	U30	None	None	None	None	None	None
I1	I2	I3	I4	I5	I6	I7	I8	I9	I10	I11	I12
I13	I14	I15	I16	I17	I18	I19	I20	I21	I22	I23	I24
I25	I26	I27	I28	I29	I30	None	None	None	None	None	None
No RT control	Cal1	Cal2	Cal3	None	None	None	None	None	None	None	None
No RNA control	None	None	None	None	None	None	None	None	None	None	None

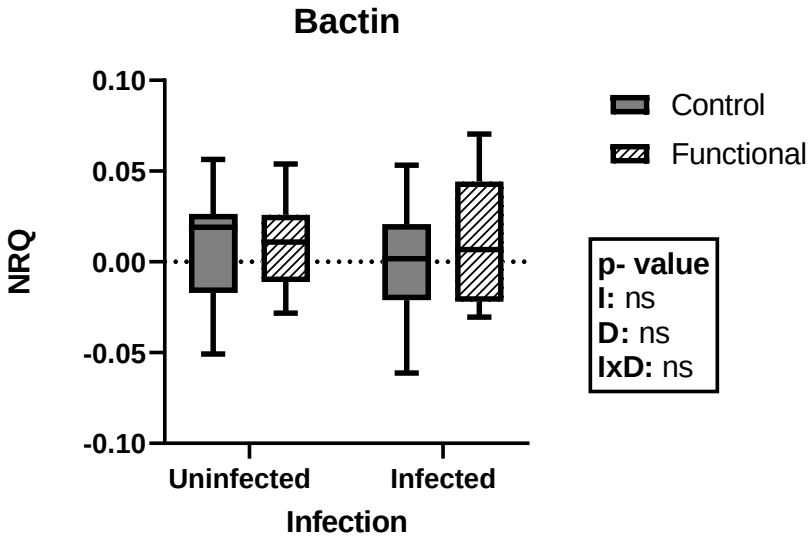
U: Uninfected; I: Infected

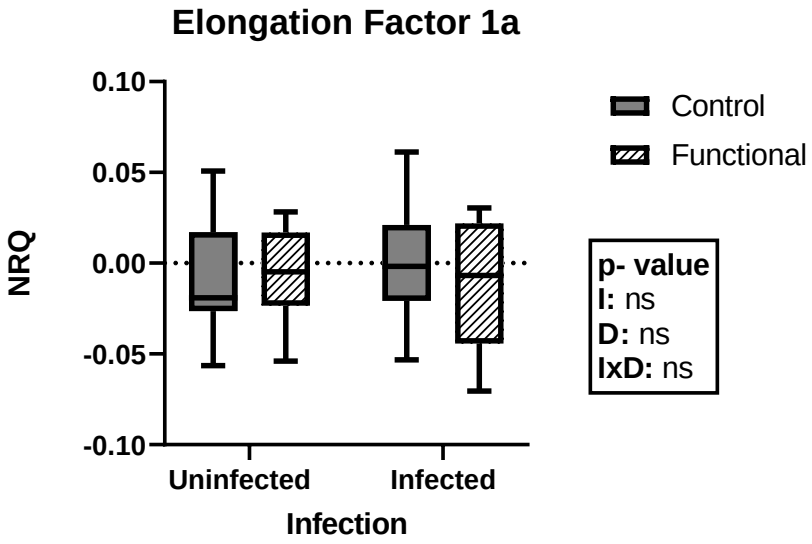
Appendix 5: Summary of mean NRQ ± SE of all analysed genes and the corresponding results for the Two-way ANOVA test and Levene's test (LV) for homogeneity of variances. Statistically significant values (p-value < 0.05) are identified with the asterisk.

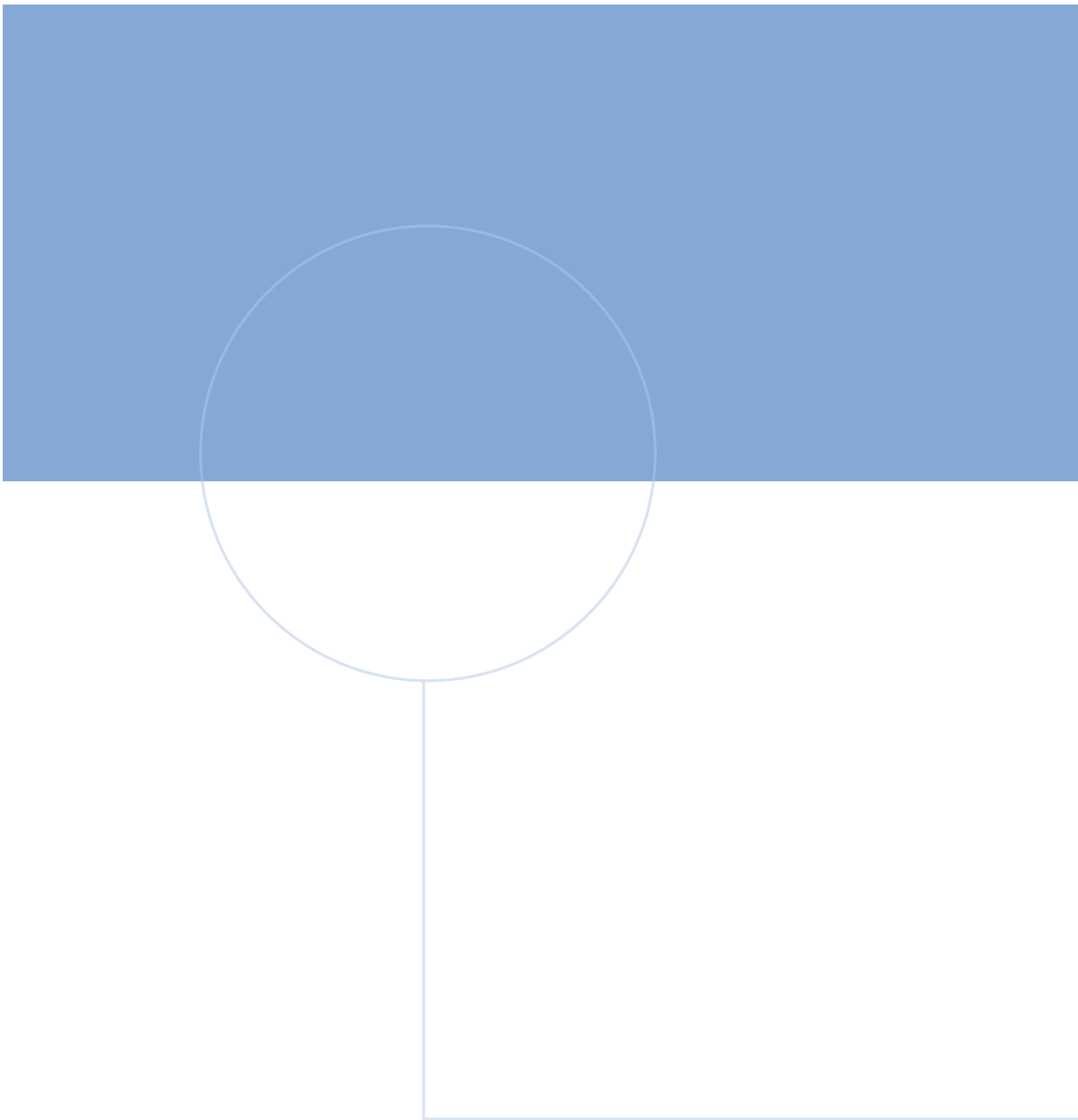
Genes	Uninfected		Infected		Statistics			
	Control	Functional	Control	Functional	Infection	Diet	D x I	LV
Cath2	-0.09±0.06	0.03±0.08	0.40±0.07	0.24±0.06	<0.001*	NS	<0.05*	NS
IL4-13a	0.01±0.07	-0.10±0.05	-0.34±0.10	-0.33±0.06	<0.001 *	NS	NS	NS
Tnfa3	-0.00±0.02	0.00±0.03	0.10±0.05	0.10±0.04	<0.01 *	NS	NS	NS
Muc18	0.02±0.03	-0.10±0.05	0.09±0.03	0.00±0.03	<0.05*	0.01*	NS	NS
Muc5	0.03±0.07	-0.03±0.10	0.09±0.13	-0.11±0.10	NS	NS	NS	NS
Bdef3	0.13±0.09	0.07±0.06	-0.05±0.07	-0.09±0.11	NS	NS	NS	NS
Bdef4	-0.00±0.03	0.00±0.03	0.02±0.05	-0.04±0.04	NS	NS	NS	NS
MHC IIB	0.02±0.02	0.00±0.02	0.00±0.02	-0.00±0.04	NS	NS	NS	NS
HSP70	0.01±0.07	-0.05±0.04	0.05±0.09	-0.10±0.10	NS	NS	NS	NS
IL1-B	0.03±0.05	0.02±0.04	-0.06±0.05	-0.00±0.08	NS	NS	NS	NS

D x I: interaction factor between diet and infection; LV: Levene’s test.

Appendix 6: Boxplot of the two housekeeping genes with the corresponding p-value result obtained in the Two-way ANOVA. Data are normalised relative quantities ± SD. ns: non-significant (p-value ≥ 0.05).







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Science and Technology