Norges miljø- og biovitenskapelige universitet

Master's Thesis 2018 60 ECTS

Department of Animal and Aquacultural Sciences (IHA)

Modulation of selected inflammatory responses and non-specific defenses in Atlantic salmon induced by use of beta-1, 3 / 1, 6-glucans (Macrogard®).

Ana Carolina Sulén Távara

Master of Science in Aquaculture

Faculty of Biosciences

Modulation of selected inflammatory responses and non-specific defenses in Atlantic salmon induced by use of beta-1, 3 / 1, 6-glucans (Macrogard®).

Master's Thesis in aquaculture
(60 credits)

By
Ana Carolina Sulén Távara

Supervisors

Dr. Trond Storebakken
Dr. Paul Midtlyng
Dr. Hetron Mweemba Munang´andu

Department of Animal and Aquaculture Sciences
Norwegian University of Life Sciences (NMBU)
Post Box 5003
1432 Ås

June 2018

ACKNOWLEDGEMENTS

The submission of this master thesis represents the end of my journey in the program Master of Science in Aquaculture at the Norwegian University of Life Sciences.

First, I want to sincerely thank my main supervisor Professor Dr. Trond Storebakken for helping me understand the importance of a well-designed salmon diet to achieve not only growth thus general welfare in fish and for the guidance during the process. I really appreciated his assistance and enthusiasm.

I am grateful for my co-supervisors Professors Dr. Midtlyng & Dr. Mweemba Munang´andu for being a key part of my study, with their experience and knowledge was possible to target an interesting aspect of the diet consequences and benefits in the salmon immune system, your constant willingness to help and advice has been of great value. I fear that without them this thesis would be a far more boring reading material.

I would like to express my gratitude to Prof. Erik Ropstad for allowing me to complete the Master Program at NMBU, while being employed in the Section of Experimental Biomedicine, School of Veterinary Medicine. Big thanks to Prof. Henning Sørum from the Microbiology department for his kind and patient explanations.

Dr. Saurabh Dubey for his amazing help during the whole process, I really owe you.

Thanks to the Norwegian University of Life Sciences for the opportunity to study in Ås and to improve my knowledge. Especially I want to thank our study advisor from the IHA department Stine Telneset for helping me with every relevant question regarding my study program. I am thankful to the Aquamedic A.S. for giving me the opportunity to carry out the fish trials in cooperation with them and to let me be a part of the study team.

To all my friends in Norway. Thank you all for motivating me and for having amazing moments together. I´m happy to have such good friends like Lucía, Kim, Franzis & Bjørn R.

I would like to thank my entire family who are always there for me and support me in everything I try to do. A special thanks to my husband Ragnar Løndal for enduring these last months, your words made me stronger in the difficult moments and I hope to make it up to you! Luis Iturrino and Gloria Tavara, my parents for always believing in me and giving me their blessings, you showed me the pathway, and thank you both for bring Luis Carlos in our life.

Everything good has to come to an end and I am grateful and blessed for every friend I made during my Master study in Norway.

Abstract

The anadromous nature and long production cycle of Atlantic salmon (*Salmo salar* L) demands for vaccination regimes to maintain long-term protective immunity in vaccinated fish. While prime vaccination is largely based on injectable vaccines, that in order to produce sufficient protection need to be formulated with potent adjuvants, these vaccines also produce undesirable side-effects in the form of inflammatory processes at the site of injection. In many species, immune responses can be modulated by beta-glucans, and this practice have gained prominence also because these subtances prove to be potential adjuvants for oral vaccines. In scientific reports from mammalian studies, beta-glucans have also been reported to modulate inflammatory processes. Hence, the main objective of the this study was to investigate the ability of (1,3)(1,6)-beta-D-glucans (BDG) to enhance non-specific immune mechanisms, and to modulate inflammatory reactions to vaccination in Atlantic salmon (*Salmo salar* L). Data generated in this study shows that mucus and serum from vaccinated fish fed with beta-glucan diet had antibacterial properties over serum from control fed fish, based on the ability to inhibit the propagation *Micrococcus luteus*, *Citrobacter freundii* and *Yersinia ruckeri* at different temperature *in vitro*. As for bacteria neutralization assays, serum lysozyme showed significant inhibition of *M. luteus* during the early post vaccination period that gradually declined to the same level as the control group as the post vaccination period increased further. On the contrary, mucus ldid not inhibit *M. luteus* growth. The kinetic of immune and inflammatory gene expression showed an inverse relationshion between the beta-glucan fed fish and control group in that there was a significant upregulation of genes such as TNFa-3, IL-6 and IFNγ in the early timepoints soon after vaccination in the beta-glucan fed group unlike the control group that had insignificant expression of these genes. Overall, this study shows that the β-1,3/1,6 glucan administered in the current study (Macrogard®) is a potent immunostimulant able to enhance the innate immune responses in vaccinated fish. As for modulatory effects on inflammation, the observed differenes yielded promising observations, suggesting that further *in vivo* studies should be carried out. Future studies should seek to determine the optimal dose and duration of exposure able to produce highest protection in vaccinated fish leaving both animal welfare and economical benefits to the Norwegian aquaculture sector.

CONTENTS

KNOWLEDGEMENT **1**

ABSTRACT **4**

ACRONYMS **7**

1.0 INTRODUCTION **8**

1.1 GLOBAL IMPORTANCE OF AQUACULTURE 8

1.2 ATLANTIC SALMON (*SALMO SALAR* L) FARMING 8

1.2.1 Production cycle *9*

1.2.2 Breeding and genetic selection of Atlantic salmon in Norway *14*

1.2.3 Feed ingredients and feed composition *15*

1.3 DISEASE CHALLENGES IN SALMON FARMING 17

1.3.1 Main diseases in salmon aquaculture in Norway *17*

1.3.3 Viral diseases associated with inflammation *19*

1.3.4 Fish mortality traduced as biological and economical losses *20*

1.4 BETA -GLUCAN 22

1.4.1 Sources and chemical structure of beta-glucans *23*

1.4.2 Beta-glucans as immunostimulants in fish *24*

1.4.3 Use of beta-glucans in fish feeds *27*

1.5 INFLAMMATORY PROCESSES AND THE IMMUNE SYSTEM 27

1.5.1 A brief overview of the innate immune system *28*

1.5.2 The adaptive immune system in brief *28*

1.5.3 Mucus as an immunological factor in fish *29*

2.0 OBJECTIVE **31**

2.1 SUBGOALS 31

3.0 MATERIAL & METHODS **32**

3.1 EXPERIMENTAL FISH AND REARING CONDITIONS 32

3.2 PREPARATION OF EXPERIMENTAL AND CONTROL FEED, AND FEEDING REGIME 32

3.3 ALLOCATION TO EXPERIMENTAL TANKS, AND VACCINATION 33

3.4 SAMPLE COLLECTION AND CONSERVATION 34

3.5 *IN VITRO* ANTIMICROBIAL EFFECT OF PLASMA AND MUCUS 35

3.5.1 Lysozyme assay *35*

3.5.2 Agar plate bacterial neutralisation assays *35*

3.5 GENE EXPRESSION ANALYSIS USING REAL TIME POLYMERASE CHAIN REACTION (RT-PCR) 36

3.7 STATISTICAL ANALYSIS 36

4.0 RESULTS **37**
4.1 REARING CONDITIONS, GROWTH AND SURVIVAL 37
4.2 *IN VITRO* INHIBITION OF BACTERIAL GROWTH 38
4.3 LYSOZYME ASSAY 41
4.5 GENE EXPRESSION 43
4.5.1 Expression in head kidney *43*
4.5.2 Expression in spleen *45*
4.5.3 Expression in distal intestine *46*
5.0 DISCUSSION **48**
5.2 LYSOZYME ACTIVITY 48
5.3 GENE EXPRESSION 49
5.5 THE ROLE OF ORAL BETA-GLUCAN IN AQUACULTURE 51
6.0 CONCLUSIONS AND FUTURE PERSPECTIVES **52**
REFERENCES: 53
8.0 APPENDIX LIST **62**
APPENDIX 01: FIRST WEIGHT REGISTRATION OF THE EXPERIMENTAL FISH 62
APPENDIX 02: FIRST WEIGHT REGISTRATION OF THE EXPERIMENTAL FISH (CONTINUATION) 63
APPENDIX 03: EXPERIMENTAL FISH WEIGHT FROM EACH SAMPLING DATE 64
APPENDIX 04: PROTOCOL FOR RNA EXTRACTION 65
APPENDIX 05: RNA CONCENTRATION USED FOR THE STUDY 66
APPENDIX 06: VACCINE PRODUCER'S DESCRIPTION 67
APPENDIX 07: PROTOCOL 69
APPENDIX 08: LINKS 73
APPENDIX 09: LIST OF PRIMER USED FOR REAL TIME PCR 74
APPENDIX 10: COATING PROCEDURE 75

Acronyms

APCs	Antigen-presenting cell
ASRV	Atlantic salmon reovirus
BA	Antibacterial substance
Bcl-2	B-cell lymphoma 2
BDG	(1,3) (1,6) -beta-D-glucans
BKD	Bacterial kidney disease
CMS	Cardiomyopathy syndrome
cDNA	Complementary DNA
CFU	Colony-forming unit
Dpv	Days Post vaccination
ELISA	Enzyme-linked immunosorbent assay
FAO	Food and Agriculture Organisation
FCR	Feed convertio ratio
FITC	Fluorescein isothiocyanate
FOXP3	forkhead box P3
GALT	Gut-associated lymphoid tissue
GIT	Gastrointestinal tract
HSMI	Heart and skeletal muscle inflammation
IFNγ	Interferon gamma
IL-6	Interleukin-6
IL-10	Interleukin-10
I.P.	Intraperitoneal injection
iNOS	Nitric oxide synthase
LPS	Lipopolysaccharides
MOS	Mannan oligosaccharides
NVI	Norwegian Veterinary Institute
NO	Nitric Oxide
OD	Optical Density
PAMP	Pathogen-associated molecular pattern
PRV	piscine orthoreovirus
NSFA	Norwegian Food Safety Authority
QTL	Quantitative Trait Locus
PRRs	Pattern recognition receptors
RAS	Recirculating aquaculture systems
RPS	Relative Percentage Survival
ROS	Reactive Oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
TAN	Total ammonia nitrogen
TGFβ	Transforming growth factor beta
TNFα	Tumor necrosis factor alpha

1.0 Introduction

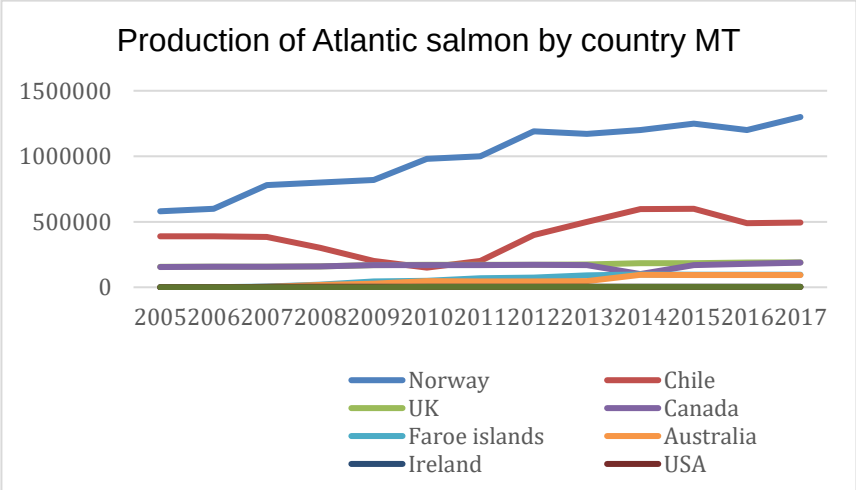
1.1 Global importance of Aquaculture

Production of farmed fish is one of the fastest growing food industries globally and it is rapidly increasing in both value and production rate [1, 2]. Global statistics predictions by the Food and Agriculture Organization (FAO) of the United Nations (UN) projected aquaculture production capacity of 73.8 million tonnes in 2014, with a total value of USD 160.2 billion [3] with the share for finfish estimated at 49.8 million tonnes at a sale value of US$ 99.2 billion. Annually, world fish aquaculture production increased by 5.8% from 2005-2014 [3]. Aquaculture accounts for almost 50% of world seafood consumption. As result of dependence on fish production as an important protein source, it is expected that aquaculture will continue to rise as the human population continues to expand [3, 4], which in turn will provide economic benefit at national, regional and household levels through increased food production and job creation [3, 5-7]. According to FAO [3], 56.6 million people were engaged in fisheries and aquaculture as a source of income and livelihood by 2014. Thus, fish farming will continue to provide increased social and economic welfare in the world through increased local employment. The rise of aquaculture has also benefited trade, aquaculture specializing techniques and diets in the developing world, with global per capita fish consumption estimated at a record 20 kilograms in 2014 [3].

1.2 Atlantic salmon (*Salmo salar* L) farming

Atlantic salmon (*Salmo salar* L) farming is one of the most successful intensive aquaculture industries in the world. Initial efforts of farming salmonids began in Norway in the 1960s. The first regulation of Norwegian aquaculture production was introduced in 1973 [8]. After a period of steady growth in the 1970s and early 1980s, the Norwegian salmonid aquaculture industry faced challenges towards the end of the 1980s [8]. A need for regulation of the industry, led to the introduction of feed-quotas in 1996. From 2005, a Maximum Allowed Biomass (MAB) system replaced the feed quota system. Currently, salmon is farmed in 16 countries worldwide, with Norway as the largest producer followed by Chile [9]. Most salmon from Norway is exported, and the EU is the primary export market with France in lead. Norway mostly exports salmon as fresh/chilled whole, which made up 75% of total export value in 2008[10]. Imports of salmon from countries outside of the EU (extra-EU trade), with Norway as a major supplier, grew substantially from 2009 to 2014 both in volumes and values with a parallel 36% rise in average prices that moved from 3.90 Euro/kg to 5.30 Euro/kg [11] (EUMOFA, 2014, 2015) The extension of the Russian import ban to December 2017 could have also led to growth of the Norwegian exports to the EU countries in 2016. However, volumes of the salmon imports to the

EU, hit by a high price increase, actually declined by 4% in 2016 compared with 2015 but, at the same time, values of the salmon imports grew remarkably by 25% and were registered at the highest amount ever. The increase of the average price of salmon imported in the EU in 2016 compared with 2015 is 27% which is from 5.22 Euro/kg to 6.62 Euro/kg (EUMOFA, 2017) [12]. The European Union market is the largest for salmon consumption in the world [13]. The nutrition value of farmed salmon has become an important factor for the success of the salmon farming industry. In order to enhance its success, well-designed feeding regimes must be used, not only giving the basic nutrition for the survival of fish but also the development of functional feeds by utilising micro-ingredient like for example 1,3/1,6-beta-D-glucans (BDG) to improve natural resistance or to support vaccination or other measures to control infectious diseases in the Atlantic salmon industry.



Source: http://www.kontali.no/?div_id=1&pag_id=1

Figure 1. Atlantic salmon production country wise

1.2.1 Production cycle

Salmonids are anadromous fish, meaning that in the wild they spawn, hatch and have the first growth phase in freshwater, before the fish eventually migrates to seawater. The process of transformation the juveniles experience before migrating is called smoltification. After 1-4 years, depending on the species, wild salmonids will return to the river where they were born to spawn [10]. With this basis, the production process of farmed salmon can be divided into four steps: Broodstock and production of fertilised eggs (embryos), start feeding and rearing of fry, production of seawater ready smolts and their transfer to marine farming sites, and seawater ongrowth plus harvest [10]. This production cycle takes place in four-farm type's/establishments; broodstock sites and incubation hatcheries, juvenile and smolt production units; marine ongrowing sites, and slaughter houses/processing plants.

Broodstock and egg production

Their main purpose is to produce eyed eggs of good quality to supply the smolt producers.

The availability of good-quality seed stock is still a constraint to the development of aquaculture [14, 15]. This type of farm is characterized for being inland and the availability of high quality water source. The operations include the selection of broodstock, using genotypic and phenotypic criteria, and tanking pedigree and inbreeding risks into account. After this selection, the fish is transported to land in wellboats and delivered to freshwater tanks for maturation. The selected group are under surveillance during few weeks, once their belly turns tender and the eggs are falling from the fish at soft pressure, and then is the moment for stripping. **Stripping/ Egg collection,** procedure takes place after anaesthesia or stunning; the fish is bled, after that the abdominal area is cleaned from mucus and blood a longitudinal incision will help to extract the eggs without damage or contamination. The fish weight and length are registered and samples are collected for further analyse. Selected males who are ready to provide sperm can survide the procedure, only exposed to the squizing technic, sperm from the male fish should be extracted early the same day, and incubated in numerated containers the main analyse is density and motility; the container will be classified and codified. Once the sperm and eggs pass the health and quality control, the next step is the **Fertilization**, eggs and sperm mixes together and immediatelly after the fecundation the eggs are rinsed with fresh water containing 9‰ of pure salt, to after be **disinfected** for 10 minutes with an iodine solution (Buffodine®) according to instructions of the producer (appendix 8) .

The fertilized eggs are sensitive to transportation especially during the third day after fertilization, as opposed to newly fertilized eyed eggs are more resilient to transport. Aquagen AS is one of the main Norwegian distributors of eyed eggs, their working routine includes the grading prior to despatch. When the eggs have been incubated for approximately 320-degree days, they exposed to a mechanical impact by a process called **shocking**. This involves the pouring of the eggs from one vessel to another from a defined height. Unfertilized and weakened eggs cannot stand this treatment, and their yolk coagulates and turns white. Following shocking and continued incubation, prior to delivery to customers, the eggs are graded to remove dead eggs and live eggs which show deviations from normal appearance and development (unfertilized eggs, micropthalmic eggs, etc) this selection was previously done manually, until now where the use of cutting edge optical technology can sort the eggs automatically by using a machine that can assess 130 000 eggs per hour (Appendix 8, AquaGen).

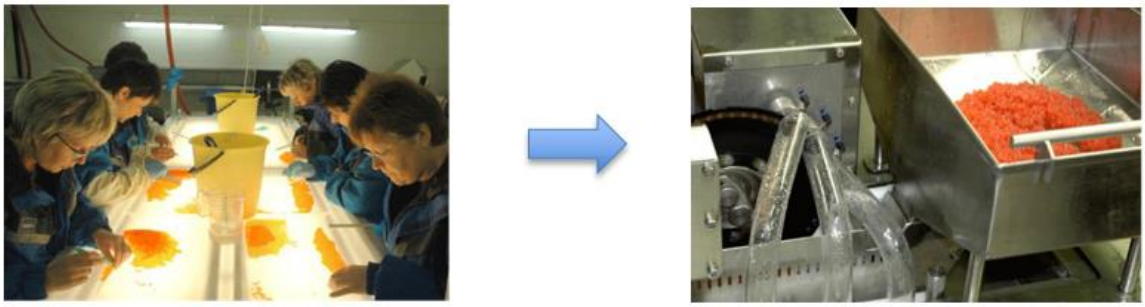


Fig 2: Selection of eyed eggs, new technology.

The fertilized eggs represent only 0,8% of the value chain for salmon production in Norway

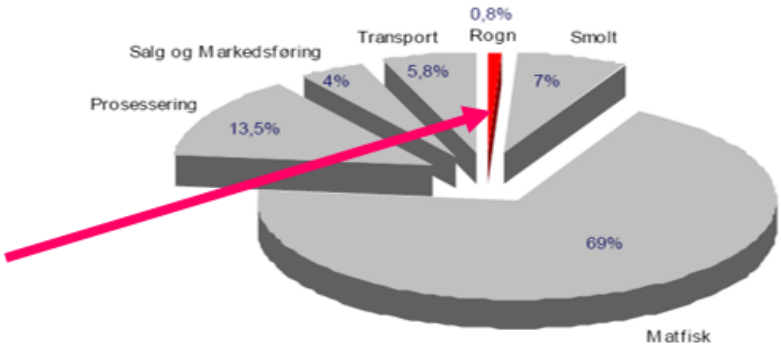


Fig.3 Elements included in the value chain for salmon production in Norway, and their economic importance. (Kontali 2006)

There are ways to manipulate the hatching time of Atlantic salmon, one is achieving early sexual maturation and stripping during the months of September and October by light and temperature regimen (Fremskynt sommer/høst). Natural light and temperature will give spawning in November and December (Naturlig light & temperature), while light and temperature can also be used to delay maturation until is last week of December until the first week of February (Delayed Summer/Autun). A second way to tailor hatching and start feeding is by manipulating the incubation temperature to targeting delivery of the eyed eggs to the hatchery in November (Early eggs), to middle of December to third week of February (Normal eggs), and march (Late eggs) or to end of April and May (Kjølet sein rogn).

The most important working operations for the eyed eggs producers are fungus control, removing dead eggs, quality grading, and maintaining water quality and water velocity control.

Fig 4: Eggs ready to be delivered to the hatchery.

Source (AquaGen)

The yolk-sack larvae are hatched after an incubation period of approximately two months, dependent of the temperature during incubation, the literature indicates that incubation temperatures should generally be in the range 4°–11°C with constant fresh water flow. The parent population should be screened to ensure that specified bacterial and viral infection are absent. As for *Renibacterium salmoninarum* (causing bacterial kidney disease), and infectious pancreatic necrosis virus (IPNV). After arrival to the hatchery, eggs are disinfected in iodine solution before being transferred to an incubator supplied with continuous water flow (0.8 L/min per liter of eggs) with water temperatures normally ranging from 5 to 7°C.

Juvenile/Smolt production

There are around 190 hatcheries and juvenile salmonid farms in Norway. The main strategy for a successful hatchery is to keep a high degree of hygiene, each procedure must be controlled avoiding contamination, the detailed labour of picking out dead eggs must be performed daily because dead eggs are nutrition for fungus growth like *Saprolegnia sp*., the dead eggs are getting covered by fungi reducing dramatically the oxygen supply for the rest of the eggs.

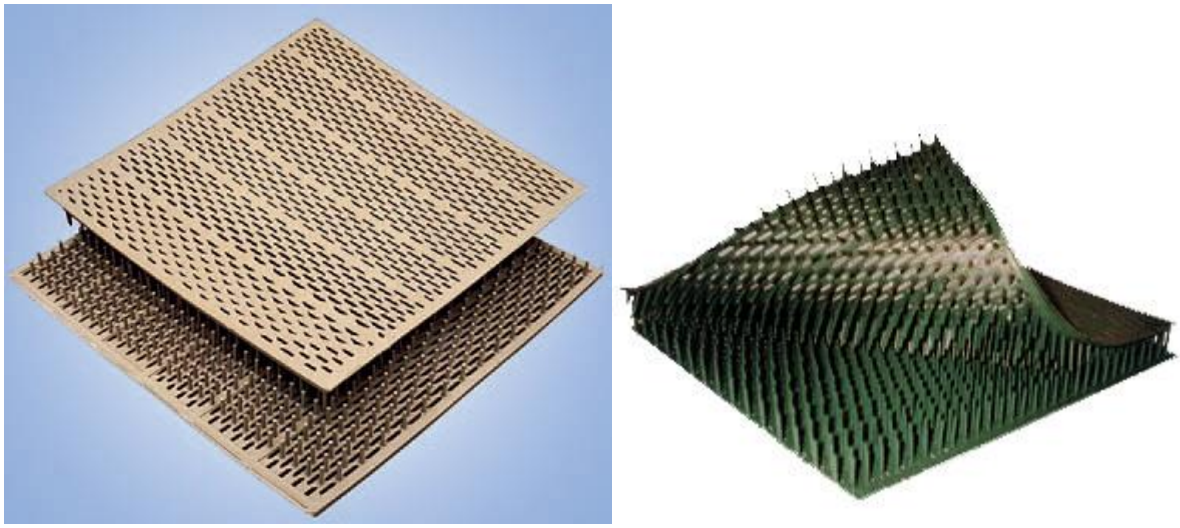


Fig. 5: Hatching substrate (Source: http://www.aquaculture-com.net/breeding.htm)

Water flow and oxygen level during incubation should be approximately 0,8l/min per liter of eggs, with a temperature of not over 8 degrees, the pH should be stable around 6 -7.

After the yolk-sack larvaes have hatched, they are referred to as **fry**. The fry feed on the contents of the yolk-sack for the first 2 months, before they start to take and digest feed. Asche & Bjørndal (2011) [13] describe this period as highly important; a faulted transformation process into using feeds have historically led to high mortality. The tanks at start feeding are small 1-4 m^2, the fry does not require a huge volume, the area used is restricted while they are lying on the bottom of the tank and the water level is normally reduced to 15 – 30 cm. A low water velocity 3-4 cm/sec is recommended, fry is not suited for faster swimming. There is an opportunity of temperature and light different regimes under start feeding, for example 24h light / temperature 8-12 ºC. The typical start feeding behaviour is burst swimming which show us that the fish is ready for the next phase. Current survival rate at hatcheries is over 70% [16].

Fig. 6: Fry in the left after 240-300 Day/degrees, and the fry of the right after 350-400Day/degrees after start feeding. (Courtesy of Bjørn Frode Eriksen Aquaculture sessions)

Before the yolk-sac completely disappears, the tiny fish must become capable of feeding themselves. When they have developed to the point where they are capable of feeding themselves, the fish are called **fry**. When, in addition, they have developed scales and working fins, the transition to a juvenile fish is complete and it is called a **fingerling**. Fingerlings are typically about the size of fingers. The fingerling stage, also known as juvenile fish "Parr" they are camouflaged with a spotty pattern and vertical bars.

When the fingerlings or parr have grown to a desirable size (60-80g), the smoltification process takes place. Smoltification is a physiological process where the fish are gradually adapted to seawater. Smoltification and seawater adaptation of Atlantic salmon are associated with profound alterations in the endocrine status, osmoregulation and behaviour. Morphological changes are

that the parr marks disappear, fish is getting silver/blank on the belly and sides and black at the back, condition factor is reduced (from1,2 -1,3 to 0,9-1,0). These changes go along with hormonal changes. [17].

There are different models for smolt production and each have different requirements
½ year smolt, often called "S+" Characterized for rapid growth achieved by recirculated warm water, smoltification induced by light manipulation. Sea transfer from August to October.
1 year, "S1" predominantly natural water temperatures, sea transfer from March-May.
2 year, "S2" No heating, slow growth (not common production today).
Salmon exposed to swimming speeds 1-2 body length per second compared to static water reached better growth, better FCR, more uniform growth rate for the total fish group, fewer aggressive interactions, reduced fin and body damages, reduced rate of oxygen consumption, more effective swimming pattern, increased aerobic capacity, recover more quickly after stress/exhaustion [18].

Seawater production of harvestable fish

After the fish has completed the smoltification process, the smolts are transferred to the grow-out sites by wellboats. The grow-out phase in seawater takes place in marine net pens. Improvements of pens, increased pen sizes and automated feeding systems have enabled the scale of each site to increase. A standard site has seen its output increase from 100 to over 5000 tonnes of fish per year from the late 1980s to today. The fish typically spend between 14-24 months in the seawater grow-out stage (Marine Harvest, 2017). When the production cycle of a given site has been completed, the site is fallowed. The length of the production process in seawater cannot be extended deliberately, as the fish will start to mature. The viral diseases mentioned below predominately affect salmon during seawater grow-out stage of the production cycle.

1.2.2 Breeding and genetic selection of Atlantic salmon in Norway

The main objective of research in selective breeding and genetics is to produce knowledge that can be used to make aquaculture more cost-effective. Since the mid-1970s this has contributed significantly to reduced production costs, giving Norwegian farmers the advantage over other international producers. Everyone oft he broodstock must pass the first filter or family selection this is checking contantly the information oft he desirable and previously measured traits. The second steep will include as first trait the growth feature for its commercial implicance and potencial to make more cost- efficient the aquaculture management. The QTL's are base on

DANN samples, this genetic markers are directly related to important, thousands of genetic markers are used to reach optimal performance of the individual (Appendix 8). The main focus of Salmon breeding companies in Norway is to improve resistance to ISA (infectious salmon anaemia) furunculosis, and IPN (infectious pancreatic necrosis), The tool is working with family-based challenge test data, markers for important quantitive traits, also the use of genomic selection methods.

1.2.3 Feed ingredients and feed composition

The digestive enzymes of salmon are active at low temperatures with a digestive physiology that is different from the herbivorous species like in carps and tilapia. Salmon are unable to digest most of the starch materials and use lipid and proteins as the main source of energy. The typical nutritional requirements for farmed salmon of different life stages are listed in Table 01. During development of commercial farming, the compositon of feeds for seawater grower salmon have changed considerably towards lipid rich, high-energy feeds, through time (Figure 07)

Table 01. Summary of dietary nutrient requirement and utilaisation of Atlantic salmon[19, 20]

Nutrients		Life stage/size class					
		Fry	Fingerling	Juvenile	Grower	Marine grower	Broodstock
Crude protein, % min		50	45–50	45	42–45	40–45	45
Amino acids, % min of dietary protein							
	Arginine	2	2	2	1.6	1.6	1.6
	Histidine	0.7	0.7	0.7	0.8	0.7	0.7
	Isoleucine	0.8	0.8	0.8	0.8	0.8	0.8
	Leucine	1.4	1.4	1.4	1.4	1.4	1.4
	Lysine	2	1.8	1.8	1.8	1.8	1.8
	Methionine	1.1	1	1	1	1	1
	Phenylalanine	1.2	1.2	1.2	1.2	1.2	1.2
	Threonine	0.8	0.8	0.8	0.8	0.8	0.8
	Tryptophan	0.2	0.2	0.2	0.2	0.2	0.2
	Valine	1.3	1.3	1.3	1.3	1.3	1.3
Crude lipid, % min		16–18	20	20	20–24	24–30	24
Essential fatty acids, % min							
	18:2n-6						
	20:4n-6						
	18:3n-3						
	20:5n-3	0.5	0.5	0.5	0.5	0.5	0.5
	22:6n-3	0.5–1.0	0.5–1.0	0.5–1.0	0.5–1.0	0.5–1.0	0.5–1.0
Carbohydrate, % max		10	10	12	12	12	12
Crude fibre, % max		2	3	3	3	3	3
Digestible energy, min kJ/g		19	19	19	20	20	19
Protein to energy ratio, mg/kJ		23–24	22–23	21–22	20–21	17–18	18
Minerals							
Macroelements (%)							
	Phosphorus, min	0.7	0.7	0.6	0.6	0.6	0.6
	Magnesium, min	0.05	0.05	0.05	0.05		0.05
	Sodium, min	0.06	0.06	0.06	0.06		0.06
	Potassium	0.7	0.7	0.7	0.7	0.7	0.7
Microelements, min mg/kg dry diet							
	Iron	60	60	60	60	60	60
	Copper	3	3	3	3	3	3
	Manganese	15	15	15	15	15	20
	Zinc	50	50	50	50	50	50
	Cobalt						
	Selenium	0.3	0.3	0.3	0.3	0.3	0.3
	Iodine	1	1	1	2		2
Vitamins, min IU/kg							
	Vitamin A	2 500	2 500	2 500	2 500	2 500	2 500
	Vitamin D	2 400	24 00	2 400	2 000	2 000	2 000
Vitamins, min mg/kg							
	Vitamin E	50–100	50–100	50–100	50–100	100	100
	Vitamin K	1	1	1	1	1	1
	Thiamine	10	10	10	10	10	10
	Riboflavin	5	5	5	5	5	5
	Pyridoxine	8	8	8	6	6	8
	Pantothenic acid	20	20	20	20	20	20
	Niacin	10	10	10	10	10	10
	Folic acid	2	2	2	2	2	3
	Vitamin B12	0.02	0.02	0.02	0.02	0.02	0.02
	Choline	800	800	1000	1000	1000	1000
	Inositol	300	300	300	300	300	300
	Biotin	0.15	0.15	0.15	0.15	0.15	0.15
	Ascorbic acid	50	50	50	50	50	50

Disease resistance in fish encompasses a variety of mechanisms including maintenance of epithelial barriers and the mucus coat; nonspecific cellular factors such as phagocytosis by macrophages and neutrophils; nonspecific humoral factors such as lysozyme, complement, and transferrin; and specific humoral and cellular immunity. Micronutrients for disease resistance include vitamins C, Bg, E, and A and the minerals iron and fluoride.

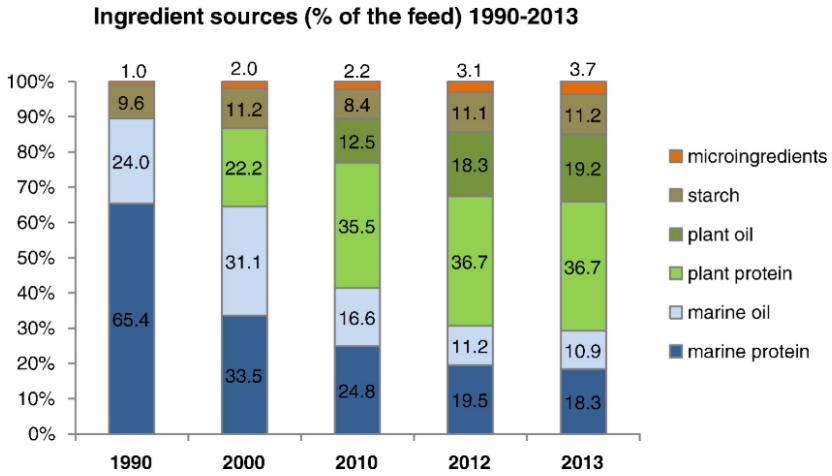


Fig. 7 Nutrient sources in Norwegian salmon farming from 1990 to 2013. Each ingredient type is shown as its percentage of the total diet [21]

Atlantic salmon feeds formulated for various stages of development and production cycle in freshwater and seawater are broadly classified as freshwater (starter, grower, smolt transfer), seawater grower and broodstock feeds. Freshwater feeds contain 45–54 percent protein and 16–24 percent lipid [21]. The protein content is decreased after salmon fry reach fingerling size. Feed manufacturers use seawater transfer feeds for salmon going through parr-smolt transformation. These diets contain salt, betaine, amino acids, nucleotides and other supplements to improve the osmotic adaptation of smolts to seawater and for better survival. Smolts are fed marine grower feeds after the seawater acclimation is complete. The protein content is reduced from 45–48 percent to 36–42 percent and lipid content increased from 24 to 30–40 percent during their seawater grow-out phase to market-size salmon (~ 4 kg) [22]. Most feeds used are highly digestible and the fines are negligible, which allows minimum impact of aquaculture feeds on the environment (FAO).

1.3 Disease challenges in salmon farming

1.3.1 Main diseases in salmon aquaculture in Norway

Intensive fish farming, enclosures made of modern cages increase the chances of many diseases to gain entry in the net pens or cages used for salmon culture. To overcome with this problem of high stocking density circumvent the increasing disease burden brought about by increased stocking densities aimed increasing the out of farmed salmon per cubic meter of cage space, which in turns exacerbates the transmission index of infectious diseases between fish. Chemotherapy, immunization and intensive treatment regimes have been designed as solution to fight these outbreaks. Although vaccination has been instrumental at reducing disease outbreak

occurrence, the continuous emergence of new diseases some of which have been difficult to find effective protective vaccines, it has become imperative that new approaches in boosting the host immune system against these diseases are devised. It is in this perspective that the use of beta-glucans may be used as an additional support to stimulate the fish innate immune system against infection. Disease prevention and control authority in Norway is held by the Norwegian Food Safety Authority (NSFA), and a number of infectious diseases (but by far not all) are listed in the legislation.

Overview of listed fish diseases in Norway

List 1 - Exotic diseases	List 2 – Non-exotic diseases	List 3 – National diseases
Epizootic haematopoietic necrosis	Viral haemorrhagic septicaemia (VHS)	Bacterial kidney disease (BKD)
	Infectious hematopoietic necrosis (IHN)	Infection with nodavirus
	Infectious Salmon Anemia (ISA)	Furunculosis ssp.
		Pancreas disease (PD)
		Systemtic infection with flavobacterium
		Salmon sea lice

Source: Norwegian Food Safety Authority (u.d).; Aukner & Haatuft, (2015)

Table 02. Diseases subject to Norwegian fish health legislation.

1.3.2 Prevalent viral diseases of Norwegian farmed salmon

In current Norwegian fish farming, the viral diseases are -besides sea lice- , the biological challenge that currently have the greatest effect on fish health [23].

Unlike the situation with the bacterial infections, vaccines developed against the viral infections of salmonids have not been able to provide effective disease control. However, zoo-sanitary strategies (mandatory rapid harvest) has been able to keep the incidence infectious salmon anaemia (ISA) very low, and recent breakthroughs in marker-assisted selection (so-called QTL based selection) has dramatically reduced the incidence of infectious pancreatic necrosis IPN (Table 03). Nevertheless, challenges remain big in the control of viral infections like Pancreas Disease (PD), heart and skeletal muscle inflammation (HSMI) and cardiomyopathy syndrome (CMS), all of which have a pathogenesis characterised by inflammatory damages involving heart and/or skeletal musculature.

Table 03: Frequency of different viral diseases in farmed salmonids 2001-2016. Adapted from Source (NVI, Oslo)

	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016
ISA	21	12	8	16	11	4	7	17	10	7	1	2	10	10	15	12
PD	15	14	22	43	45	58	98	108	75	88	89	137	99	142	137	138
HSMI				54	83	94	162	144	139	131	162	142	134	181	135	101
IPN		174	178	172	208	207	165	158	223	198	154	119	56	48	30	27
CMS				88	71	80	68	66	62	49	74	89	100	107	105	90

1.3.3 Viral diseases associated with inflammation

Cardiomyopathy syndrome (CMS)

CMS mainly affect the large size of salmon in Aquaculture. It was reported in mid 1980 in Norway [24], while It aslo been found in Canadian and Scottish farmed salmon [24]. This disease affected the farmed salmon in Norway in 2003. [25]. The disease appears withouth any sign in 2-5 kg farmed fish from 12 to 15 month after transfer to seawater, cause huge economic losses [26]. The characteristics of this disease using histopathology showes severe inflammation of the heart musculature due to mononuclear cell infiltration in the atrium and ventricle [27], that may eventually leading to tissue rupture and sudden death.

Heart and skeletal muscle inflammation (HSMI)

It also cause disease in farmed salmon in where cardiac inflammatory lesions are the main sign. HSMI is one of the actual most important fish diseases in Norwegian aquaculture in the last decade. This disease mainly affects smaller fish size ranges from 0.3 to 1 kg, at 5 to 9 months after transfer to sea water [24]. The mortality generally stays below 20%, but the morbidity estimated by histopathology are high in affected cages. HSMI shows moderate to severe myocarditis with inflammation in the ventricle. Other lessions produced are moderate to severe epi- and endocarditis. Sadly the fish affected with CMS are not able to recover, at the other side, fish with HSMI seem to have opportunity for recovering with time[24].

Pancreas disease (PD)

It is a notifiable viral disease occurring in salmon farmed at sea, caused by *Salmonid alphavirus* (SAV) [23]. Six subtypes of SAV virus have been described worldwide, although only two subtypes affect Norwegian salmon farming operations. Until 2010, SAV3 had endemic along the Southwestern coast of Norway, when marine SAV2 was diagnosed in Central Norway [23]. There are believed to exist differences in the mortality between outbreaks of SAV2 and SAV3 [23]. There are usually no external symptoms of a disease outbreak, but diseased fish will experience rapidly reduced appetite and growth. The disease first degrades and destroys the pancreas, which can lead to lower nutritional content in the harvested fish [28]. Some fish that survive outbreaks can become "loser fish", meaning that their pancreas does not recover and they are not able to utilize or absorb nutrition [9]. There are currently around 130-140 outbreaks of PD in Norway per year[29], two thirds of which are SAV3 outbreaks. Licensed vaccines against PD have on the market for many years, but the effect of the vaccination has been questioned [29]. One study has reported that vaccination against PD has a positive effect in reducing the number of outbreaks, and to decrease the mortality and fish downgrading at slaughter [30]. PD vaccines are now integrated in multi-component vaccines.

1.3.4 Fish mortality traduced as biological and economical losses

The health related losses in Norwegian salmon industry are indicator of the salmon farms disease situation and health management. Norwegian farmed Atlantic salmon is the targeted population for numerous diseases previously mentioned (Table 2). The way to quantify the health related losses by their biological effects, identify risk factors and asses the economic impact, became useful methods for further application in aquaculture health management [31]. A major part of the observed loss is caused by various diseases, representing a substantial portion of the cost of diseases in the salmon industry [32]. For population diagnostic work, the regulations require Norwegian salmon farmers to perform routine health inspections and investigate causes of death if daily mortality at the pen level exceeds 0.5% or an infectious disease is suspected [33]. The introduction of highly efficient , multivalent vaccines in the early 1990s was a major contributor to dramatically reducing the impacts from these (bacterial) disease, and led to an immediate and almost complete reduction in the use of antibiotics in the salmon industry [34, 35]. Actual salmon industry is challenged with emergent viral diseases which arising unexpectedly, and calling for immediate reaction. Infectious salmon anemia (ISA) initially detected in 1984 caused severe outbreaks in Norway in the late 1980s and early 1990s [36] but was eventually controlled by depopulation strategies. Infectious pancreatic necrosis (IPN) caused significant economical

losses in Norwegian salmon aquaculture, with it peak in 2009 having 223 cases detected, there has been a drastic reduction with only 138 cases [23] in 2016. The success in IPN control was due to the implementation of genetically resistant salmon and zoosanitary measures like removing the reservoir in fresh water facilities [23] .

Pancreas disease (PD) causes lesions in the exocrine pancreas tissue that can persist over several months and that reduce the ability to digest the feed [37] resulting in reduced feed intake, slow growth rates and increased feed convertion ratio (FCR).The mortality levels in association with PD vary from insignificant up to 60% [29]. In Norwegian salmon farms specific mortality due to PD has been estimated as 17,6% (minimum 6.5 and maximum 41,3%) [38]. Functional feeds can be used after a salmon population is infected, during the outbreak, and /or during the recovery from an outbreak. Various functional feed have been available since 2006 [30]. Functional feeds are not targeting the virus itself but are composed of ingredients that are digestible for fish with pancreatic injuries, and often include ingredients intended to enhance the immune system.The direct cost of a PD outbreak at a marine salmon farm stocked with 500 000 smolts was estimated at 14,4 million NOK [38]. This would typically occur approximately nine months after sea transfer. Simulation results [39] suggests that conducting prescheduled harvest of the salmon farm after it has been detected with PD virus and verging into an outbreak, on average provides economical benefits once the average salmon weight at the farm has reached 3,2 Kg or more. After discussed the direct costs associated with PD outbreaks in Norway, it was estimated that with 0.5 million smolts at a given site, and with the salmon prices at the time, costs of 15.6 NOKm could be identified at a farm level. Pettersen et. al. (2015)[39] completed a similar exercise, though with a farm size of 1 million smolts and updated salmon prices, and found costs equal to 55.4 NOKm on a site level. Pettersen et. al. (2015) [39] also included the preventive cost associated with functional PD-feed, which accounts for some of the increase.

Heart and skeletal muscle inflammation (HSMI)

This disease was first decribed in 1999 [40] spread a long the coast with 162 registered outbreaks in 2007 and stills causes severe economic losses to salmonid aquaculture, with 134 fish farms registered with outbreaks in 2013 and the last report shows 101 registered outbreaks in 2016 (Source NVI, Oslo) [23]. Diseases in animal productions cause direct effects (costs) as biological losses, extraordinary costs, costs of treatment, and costs of prevention as well as indirect effects (hidden costs), impaired human health, reduced animal welfare, environmental effects, effects on the market and other society effects[41]. Studies have documented the relationship between the PRV virus and the development of melanin focal changes in Atlantic salmon. In Norway,

these focal changes have increased from affecting 13% of harvested fish in 2011 to 19% in 2015, meaning that currently one in five Norwegian Atlantic salmon have one or more dark patches at harvest [23]. Walde and Alarcón (2016) reported that in 2010, the problem had been estimated to cost approximately 500 NOKm [23]. HSMI is associated with PRV, and is therefore believed to be a key reason for the development of melanin patches in the white skeletal muscle [42].

Cardiomyopathy syndrome (CMS)

CMS was first diagnosed in Norway in 1985 [23]. In the year 2011, a novel virus of the *Totiviridae* family was successfully identified from fish with CMS. This virus was named *piscine myocarditis virus* (PMCV) and is the most likely causative agent. It is estimated that CMS cost the Norwegian salmon farming industry between 33.5 NOKm to 66.3 NOKm per year, based on production, cost and price data from 1999 and 2000. CMS is a disease that typically develops into a clinical disease during the second year of seawater production. This is a chronic, non-treatable disease affecting weight gain and survival rates throughout the whole seawater phase[26]. This means that with higher salmon prices, the losses become significantly more prominent.

1.4 Beta -glucan

Historically, reports of immunomodulation using polysaccharide date far back. Beta-glucans, found in mushroom species, have been used in traditional oriental medicines for centuries especially those from mushrooms such as shiitake (lentinan), maitake and reishi [56]. In the 1940s, immunomodulatory polysaccharide began to gain consideration especially in the USA and Europe where crude mixes of polysaccharides such as Zymosan from the cell walls of *Saccharomyces cerevisiae* (baker's yeast) were found to be potent stimulators of interlukin-8 (IL-8) from neutrophils and were able to induce hyperplasia and increase functional properties of alveolar macrophages [43]. Subsequently, by 1969-1970 the active component in the both oriental medicinal mushrooms and crude polysaccharide mixes was identified as beta-glucan excerting immunostimulatory effects [44, 45]. In recent years, beta-glucan have have been shown to reduce the susceptibility of livestock to various infections and have thus contributed to reduction of the consequences of infectious diseases in aquaculture. Their inherent strength is that they enhance the ability of animals to cope with stressors such as handling, environmental changes, diet, and translocation ultimately contributing to increased survival, growth and the quality of animal production [45-48].

1.4.1 Sources and chemical structure of beta-glucans

Glucans are natural components of the cell wall matrices of various fungi, algae, bacteria and plants whose major role is to maintain the structural shape of the cell wall and rigidity. Glucans can also be found in the growth or fermentation medium of cells, making extraction and characterization a little easier [49]. They are heterogeneous, highly conserved glucose polymers that can be grouped into α and beta-glucans based on their relative stereochemistry identified by the position (axial or equatorial) of the hydroxyl (OH) group joined to carbon number 1 (C1) in the basic pyranose structure (Figure 8). Upon condensation, glycosidic bonds between α- glucose molecules result in the formation of α-glucan chains or simply α-glucans. Consequently, glucose molecules with equatorial orientation of the C1 hydroxyl (OH) groups form beta-glucans upon condensation [50].

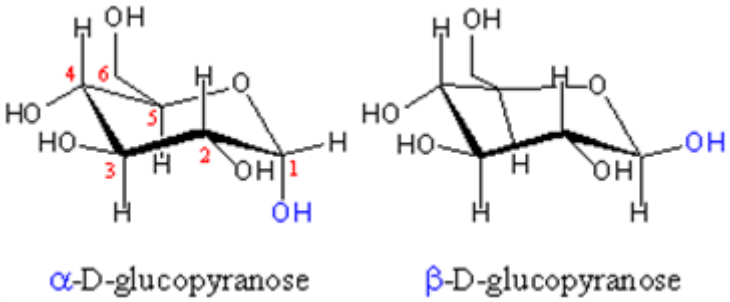


Fig.8. Glucose monomer stereoisomers

This figure shows two monomer of glucose with the same chemical formula and constitution, but with different 3-dimentional orientation of only one side chain. The difference lies in the orientation of the hydroxyl group (blue colored OH) on the first carbon atom in the ring being named α and β-D-glucopyranose, repsectively. A chain of D-glucopyranosyl units forms the main backbone og all glucan macromolecules. In beta-glucans the main chain is coiled into a triple helix and stabilised by side chains[50].

Beta-glucans comprise of a wide variety of structurally diverse molecules that are either short or long, linear or branched, soluble or insoluble in nature. The common denominator among all these molecules is that they are polymers made of repeating units of glucose, linked by β-glycosidic bonds (figure 08) [51, 52]. Although all beta-glucans are immunostimulants, they have clear differences in functional activities based on their diverse structure [44, 52]. For example, it has been shown that large molecular weight beta-glucans have stimulatory effects on leukocytes that include the induction of phagocytic, cytotoxic and antimicrobial activities [52]. The most-studied and widely used beta-glucans are large molecular weight *S. cerevisiae* and *Candida albicans* yeast-derived β-1,3/1,6-glucans. There is no consensus as to which glucan is the 'best' although generally, higher ordered (triple helix), high molecular weight (100-200kDa) beta-glucans with 1,6 linked side chains are considered among the strongest immunomodulators. [45, 53-55].

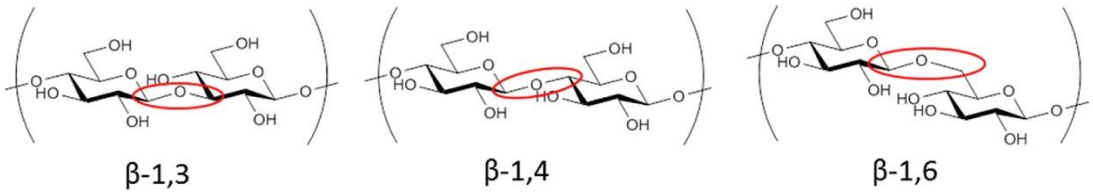


Fig. 9. Representation of the basic B glucan structure [56]

1.4.2 Beta-glucans as immunostimulants in fish

Guselle, et al., (2010) tested the efficacy of the intraperitoneal and oral administration of another β-(1,3)/(1,6)-D-glucan Product ProVale® concluding that there is an inhibition in the xenoma formation by the Microsporidian *Loma salmonae* on Rainbow Trout Gills. The most protective IP dose of commercial ProVale was 10 mg/kg when compared with the laboratory-grade IP dose of Sigma beta-glucan (4 mg/kg) [57]. Cases like the I.P. injection of the β-(1,3)/(1,6) glucan dissolved in PBS or encapsulated in nanoparticles in *Salmo salar* has proven that two days post-injection, was registered significant up-regulation in TNFα, IL-1β and IL-10 expression and encapsulated beta-glucan invoked a stronger increase in IL-1β expression [58]. Paredes, et al.,2013 experimented on Atlantic salmon macrophage function, analyzing the Immunomodulatory effect of prolactin by injecting I.P. β-1,3/1,6-glucan, he found that Macrophages show significantly increased oxidative burst, lysozyme and phagocytic activity at 10 and 20 days post injection [59].

In the case of orally administered β-glucans, the immunostimulatory effects are believed not only dependent on the branched molecular structure but may on their non-digestible nature [60]. The efficacy of β-1,3/1,6-glucan has been also tested by Kiseleva al., (2014) in *Oncorhynchus keta* (Walbaum), not only in adult fish but in a treatment of the eggs and gametes, resulting in increased embryo and juvenile survival and increased resistance against *Saprolegnia* spp. Infection. The popular model fish *Danio rerio* object of another study of β-1,3/1,6-glucan looking for disease resistance, by I.P. injection. The experiment proved increased myelomonocytic cell counts, pro-inflammatory cytokine and chemokine expression, and increased resistance against *Aeromonas hydrophila* challenge [61].

Table 04. Effects of beta-glucan on in vitro immune parameters in different fish species after oral delivery

Fish	**Stimulant**	**Results**	**RF**
Oncorhynchus mykiss	β-1,3/1,6-glucan (lentinan)	Decreased expression of pro-inflammatory genes in response to LPS	[62]
Oncorhynchus mykiss	β-1,3/1,6-glucan	Increased gene expression of cathelicidins 2 and IL-1β in gut epithelial cells	[63]
Oncorhynchus mykiss	β-1,3/1,6-glucan	Increased number of mucus secreting cells in the intestine	[63]
Oreochromis niloticus	β-1,3/1,6-glucanor or laminaran	Increased oxidative burst and neutrophil adhesion cells in IMC fish fed beta-glucanor laminaran	[64]
Epinephelus coioides	Mixture of β-1,4; β-1,3 and β-1,6-glucans	Increased lysozyme activity, alternative complement activation, phagocytic activity and oxidative burst	[65]
Sparus aurata	β-1,3/1,6-glucan (99% purity)	Increased IL-1β and IFNγ expression Increased phagocytosis and phagocytic index	[66]
Pagrus major	Heat killed lactobacillus plantarum (HKLP) in combination with commercial β-1,3/1,6 glucan	beta-glucanssignificant increase the effect of HKLP, with respect to the lysozyme activity, the bactericidal effect,	[67]
Cyprinus carpio	β-1,3/1,6-glucan	Increase oxidative burst, lysozyme activity and also incresde protection against Aeromonas hydrophila	[68]
Cyprinus carpio koi	β-1,3/1,6-glucan Chitosan or Raffinose	Increased white blood cell count (WBC) Increased oxidative burst, lysozyme activity, phagocytosis, bactericidal effect	[69]
Cyprinus carpio	β-1,3/1,6-glucan	Down-regulation of pro-inflammatory genes in gut and head kidney	[70]
Cyprinus carpio	β-1,3/1,6-glucan	No apoptosis in head kidney cells Up-regulation of several anti- and pro-apoptotic genes	[71]
Cyprinus carpio	β-1,3/1,6-glucan	Increased expression of β-defensin 1 and 2 and mucin5b in skin and β-defensin-2 in gills	[72]
Cyprinus carpio	β-1,3/1,6-glucan	Increased basal CRP levels and alternative complement activation	[73]
Cyprinus carpio	β-1,3/1,6-glucan	Reduced expression of immune-regulatory genes in the midgut (IL-1β, IL-10 and TNFα)	[74]
Cyprinus carpio	β-1,3/1,6-glucan	Increased serum complement activity and alternative complement activation	[75]

J.H.Laurisen et al., (2010) [76] conducted an study where the glucan fed trout show increased resistance to challenge with *Ichthyophthirius multifiliis* (white spot disease). Refstie et al., (2010) [77] found that there are significant lower salmon lice *(Lepeophteirus salmonis*) infestation when he proved the effects of dietary yeast cell wall beta-glucans and MOS on performance, gut health, and salmon lice resistance in Atlantic salmon (*Salmo salar*) fed sunflower and soybean meal. Also Covello, et al., [78] discover through histology that there are no adverse effects of glucan enriched feed on intestines in sea lice (*Lepeophtheirus salmonis*) burdens on Atlantic salmon (*Salmo salar*).

Among the earliest studies to be carried out in fish was the intraperitoneal injection of a β-1,3/1,6 glucan from *S. cerevisiae* (Macrogard®) in Atlantic salmon in which it enhanced resistance against different bacterial pathogens. Beta-glucans are an example of immunostimulants that have been used to enhance fish vaccine efficacy and activate shrimp hemocytes in vitro [79] Robertsen et al.(1990) showed that Macrogard enhances the resistance of Atlantic salmon presmolts to three different bacterial pathogens. Macrogard® has been also used as an adjuvant in injectable furunculosis vaccines [80]. They used intraperitoneal injections of a suspension in physiological saline at 0,2 ml per 20 g fish and obtained different dose rates by varying the concentration using 2mg per fish it was found that maximum protection developed at 3rd week [47]. When Macrogard® was used with an injectable furunculosis bacterin, glucan was found to enhance both humoral antibody formation and protection specifically. A comparative study has indicated that glucan-adjuvanted furunculosis vaccines posses a protection and side-effect profile similar to or slightly higher than aluminium-salt adjuvants [81]. The potential of beta-glucanparticles (GP) as an oral antigen delivery system and their adjuvant characteristics. GP are efficiently internalized by human intestinal epithelial cell lines (Caco-2 and HT-29 cells), without exerting negative effects on cell viability [82]. There are comparable few publications showing that oral administration of beta-glucansin salmonids can increase resistance against clinical disease. For example, beta-glucan was believed to increase the resistance of Atlantic salmon to *Lepeophtheirus salmonis* unlike in *Caligus elongates* sea lice in which treatment led to higher infestation [83, 84]. Surprisingly, there is no independent confirmation of this rather remarkable finding, given the current crisis in control of salmon lice. However, in a published Chilean study there was no positive effect of beta-glucan supplemented feed against *Caligus elongateu* sea lice infestation in [83, 84].

Non-digestible beta-glucans have been postulated to induce changes in the composition of the gut microbiota that indirectly influence the immune system [85] Studies in salmonids on the uptake of laminaran, which is a linear β-1,3-glucan absorbed via the posterior intestine resulted in systemic accumulation leading to deposition of beta-glucanin the heart and spleen [86], whereas anal intubation of FITC-labelled yeast particles showed uptake by mononuclear cells in the intestinal lumen [87]. Thus, it appears that the teleost intestine certainly is capable of absorbing beta-glucans.

1.4.3 Use of beta-glucans in fish feeds

In vivo fish studies have shown that Mcrogard®, glucan derived from *S. cerevisiae* enhance resistance against *Yersinia ruckeri*, *Vibrio anguillarum* and *Aeromonas salmonicida* in Atlantic salmon [47] Similarly, Chen and Ainsworth [88] showed that β-1,3 glucan from the cell wall of bakers' yeast reduced the level of mortality in channel catfish (*Ictalurus punctatus*) experimentally infected with *Edwardsiella ictaluri*. Early Japanese works reported that glucans derived from either *Schizophyllum commune*, *Sclerotium glucanicum* or *Lentinus edodes* would enhance resistance against *Edwardsiella tarda* infections in common carp (*Cyprinus carpio*) [89] and Duncan and Klesius [90], showed that feeding blue-green algae (*Spirulina platensis*) to channel catfish enhanced their innate immune responses although this did not improve their resistance to *Edwardsiella ictaluri* infection. Whittington et al. [91] showed that β-1,3 glucan from the cell wall of bakers' yeast enhanced the innate immune response of Nile tilapia (*Oreochromis niloticus*) and improved protection in fish vaccinated against *S. iniae* infection.

1.5 Inflammatory processes and the immune system

The immune system refers to biological processes, molecules, cell, genes, structures and pathways within an organism responsible for protecting it against invading pathogens such as viruses, bacteria, fungi and parasites. It also plays an important role in repairing and clearing of dead cells [92]. The fish immune system is in many aspects similar to other vertebrates in differentiating between self and nonself, and protecting against the latter. In general, it can be subdivided into two distinct protective mechanisms, namely the nonspecific ‘innate immunity’ and the specific ‘adaptive immunity’ [93, 94].

In fish, the innate immune response plays an important role in combating infectious diseases. Chronic and acute inflammatory responses are mediated by cellular reactions, orchestrated by chemical factors among which are cytokines (that mobilize and attract immune cells) , prostaglandins (that induces inflammatory tissue changes), nitric oxide (NO) and other reactive oxygen species (ROS), that kill invading microorganisms [95]. In vertebrates, including fish, inflammatory response to various infectious agents involves stimulation and migration of antigen presenting cells such as macrophages, monocytes, neutrophils and dendritic cells to the site of infection agent localization [96]. Chronic infections such as (HSMI) and cardiomyopathy syndrome (CMS) of salmonid are characterized by chronic inflammations. For example, HSMI characterized by epicarditis and myocarditis with mononuclear cell infiltrations [40]. The severity of the lesions in heart tissue have been shown to correlate with the intensity of the innate immune

response associated with tissue fatty acid composition [97]. There is a need for vaccines against HSMI and CMS able to prevent inflammatory reactions caused by these diseases [98].

1.5.1 A brief overview of the innate immune system

The innate immune system provides the first defence mechanisms that protect the host from pathogen infection in non-specific ways. The innate immune system recognizes pathogens through so-called pathogen associated molecular patterns (PAMPs) such as exogenous double stranded (ds) RNA, lipo-polysacharrides (LPS), peptidoglycans and β-glucans. The complement system consists of serum proteins that are central to many defense mechanisms by playing an important role in the link between the innate and adaptive immune responses in fish [99, 100]. The complement supports (complements) the activity of macrophages, monocytes and neutrophils are involved in phagocytic killing of invading pathogens that are opsonized ("marked") by antibodies. [101].

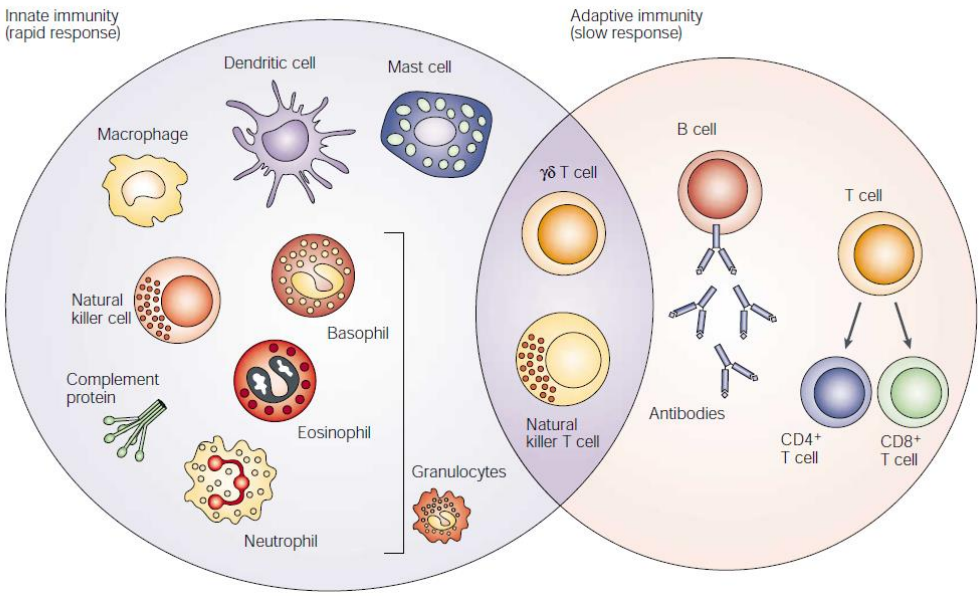


Fig. 10. The innate and adaptive response [102]

1.5.2 The adaptive immune system in brief

The acquired immune system is composed of highly specialized cells able to neutralize pathogens in systemic circulation or eliminate pathogens infected cells. It is classified into humoral and cellular immune system[103]. Humoral immunity is specialized in neutralizing pathogens in body fluids and hence the name 'humor' while the cellular mediated immune system is specialized in 'killing' and eliminating infected cells from the host [104, 105]. The PAMPs on invading pathogens are recognized by antigen presenting cells (APCs) such as macrophages, monocytes and dendritic cells. Recognition of pathogens by APCs leads to phagocytosis. Once phagocytozed, the

pathogens are processed by APCs into peptides that are presented to cells of the adaptive immune systems. This lead to activation of cells of the adaptive immune systems to produce antibodies able to neutralize or opsonise the intruder, or to directly eliminate the pathogens from the host.

1.5.3 Mucus as an immunological factor in fish

Epithelial cells form the surface lining of body tissues and their major function is to protect the inner tissues by forming a mechanical barrier on the outer surface. Their functional roles also include secretion of mucus, that is mainly composed of glycoproteins and is essential for several functions that include (i) osmotic and ionic regulation, (ii) excretion of various peptides and protection against toxins, (iii) excretion of hydrolytic enzymes, (iv) excretion of antimicrobial peptides as well as removal of microorganisms and infectious agent from epithelial surfaces. Mucus is continually produced by goblet cells in epithelial surface layers [106, 107]. In mammals, >75% of the body's immune cells are found in the epithelium especially in the gut being the largest immunological organ in the body [92]. While the outer surface epithelium of land-living mammals and birds consists of keratinized, dead cells, the surface epithelium of fish constitutes a mucosal membrane, much alike those lining the alimentary, respiratory and reproductive tracts of terrestrial vertebrates. Defense system components found in mucus include amongst others lysozyme [108], immunoglobulins [109] and complement [110] as well as proteolytic enzymes [111]. Mucosal epithelial surfaces covered by mucus in fish include the skin, gut and gills. Variable antibacterial activities in mucus have been demonstrated in different fish species [112, 113].

Table 5. Effects of beta-glucanon different bacteria infections

Fish species	Bacteria	Immunostimulant	Effect	Rf
Cyprinus carpio	*Aeromonas hydrophila*	l,6-branchcd-β-l,3-glucans	Protection	[114]
Cyprinus carpio	*Edwardsiella tarda*	l,6-branchcd-β-l,3-glucans	Increasedsurvival	[114]
Cyprinus carpio	*Aeromonas hydrophila*	MacroGard®	Protection of neutrophil extracellular traps	[115]
Cyprinus carpio	*Aeromonas hydrophila*	Betaglucan (*Saccharomyces cervisiae*)	Increase in total blood leucocyte counts, neutrophils and monocytes	[116]
Cyprinus carpio	*Aeromonas salmonicida*	beta-glucan(MacroGard®)	Increase in expression of tnfα, il1β, il6 and il10	[70]
Salvelinus fontinalis	*Aeromonas salmonicida*	β-glucan	macrophages showed increased phagocytic and bactericidal activities	[117]
Salmo salar	*Vibrio anguillarum*	M-Glucan	Resistance	[47]
Salmo salar	*Yersinia ruckeri*	M-Glucan	Resistance	[47]
Salmo salar	*Vibrio salmonicida*	M-Glucan	Resistance	[47]
Oncorhynchus mykiss	*Piscirickettsia salmonis*	MacroGard®	Increase survival	[118]
Salmo salar	*Aeromonas salmonicida*	β-1,3-M-Glucan as adjuvant	Increase in antibody level	[119]
Oncorhynchus mykiss	*Flexibacter columnaris*	β-glucan	Increase survival	[120]
Paralichthys olivaceus	*Edwardsiella tarda*	β-glucan	Increase protection	[121]
Gadus morhua	*Vibrio anguillarum*	yeast derived mannan oligosaccharide or β-Glucan	Increase in Il-1β expression	[122]

1.7 Effect of beta-glucan in viral and parasitic disease

In aquaculture, control of viral diseases remains a major challenge given that there are still several serious diseases for which protective vaccines have not been developed. To enhance vaccine efficacy, there are deliberate efforts to include immunostimulants in vaccine formulation of which beta-glucans seem to be one of the the most effective adjuvants. For example, a recent study showed that glucan-enhanced protection against viral hemorrhagic septicemia [123]. Studies on carp infected with ectoparasites showed that feed containing 0.3% of glucan increased the red blood cells, neutrohils and monocyte and decreased the lymphocytes counts that corresponded with increase in the survival rate from 77-91% [124]. Studies on the Southern bluefin tuna *Thunnus maccoyii* showed reduced parasite prevalence after feeding with glucan [125]. In rainbow trout the glucan supplementation of feed showed reduced skin-parasitic ciliate *Ichthyophthirius multifiliis* [126].

2.0 Objective

The objective of this study is to evaluate the modulatory effect of beta-glucan (β-1,3/1,6 glucan; Macrogard®) on selected immune- and inflammatory responses in Atlantic salmon (*Salmo salar* L.)

2.1 Subgoals

(a) Design and carry out a feeding trial with Macrogard® and control feed involving vaccination for induction of inflammatory and immune response in Atlantic salmon.

(b) Evaluate the *in vitro* antimicrobial properties of mucus and serum obtained from vaccinated Atlantic salmon fed beta-glucandiet (β-1,3/1,6 glucan; Macrogard®) vs. from control fed salmon, including a standardized lysozyme activity assay.

(c) Investigate the expression of selected immune-and inflammatory response genes in fish fed with beta-glucan supplemented diet (β-1,3/1,6 glucan; Macrogard®) compared to the control group receiving feed without β-glucan.

3.0 Material & methods

3.1 Experimental fish and rearing conditions

Unvaccinated (102) Atlantic salmon with an average weight of 120g (see Appendix 01 & 02) were obtained from the Norwegian University of Life Sciences (NMBU) freshwater aquarium at Ås campus, Norway. The fish were evenly split between two tanks and were left for acclimatization for a month being fed 2.2% of the biomass per day using the hatchery´s standard growth diet Nutra Olympic from Skretting. Based on rearing history, health record and visual observation showing no abnormal appearances or swimming behavior, the health status was deemed excellent. In addition, six fish from the recruitment population were euthanized for pathological examination. For the entire experimental period, the water in the recirculation aquatic system (RAS) was between 15^{o}C and 12°C and was monitored by daily checking for temperature, pH, alkalinity, TAN, and nitric oxide concentrations in the flow systems (Table 6). Figure 13 shows the layout of the tanks system used in the RAS used for experimental feeding of fish.

Individual fish weights were obtained using a precision scale (model OHAUS NV3100M; max.load:3.1kg; 0÷40°C).

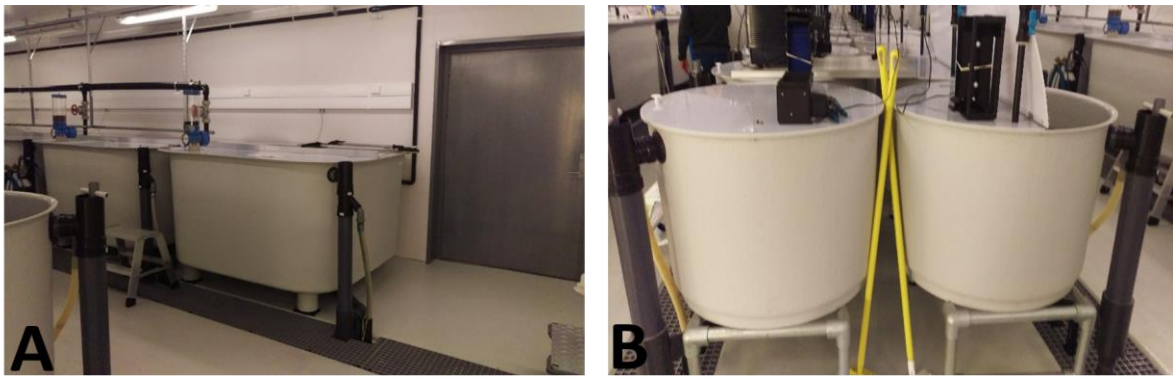


Fig. 11. Display of the tanks used in the A) before start of Macrogard feeding. & B) After start of Macrogard feeding,

3.2 Preparation of experimental and control feed, and feeding regime

Test feed with supplementation with beta- glucan and control feed was provided by Aquamedic AS, Oslo, Norway. MacroGard® (a beta 1,3/1,6 glucans produced from a strain of the yeast *Saccharomyces cerevisiae)* was used at beta-glucansource ingredient. The base diet formulation (without top coating) was Nutra Olympic 2 mm, produced by Skretting Norway

(https://www.skretting.com/). The experimental feed was top coated with Macrogard® at an inclusion rate of 0,1% (1 part per thousand), using cod liver oil (Møllers tran) as a sealant.

Top coating procedure was conducted in Forskningsparken Oslo installations following the coating protocol (Appendix 10) established by Aquamedic AS.

Control or experimental diets were fed was delivery by the automatic feeders at 1.8% of the predicted biomass per tank per day. Approximately 18 hours prior to, and on the days of random allocation or vaccination, administration of feed was temporarily suspended.

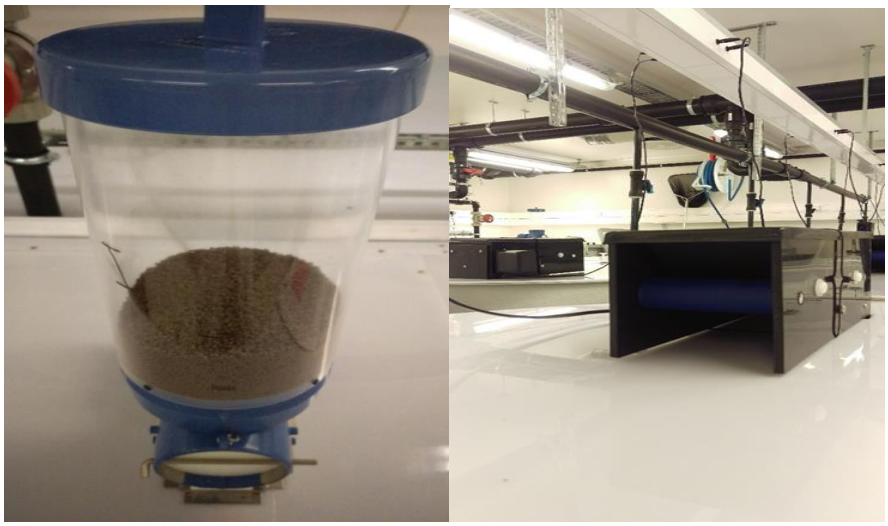


Fig. 12. Feed dispensers used during the priming period (A) and following vaccination (B).

3.3 Allocation to experimental tanks, and vaccination

After acclimation, the fish from the recruitment tanks were individually weighed under anaesthesia and sequentially allocated one-by-one to each of the four experimental tanks. (Appendix 01-03) The final fishes to be allocated were selected by size to minimize biomass differences between the four tanks. After one month of priming with either experimental or control diet, a commercially available, multivalent salmon vaccine (Aquavac® PD7 vet) was administered by intraperitoneal injection according to manufacturer's instructions (MSD, Animal Health, AN, Boxmeer, Nederland. Appendix 06). Each fish was injected with 0.1 mL of the vaccine. This and similar vaccines are known to induce mild to moderat inflammatory responses in the abdominal cavity (Midtlyng et al. 1996). Figure 13 shows the layout of the study plan and sampling time.

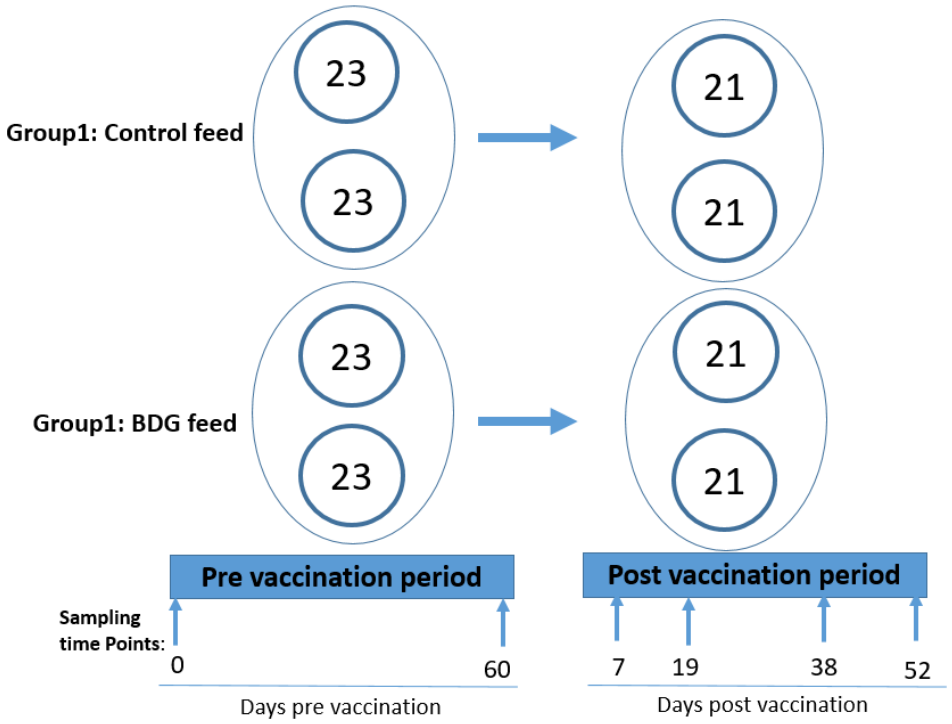


Fig. 13. The layout of the study plan and sampling time point. Control feed and beta-glucan (BGD) groups located, 92 fish sub-divided into two replicates

3.4 Sample collection and conservation

Samples were collected at five different time point namely at 77 days after onset of test or control feeding (unvaccinated fish) followed by 7, 19, 38 and 52 days post vaccination (dpv). At each sampling occasion, four fish from the each of the tanks were drawn. In order not to avoid too large biomass per tank, five fish were withdrawn per tank during the first sampling after vaccination.

For sampling, the fishes were netted one by one, stunned by a sharp blow to the head, and blood and mucus and samples were collected before opening the abdominal cavity for sampling internal organs. Blood was collected into heparinized vacutainer tubes from the caudal vein. Mucus was carefully scraped from the body surfaces using a sterile glass slide and collected into sterile 1.5 ml Eppendorf tubes. The tube having mucus were stored instantly on dry ice at the time of sample collection to avoid contamination. No mucus was collected from areas around the anal opening or the incision point for blood sampling. Tissue samples collected included the spleen, head kidney and distal intestine. Every tissue was carefully extracted, sectioned, and a portion of $0.5cm^2$ held in RNA Later in Eppendorf tubes until cover the complete tissue, every tube was labeled and immediately preserved in the cooler for after being freeze down to -80ºC until being processed for analysis. Another $0.5cm^2$ portion was also kept in formalin for further studies.

Sample processing on the site

The NMBU Fish laboratory at Ås offered the best condition for the sampling extraction and preserving process, once that the blood was extracted the tubes were centrifuged and the plasma collected, rapidly placed in a cooler box filled with dry ice, these plasma tubes transported to Oslo NMBU campus Adamstuen where frozen at -80ºC. The remaining samples where kept also in the same freezer waiting for processing.

3.5 *In vitro* antimicrobial effect of plasma and mucus

To test the inhibitory capacity of plasma and mucus collected from vaccinated and unvaccinated fish in the control and beta-glucan groups, the antimicrobial susceptibility assay was carried out against six bacterial strains namely;

- *Micrococcus luteus* **(**standardized lysozyme assay**)**
- *Escherichia coli*,
- *Klebsiella pneumoniae*,
- *Yersinia ruckeri*,
- *Salmonella enterica* subsp. *diarizonae*,
- and *Citrobacter freundii*

3.5.1 Lysozyme assay

Overnight grown *Micrococcus luteus* was put in fresh Mullar Hinton broth at a ratio of 1:10. 100 µL diluted bacterial inoculum was transferred to each well. 50 µL mucus or plasma from beta-glucan and control feed groups was added to assess lysis and inhibition of the bacterial growth. Lysozyme (Sigma, Aldrich) was used as a positive control and only Mullar Hinton broth without any bacteria was used as a negative control. The 96 well plate containing media and mucus was incubated at 37^0C. Reading of results was done after 24 hours using an ELISA reader (TECAN, USA) at wavelength (optical density -OD) of 620 nm.

3.5.2 Agar plate bacterial neutralisation assays

Concentration of over night grown bacteria in Mullar Hinton broth were adjusted to 10^6 CFU/ml for *in vitro* antibacterial susceptibility test using fish mucus. 100 µl of 10^6 CFU/ml of all the above mentioned bacteria were spread on Mullar hinton agar plate. Four wells were made in all the plates at equal distance and 50 µl of mucus and serum sampled from the control and beta-glucan fed groups as well as controls of lysozyme and antibacterial substance, respectively. All plates subjected to different treatments were incubated at three different temperature 37^0C, 30^0C and 25^0C, respectively using an ELISA plate reader (TECAN, USA).

3.5 Gene expression analysis using real time polymerase chain reaction (RT-PCR)

Every tissue collected were extracted from RNA later tubes over to individual disposable plates with aseptic suraface, to be sectioned with the scalpel, in order to prepare the sample for RNA extraction. The gene expression analyses was carried out following the method described previously by Mundang'andu et al. 2013 [127], and with kind guidance and support from Saurabh Dubey. Extraction of total RNA from head kidney, spleen and gut tissues was carried out using a modification of the Trizol® (Thermo) and RNAeasy Mini kit (Qiagen) protocols (Appendix 04). The resultant cDNA was stored at -80°C until use. The synthesis of cDNA was carried out in 20 μL reaction volumes using transcriptor cDNA synthesis kit (Roche). Primers for immune gene analyses as well as housekeeping genes β-actin were designed based on published sequences (appendix 09). Transcription levels for the target genes were quantified relative to internal housekeeping genes using the delta-delta method described by Rao et al [128] .

3.7 Statistical analysis

Most of the results were nominal and ordinal scaled and only a few were metrical scaled. The evaluation of the data was therefore done with descriptive statistics and only some parts with an analytical procedure where standard deviation and standard error was calculated. The evaluation was carried out in Excel 2016.

4.0 Results

4.1 Rearing conditions, growth and survival

Data from the trial site technical system showed that after peaking in September, the water temperature went slightly down and remained between 12^0 and 13^oC from mid-November (prior to vaccination) through the final sampling. The pH varied between 7.4-7.7 and alkalinity, TAN and nitric oxide varied between 1.2-1.4 mmol/L, <0.12 and <0.04, respectively (Table 6).

Table 6. Temperature and water quality parameters at different time points

	Water quality RAS 1					
Trial date	**Date**	**Temp**	**pH**	**Alk (mmol/l)**	**TAN mg/l)**	**NO2 (mg/l)**
NFR until 22.09.17	07.09.17	15,7	7,4	1,3	<0,05	<0,02
NV	12.10.17	14,3	7,5	1,2	0,08	0,03
NV	24.11.17	12,8	7,6	1,2	0,12	0,04
NV 01.12.17/PV1 08.12.17	18.12.17	12,5	7,5	1,3	0,07	0,03
PV2 27.12.17 /PV3 15.01.18/ PV4 29.01.18	23.01.18	12,0	7,7	1,4	<0,05	<0,02
	21.02.18	12,1	7,6	1,4	<0,05	<0,02
	20.03.18	12,8	7,5	1,3	<0,05	0,03

There were no observable signs of ill health, and no mortality during the course of the trial. The fish showed a normal growth pattern with an increase in average weight from slightly below 120 gram after priming with test or control feed to a mean of 307.94 grams (SD ± 246.58 grams) when they were random allocation and vaccinated. The weight measurements in sampled fish reached 485.19 grams (SD± 210.15 grams) in week 8 post immunisation and 471.56 grams (SD± 93.90 grams) at the final sampling, 9 post immunization (Figure 14).

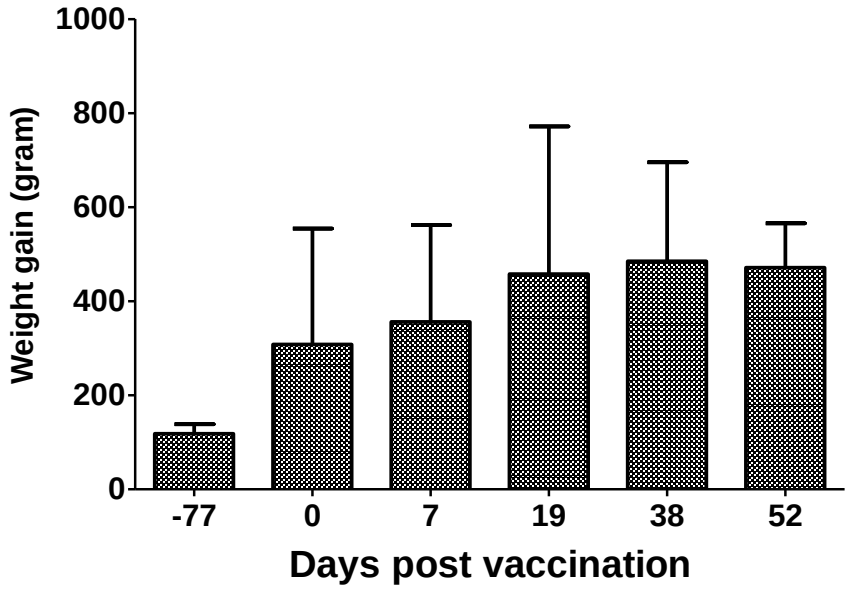


Fig. 14. Mean body weight for fish used in the experiment and of fishes selected for sampling after vaccination

4.2 *In vitro* inhibition of bacterial growth

The ability of plasma (P) or mucus (M) from fish fed beta-glucan to inhibit the growth of six different bacteria species cultured on Muller- Hinton agar plates was assessed at three different temperatures (25^0C, 30^0C and 37^0C) using a presumptive antibacterial proprietary substance (AB) and lysozyme (L) as positive controls. The growth of *M. luteus* was inhibited by both fish mucus and plasma when incubated at 25^oC, but not at higher temperatures. *C. freundii* was inhibited by fish plasma at all three temperatures tested, and growth the fish pathogen *Y. ruckeri* was inhibited when being incubated with fish mucus at 30^o and 37^oC (Figure 15 a-c; Table 7). Figures 15A-15F show the presence or absence of inhibition caused by plasma and mucus from vaccinated fish fed to beta-glucanas well as the presumptive antibacterial substance (AB) and lysozyme (L) controls.

Table 7. Inhibition (+) or absence (-) of inhibition of bacteria cultures on agar plates after 24 hours of incubation from mucus or plasma from beta-glucan fed fish.

Bacterial species	**Mucus (M)**			**Plasma (S)**			**Lysozyme (L)**			**Antibacteria (AB)**		
	25^0C	30^0C	37^0C	25^0C	30^0C	37^0C	25^0C	30^0C	37^0C	25^0C	30^0C	37^0C
Micrococcus luteus	+	-	-	+	-	-	+	+	+	+	+	+
Citrobacter freundii	-	-	-	+	+	+	-	-	+	-	-	-
Yersinia ruckeri	-	+	+	-	-	-	-	-	-	-	-	-
Klebsiella pneumonia	-	-	-	-	-	-	-	-	-	-	-	-
Escherichia coli	-	-	-	-	-	-	-	-	-	-	-	-
Salmonella enterica	-	-	-	-	-	-	-	-	-	-	-	-

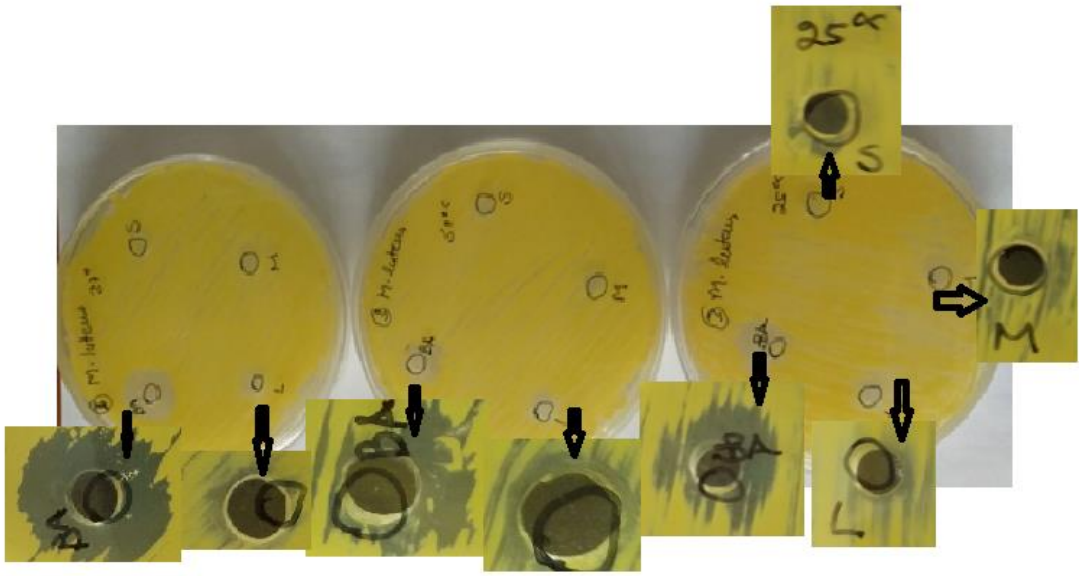


Fig. 15A. *Micrococcus luteus* bacteria cultured on Mullar Hinton agar plates 37^0C, 30^0C and 25^0C exposed to BA (antibacterial substance), L (lysozyme), M (mucus) and S (Plasma). Note that BA and L show inhibition of *M. luteus* on all three plates at all the temperatures whereas mucus and serum also show the inhibition at 25^0C.

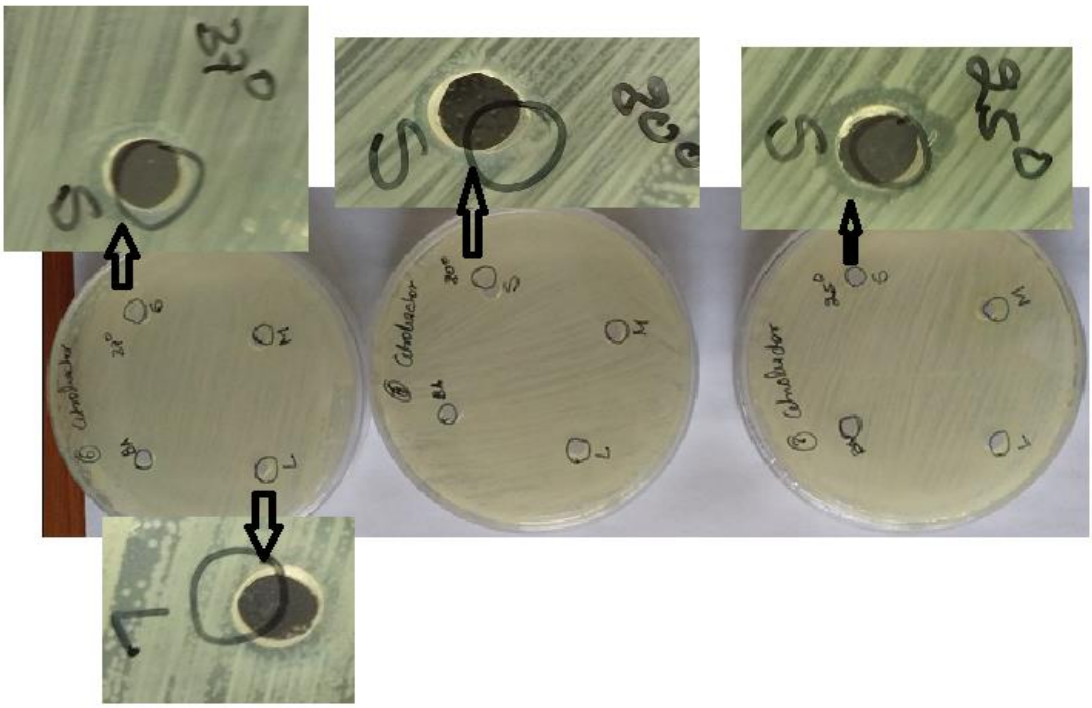


Fig. 15B. *Citrobacter freundii* grown in three Mullar Hinton agar Plate and incubated at three different temparature 37^0C, 30^0C and 25^0Cexposed to BA (antibacterial substance), L (lysozyme), M (mucus) and S (Plasma). Serum shows the inhibition on all plates at all three temperatures whereas Lysozyme only shows the inhibition at 25^0C.

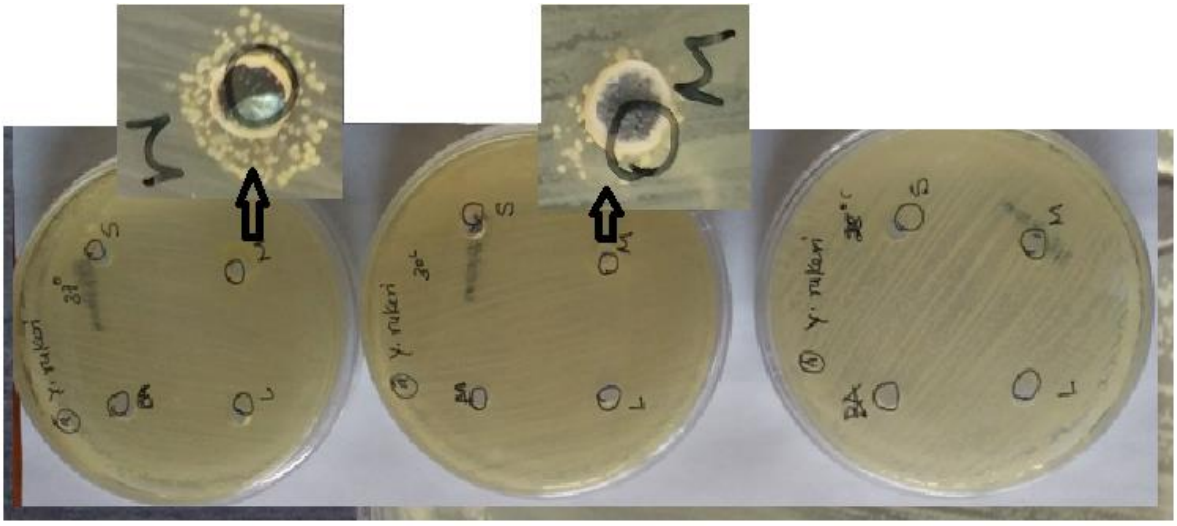


Fig. 15C. *Yersenia ruckeri* grown in three Mullar Hinton agar Plate and incubated at 37^0C, 30^0C and 25^0C exposed to BA (antibacterial substance), L (lysozyme), M (mucus) and S (Plasma). Mucus shows inhibition of *Y. ruckeri* at 37^0C and 30^0C temperature plates while there was no inhibition on the plate incubated at 25^0C.

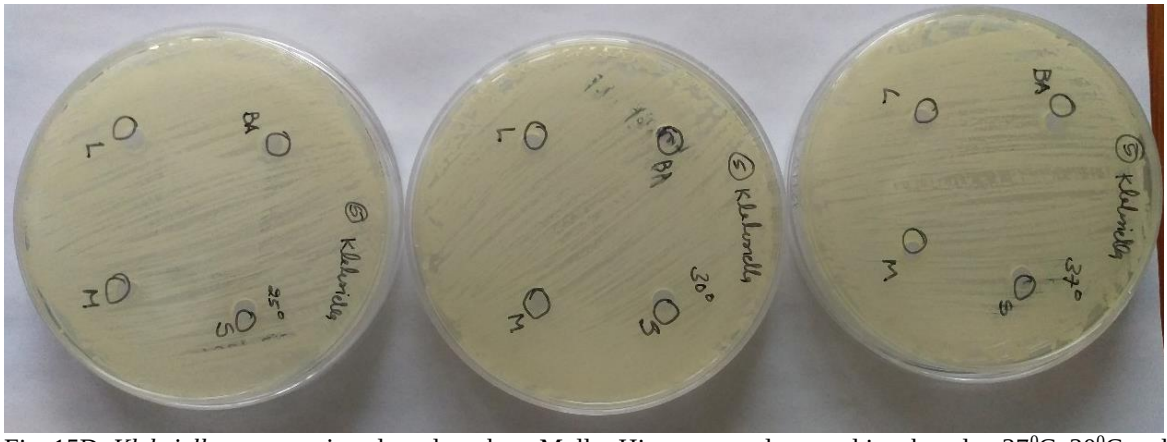


Fig. 15D. *Klebsiella pneumonia* cultured on three Mullar Hinton agar plates and incubated at 37^0C, 30^0C and 25^0C exposed to BA (antibacterial substance), L (lysozyme), M (mucus) and S (Plasma). There was no inhibition of *K. pneumonia* observed on all three plates at 37^0C, 30^0C and 25^0C.

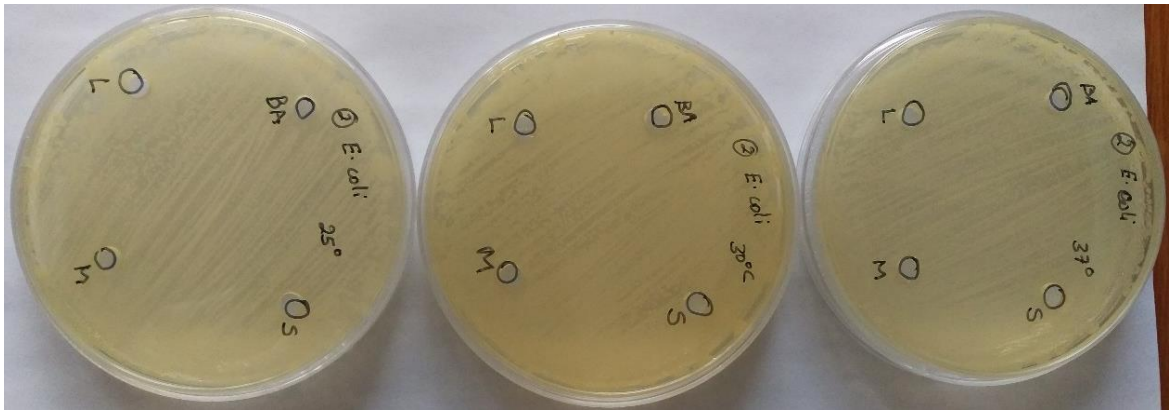


Fig. 15E. *Escherichia coli* cultured on three Mullar Hinton agar plates and incubated at three different temparatures 37^0C, 30^0C and 25^0C exposed to BA (antibacterial substance), L (lysozyme), M (mucus) and S (Plasma). No inhibition of *E. coli* observed on all three plate at different temperatures.

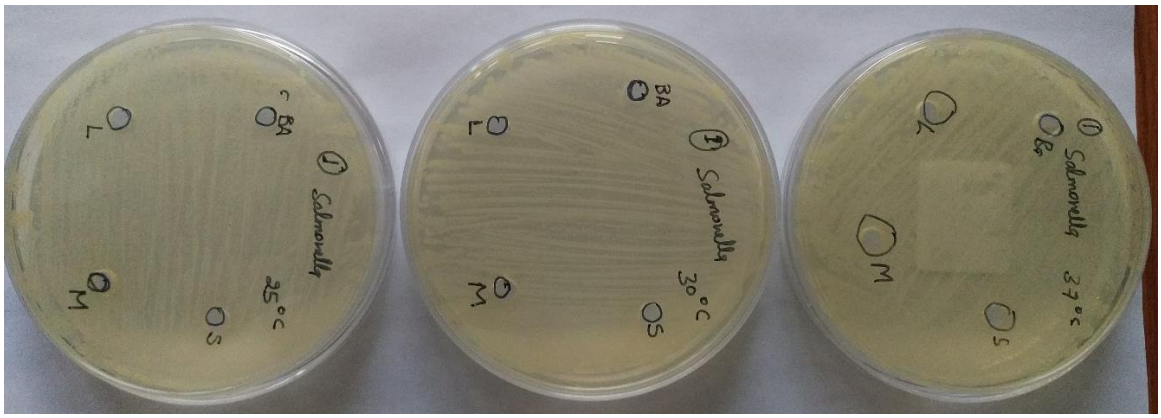


Fig. 15F. *Salmonella enterica subsp. Diarizonae* bacteria cultured on three Mullar Hinton agar Plate and incubated at 37^0C, 30^0C and 25^0C exposed to BA (antibacterial substance), L (lysozyme), M (mucus) and S (Plasma). No inhibition of *S. enterica subsp. Diarizonae* on all three plates at all three temperatures

4.3 Lysozyme assay

The results from the standardized lysozyme assay carried out using samples from (i) fish fed beta-glucan diet and, (ii) fish fed the control diet using (iii) lysozyme as positive control. Bacterial suspension alone (iv) and growth medium alone (v) were negative controls.

Only the positive (lysozyme) control showed lysis of the *M. luteus* bacterial suspension when testing plasma drawn from unvaccinated fish (0 dpv). However, 7 days after vaccination plasma from the group fed the beta-glucan diet induced a strong lysis of *M. luteus*, now clearly stronger than the effect of the lysozyme used as positive reference (Figure 16). At 19 dpv, the lytic activity of the plasma from the beta-glucan group was similar to that of the positive standard solution, but thereafter showed no lytic activity, like in the plasma from the the control group.

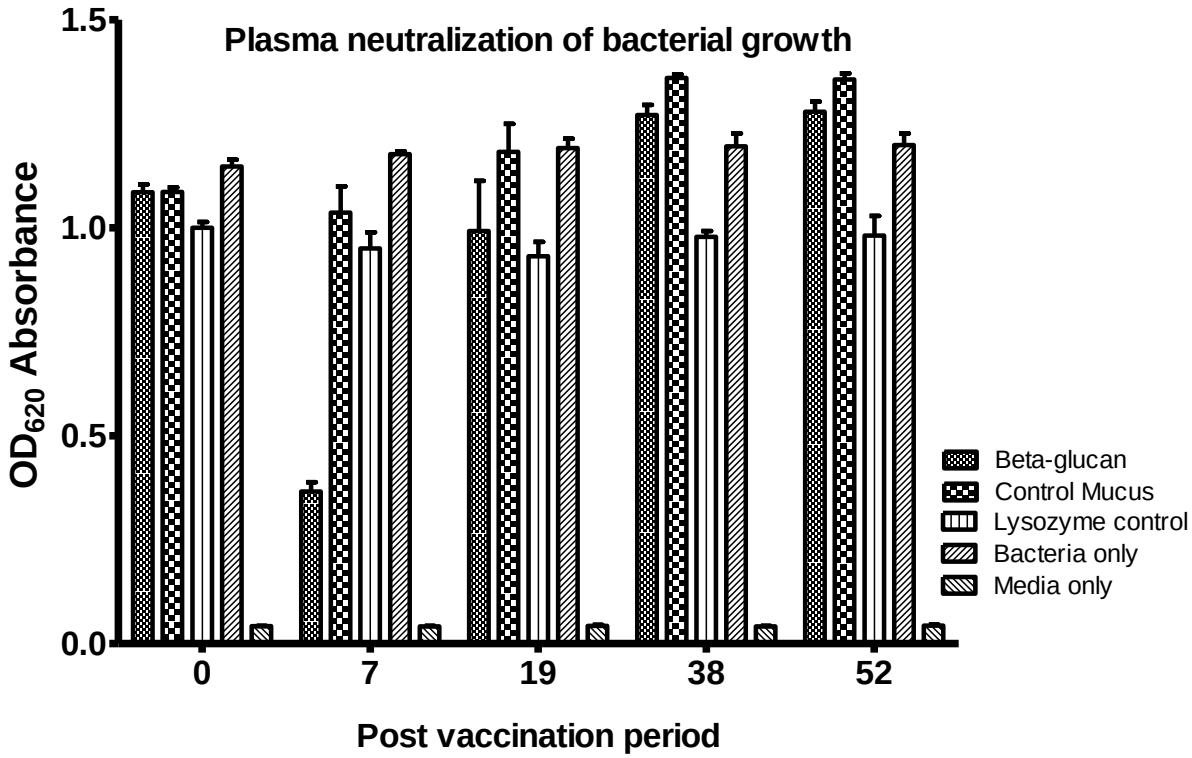


Fig. 16. Plasma neutralization of *M. luteus*. Note that there was high inhibition in the group fed beta-glucan at 7 dpv gradually reducing to the same level as the lysozyme positive control at 19 dpv. Inhibition levels in the lysozyme control group remained constant throughout the study period. No inhibition was observed in the negative control group. All bacteria cultures were read 24 hours after incubation.

Neither the beta-glucanfed nor the control groups showed any lysis of *M. luteus* growth in mucus samples collected from unvaccinated fish at 0 dpv nor in post vaccination samples collected at 7, 19, 38 and 52 dpv (Figure 17). The positive lysozyme control showed a constant level of inhibition in all assays.

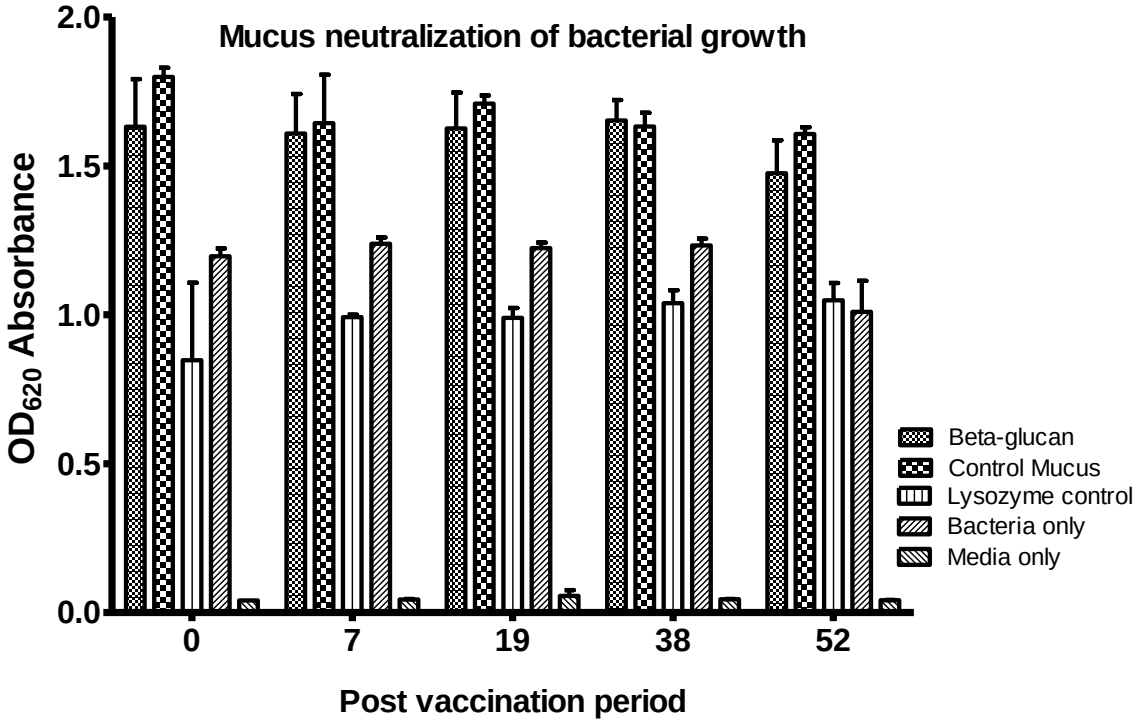


Fig. 17. Standardized lysozyme assays performed with mucus from fish fed beta-glucan diet or control diet No lytic effects could be observed, while the level of lysozyme activity was at the same in all assay setups and turbidity read in bacteria or media only wells were consistent in all setups.

4.5 Gene expression

4.5.1 Expression in head kidney

Prior to vaccination, the relative expression of all selected genes of interest was generally low. However, the samples from the control fish showed somewhat higher expression of TNFa-3 and IFN-g than samples from the group having received beta-glucan supplemented feed (Fig 18 A, B). Seven days after vaccination, however, TNFa-3, IL-1b, IL-6 and IFN-g were upregulated in the beta-glucan group as compared to the control group. At the final sampling 52 days post vaccination, the expression pattern seen prior to vaccination was re-established, with downregulation of TNFa-3 and IFN-g in the beta-glucan group compared to the controls.

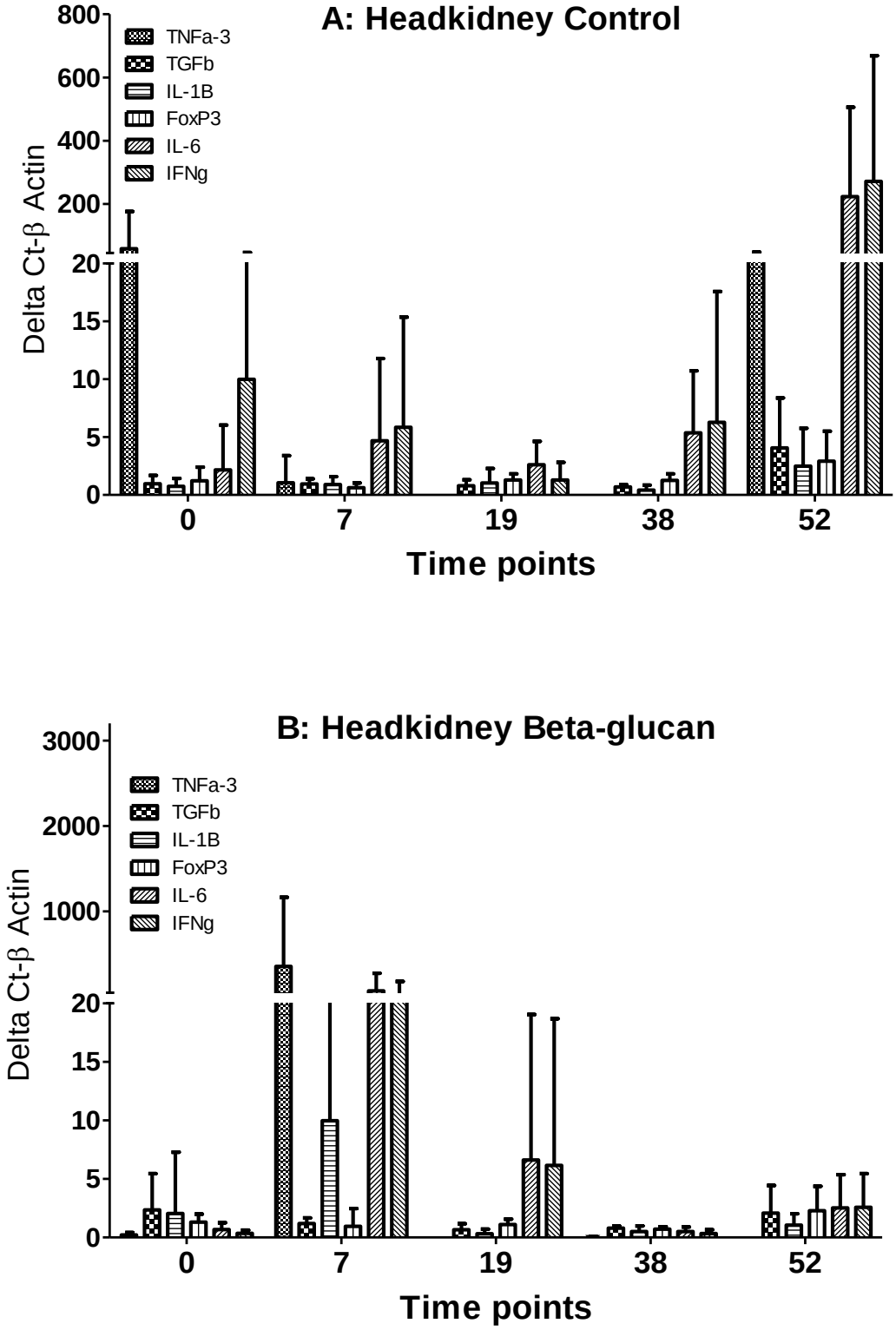


Fig. 18A, B. Expression of selected immune and inflammatory genes in head kidney samples (n=84) analysed from the beta-glucan fed and control groups, relative to expression of a housekeeping gene (beta- actin) in the same sample.

4.5.2 Expression in spleen

In the spleen, samples, the relative expression of all selected genes of interest was also generally low. However, the samples from the beta-glucan fish showed higher expression of TNFa-3 and IL-1b than the samples from the control group prior to vaccination (Fig 19 C, D). At the final sampling 52 days post vaccination, the expression pattern showed down regulation of TNFa-3, IL-6 and IFN-g in the beta-glucan group compared to the controls.

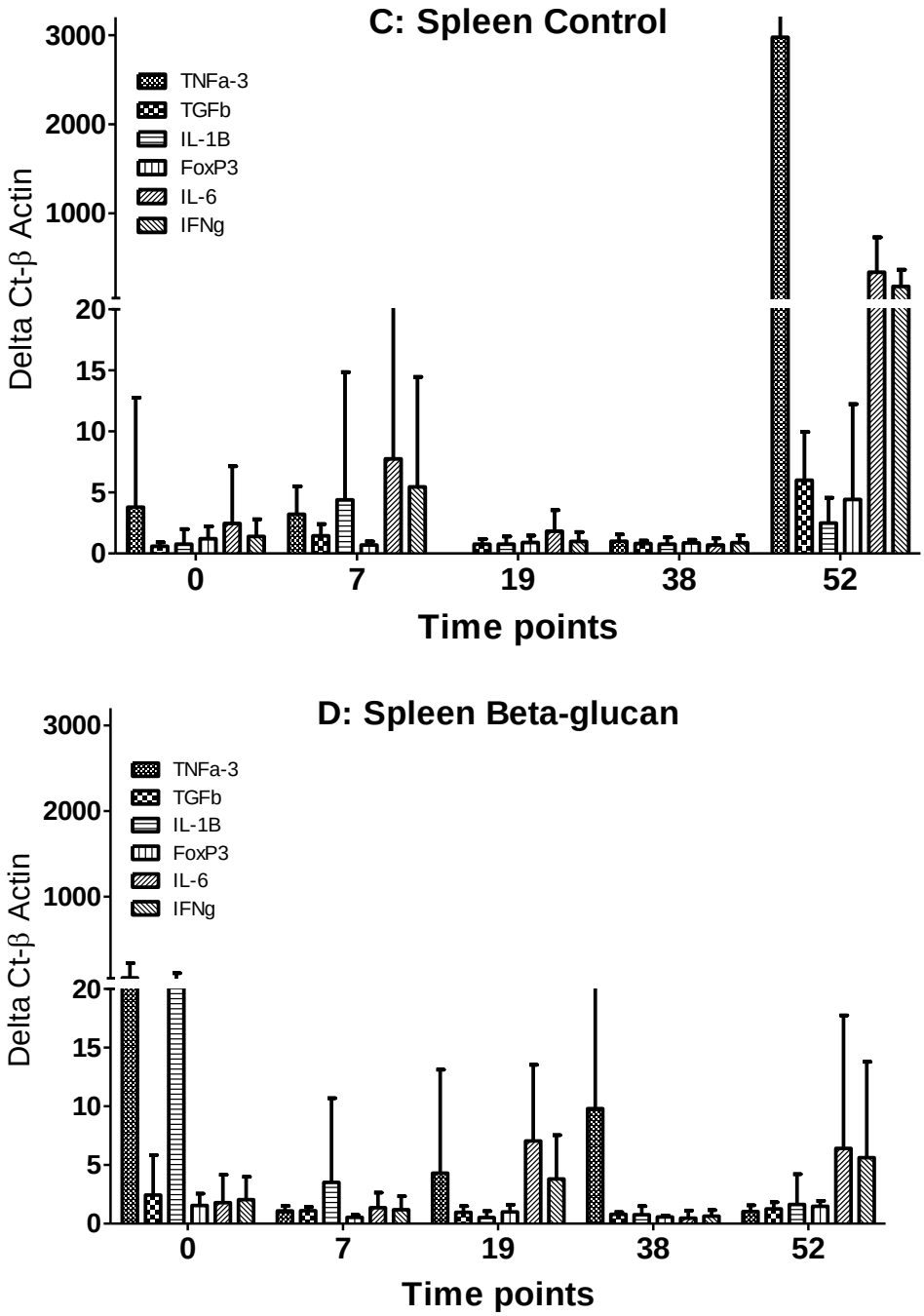
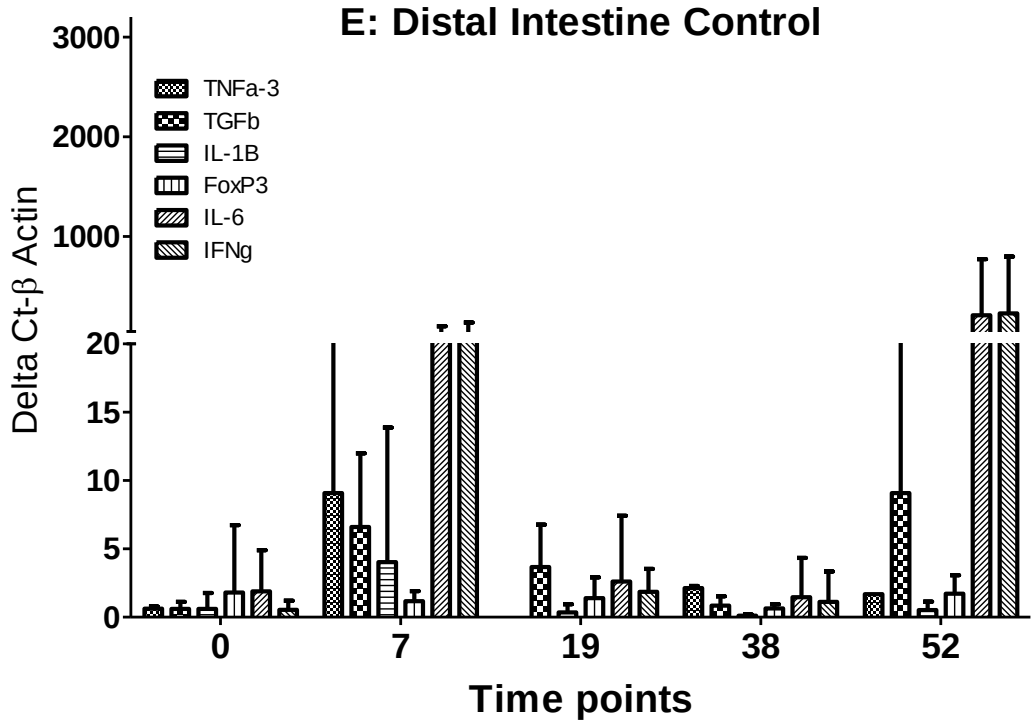


Fig. 19 C, D. Expression of selected immune and inflammatory genes in spleen samples (n=84) analysed from the beta-glucan fed and control groups, relative to expression of a housekeeping gene (beta- actin) in the same sample.

4.5.3 Expression in distal intestine

Prior to vaccination, the relative expression of all selected genes of interest was generally low, except for TGF-b and to a lesser degree TNFa-3 that were upregulated in the samples from the group having received beta-glucan supplemented feed (Fig 20 E, F). Seven days after vaccination, however, TNFa-3, IL-6 and IFN-g were upregulated in the control fish intestine as compared to the samples from the beta-glucan fed fish. In the finalsamplings, the expression pattern showed downregulation of TNFa-3, IL-6 and IFN-g in the beta-glucan group compared to the controls



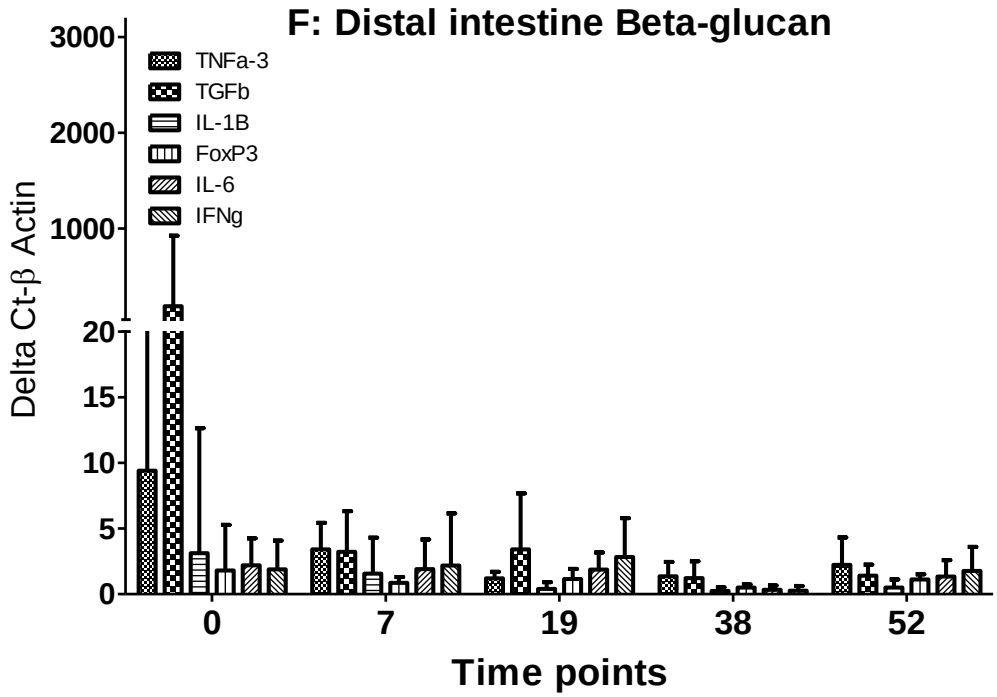


Figure 20 E, F. Expression of selected immune and inflammatory genes in spleen samples (n= 84) analysed from the β-glucan fed and control groups, relative to expression of a housekeeping gene (beta- actin) in the same sample.

5.0 Discussion

5.1 Inhibition of in vitro bacterial growth by plasma

The bacterial growth inhibition assay using Muller-Hinton agar showed that plasma from fish that had received beta-glucan was able to inhibit the growth of *Micrococcus luteus* cultured over night at 25ºC, while no inhibition was seen when the same bacterium was cultured at 30ºC or 37ºC. The standardized lysozyme assay was therefore carried out at a temperature of 25º C. In the agar assay, plasma from the glucan-supplemented feed group was also able to inhibit the growth of *Yersinia ruckeri* cultured at 30ºC or 37ºC, but not when cultured at 25ºC. *Citrobacter freundii*, an ubiquitous enterobacterium was inhibited at all temperatures tested. These results suggest that further investigation are warranted to asses if oral beta-glucansupplementation, alone are together with immunization, may play a roll in control of yersiniosis in farmed salmonids.

5.2 Lysozyme activity

In the current study, plasma taken from salmon that had received beta-glucan supplemented feed 7 and 19 days post vaccination was able to lyse the *M. luteus* strain used in the assay. In contrast, plasma from vaccinated fish having received control feed showed no such effect.

The function of serum lysozyme as a mediator of antibacterial activity was shown by Kokoshis et al. 1978 [129]. They reported that serum lysozyme activity was seven times higher than the control group in which mice treated with lysozyme enhanced their survival when exposed to systemic infection against *Staphylococcus aureus* infection. These findings showed that glucan was able to enhance the host defense against bacterial infections. In carp (*Cyprinus carpio* L), Siwicki and Studnicka [130] showed that there was a significant increase in total leucocyte count that corresponded with increase in serum lysozyme activity. By 21 days post beta-glucantreatment total leucocyte counts had decreased which corresponded with decrease in lysozyme activity to levels below the control group. Grinde [131] investigated two lysozyme variants purified from rainbow trout kidney and tested them against seven different bacterial species of which five were gram positive while another two were gram negatives. One of the variants was found to be potent against all bacterial species tested demonstrating that lysozyme could be potent against a wide range of bacteria species. The ability of serum and mucus lysozyme to reduce bacteria growth against *Aeromonas salmonicida*, *A. punctate* and *Serratia marcescens* has been demonstrated *in vitro* with high inhibition capacity at 21 days post vaccination [132].

In earlier Atlantic salmon studies reported by Engstad et al [133], glucan treatment resulted in increase the serum lysozyme activity within 1-3 weeks after exposure. Similarly, this study showed increase lysozyme activities with three weeks after vaccination in which activity against *M. luteus* was highest at 7 dpv gradually decreasing at 19 dpv vaccination reducing to similar levels as the control group at 38 and 52 dpv being similar to observations made by Engstad et al [133]. Several other studies have reported of glucan induced enhancenment of serum lysozyme activity in different fish species such as *Cyprinus carpio* [134], *Dicentrarchus labrax* [56] and *Labeo rohita* [135]. Misra et al [135] showed increased serum lysozyme activity by 42 days after feeding with beta-glucan containing 250 mg beta-glucan kg^{-1} diet in *Labeo rohita* carp. This dose resulted in highest protection against bacterial challenge and enhanced immunity, growth and survival in *L. rohita* fingerlings.

5.3 Gene expression

The results obtained in the current study suggest that the expression of TNFa-3 and IFN-g in head kidney prior to vaccination was lower in the fish receiving beta-glucan supplemented feed vs. in the group receiving control feed. However, 7 days after vaccination the expression of TNFa-3, IL-1b, IL-6 and IFN-g was upregulated in the beta-glucan group as compared to the control fish. At the final sampling 52 days after immunisation, the pre-vaccination pattern was re-established with indications of a lower expression profile of TNFa-3, IL-6 and IFN-γ in the fish that had received beta-glucan supplemented feed compared to the control group. A similar response pattern between the dietary groups could be seen also in spleen tissue and in distal intestine samples both 7 days after vaccination and at the final sampling 52 days post immunisation.

The immunomodulatory effect of beta-glucanhas been attributed to induction of immune responses such as bacterial killing and cytokine production, eventually contributing to enhance resistance to infection and survival [116, 136, 137]. Rodriguez et al [61] showed increase expression of TNFα in zebrafish pre-treated with β-glucan, which is similar with observation made in this study in headkidney samples drawn 7 days post immunisation. Also Guzmán-Villanueva et al [138] showed upregulation of IFN-γ and IL-1β transcripts in headkidney samples of seabream exposed to beta-glucan[138]. It is well established that IFNγ is a strong activator of macrophages [139]. Hence, its high expression levels during the early stages in the headkidney tissue in this study could imply that it could have played a major role in enhancing innate immune response. However, to fully understand its, future studies should include in vivo challenge of

beta-glucan fed fish in order to demonstrate its role in enhancing vaccine immunity and improving clinical protection.

The low baseline expression of the pro-inflammatory cytokines TNFa-3 and IFN- γ in fish that had received beta-glucan feed supplement prior to vaccination, and the return to lower expression level of these genes than in the control fish after 52 days are interesting, albeit not straightforward to interpret. A possible explanation is that oral administration of beta-glucan is able to lower the systemic inflammatory baseline. The gene expression results in day 7 samples suggest, however, that the responsiveness towards an inflammatory noxe (in our case the vaccination) is thereby retained or even enhanced. Given that viral diseases whose pathogenesis are associated with inflammation of the heart muscle and other tissues are widespread in salmon culture (see Introductory chapter above), further studies involving experimental infection with the relevant viral agents would be of high relevance to Norwegian aquaculture.

5.4. Effect of beta-glucan on bacterial diseases

Although little is known about the mechanism of their action in fish, some immunostimulants appear to enhance the non-specific killing of microorganisms [117]. They induce and enhance resistance against bacterial infections by stimulating the production of host defensins [48, 52, 140]. Several types of immunostimulants have been used for protection against a wide range of pathogens as feed addtives of which beta-glucans have been the most widely explored in aquaculture [141, 142]. And as such, several studies have been carried out earlier to test the potency of beta-glucans as immunostimulants in various fresh and seawater fish species [116, 143, 144]. Hence, it is not surprising that feeds containing glucan are commercially available of which the most common brands are MacroGard, Vetregard and EcoActiva [145]. Major fish species studied using B-glucan include rainbow trout (*Oncorhynchus mykiss*) [62, 144, 146] African catfish (*Clarius garipinus*) [147], Channel catfish (*Ictlurius punctatus*)[143], Atlantic salmon [148], Indian carp (*Labeo rohita*) [149], turbot (*Scophthalmus maximus* L.) [150], pink snapper (*Pagrus auratus*) [151], sea bass (*Dicentrarchus labrax*)[152], atlantic cod (*Gahus morhua* L.) [153], gilthead seabream (*Sparus aurata*) [154], large yellow croaker (*Pseudosciaena crocea*) [155], Nile tilapia (*Oreochromis niloticus*) [156], and zebrafish (*Danio rerio*) [61].

Administration of glucan in carp enhanced their survival, irrespective of the mode of delivery whether by intraperitoneal injection, bath or oral delivery [116]. Upregulation of complement and C reactive proteins was reported in carp [73, 157] while glucan-activated macrophages in trout showed increased ability to kill *Aeromonas salmonicida* [146]. Radioactively labeled

glucan was shown to translocate into the intestinal mucosa through epithelial cells although it was later cleared from blood [158]. Recent observations point to possible involvement of neutrophil extracellular traps in antimicrobial activity of beta-glucans [115].

In addition to direct stimulation of both specific and non-specific immune responses, glucan also influence the expression of immune-related genes. Macrophages from Atlantic salmon rainbow trout and Nile tilapia showed elevated levels of cytokines [159, 160].Five week-long glucan immersion of Atlantic cod exposed upregulated IL-1β expression in the anterior intestine and rectum, whereas IL-10 was downregulated. Mannan-based oligosaccharide MOS) upregulated the expression of IL-8 and IFN-γ [122]. Interestingly, an earlier study on inflammatory cytokines expressed in response to *A. salmonicida* infection showed that feeding with glucan resulted in reduction of proinflammation cytokines such as IL-1β, IL-6, IL-10, and TNF-α [70].

5.5 The role of oral beta-glucan in aquaculture

Lysozyme activity has been shown to vary with water temperature, toxicants, salinity, pH and other stressors [161]. In this study, the temperature was between $12,8^0$C and 15.7^0C, which is the normal temperature range for Atlantic salmon reared in recirculation aquaculture systems, and corresponds to the natural seawater temperatures observed along the Norwegian west coast in the summer season through the month of October. Thus, the results obtained with beta-glucan supplementation feed in this thesis are believed relevant to Norwegian salmon culture conditions.

It is important to note that responses to beta-glucan are highly influenced by the route administration, dose, duration of exposure and several factors [162]. Vetvicka and Vetvickova [163] showed that injectable beta-glucan showed high response than orally administered and longer duration of exposure showed higher reponse than shorter duration of exposure. Jafaar et al. [164] showed a dose dependent effect on protection against the skin parasitic ciliate *Ichthyophthirius multifiliis* linked to expression of lysozyme and several immune genes, including a dose dependent increase in upregulation of immune genes in fish fed high beta-glucan diet [164]. To which degree the effects of orally administeres beta-glucan observed in the current study can be further increased by higher inclusion rates remain, however, to be investigated.

6.0 Conclusions and future perspectives

In vitro studies carried out herein have shown that both mucus and serum lysozyme from Atlantic salmon fed a beta-glucan diet have antibacterial properties against *Micrococcus luteus* at 25^0C. In addition, mucus showed antibacterial properties against *Yersinia ruckeri* at 30^0C and 37^0C while serum showed antibacterial properties against *Citrobacter freundii*. Plasma from the beta-glucan fed fish showed a significant inhibition of *M. luteus* in the early time points after vaccination gradually decreasing in subsequent time points. As for gene expression studies, the general trend shows that there was significantly upregulation of genes such as TFNa-3, IL-6 and IFNγ in the beta-glucan fed group soon after vaccination unlike in the control group that had insignificant expression of these genes. In this study there was no challenge done to determine whether beta-glucan would enhance protection in fish exposed to the most prevalent viral infections of Norwegian salmon, that have a pathogenesis associated with inflammation of heart and/or skeletal musculature. The effect of oral beta-glucan supplementation on protection induced by vaccination against Pancreas Disease (PD) would be of major relevance. Hence, future studies should include the challenge of fish to determine the degree of clinical protection that can be achieved by beta-glucan supplementation of fish feeds. Future studies should also include dose dependence and duration of exposure investigations of feeding beta-glucan diets. Nevertheless, this study has shown that β-1,3/1,6-glucan is an immunostimulant that is able to enhance the activity of plasma lysozyme, its ability to inhibit bacterial growth, and to modulate the expression of selected immune- and inflammatory genes in Atlantic salmon.

References:

1. Naylor, R.L., et al., *Effect of aquaculture on world fish supplies.* Nature, 2000. **405**: p. 1017.
2. Grealis, E., et al., *The economic impact of aquaculture expansion: An input-output approach.* Marine Policy, 2017. **81**: p. 29-36.
3. FAO, *The State of World Fisheries and Aquaculture, Contributing to food security and nutrition for all Rome.* 2016.
4. Tidwell, J.H. and G.L. Allan, *Fish as food: aquaculture's contribution: Ecological and economic impacts and contributions of fish farming and capture fisheries.* EMBO Reports, 2001. **2**(11): p. 958-963.
5. Ahmed, M. and M.H. Lorica, *Improving developing country food security through aquaculture development—lessons from Asia.* Food Policy, 2002. **27**(2): p. 125-141.
6. Neiland, A.E., et al., *Shrimp aquaculture: economic perspectives for policy development.* Marine Policy, 2001. **25**(4): p. 265-279.
7. A. Lane, C.H., J. Bostock, *The Long-Term Economic and Ecologic Impact of Larger Sustainable Aquaculture.* European parliament, 2014: p. 96.
8. Aarset, B., et al., *Lovverk, teknologi og etableringsbetingelser i norsk havbruk.* Fase I. Rapport, 2004(1).
9. Vedeler, H.V., *Viral diseases in salmonid aquaculture: quantifying economic losses associated with three viral diseases affecting norwegian salmonid aquaculture*. 2017.
10. Asche, F. and T. Bjorndal, *The economics of salmon aquaculture*. Vol. 10. 2011: John Wiley & Sons.
11. EUMOFA, *EU consumer habits regarding fishery and aquaculture products - Final report.* European Market Observatory for Fisheries and Aquaculture Products 2015: p. 8.
12. Bostock, J., et al., *An assessment of the economic contribution of EU aquaculture production and the influence of policies for its sustainable development.* Aquaculture International, 2016. **24**(3): p. 699-733.
13. Asche, F.a.T.B., *The Economics of Salmon Aquaculture.* Wiley., 2011.
14. Edwards, P., D. Little, and H. Demaine, *Rural aquaculture*. 2002: Cabi.
15. Mair, G., *Genes and fish: supply of good quality fish seed for sustainable aquaculture.* Aquaculture Asia, 2002. **7**(2): p. 25-27.
16. Asche, F., A.G. Guttormsen, and R. Nielsen, *Future challenges for the maturing Norwegian salmon aquaculture industry: An analysis of total factor productivity change from 1996 to 2008.* Aquaculture, 2013. **396**: p. 43-50.
17. Carey, J.B. and S.D. McCormick, *Atlantic salmon smolts are more responsive to an acute handling and confinement stress than parr.* Aquaculture, 1998. **168**(1-4): p. 237-253.
18. Jagan, G., et al., *Water velocity in commercial RAS culture tanks for Atlantic salmon smolt production.* 2018. **81**.
19. Lall, S.P.A., J.S, 2005. , 63: 73–90., *Amino acid nutrition of salmonids: dietary requirements and bioavailability. Proceedings Mediterranean Fish Nutrition.* Cahiers Options Mediterranean,, 2005. **63**: p. 73–90.
20. Lall, S.P., *Trace mineral requirements of fish and crustaceans. In P. Schlegel, S. Durosay & A.W. Jongbloed, eds. Trace elements in animal production systems,.* Wageningen, Academic Press., 2008. : p. 203–214.
21. Ytrestøyl, T., T.S. Aas, and T. Åsgård, *Utilisation of feed resources in production of Atlantic salmon (Salmo salar) in Norway.* Aquaculture, 2015. **448**: p. 365-374.
22. Lall, S.P., *Studies on mineral and protein utilization by Atlantic salmon in seawater*. Fish. Mar. Service Technol. Report, 1977. **688**: p. 1-16.
23. Hjeltnes, B.e.a., *The Health Situation in Norwegian Aquaculture 2016, Oslo.* Norwegian Veterinary Institute 2017.

24. Løvoll, M., et al., *A novel totivirus and piscine reovirus (PRV) in Atlantic salmon (Salmo salar) with cardiomyopathy syndrome (CMS).* Virology Journal, 2010. **7**(1): p. 309.
25. T Poppe, T. and S. L Seierstad, *First description of cardiomyopathy syndrome (CMS)-related lesions in wild Atlantic salmon Salmo salar in Norway.* 2003. **56**: p. 87-8.
26. Brun, E., et al., *Cardiomyopathy syndrome in farmed Atlantic salmon Salmo salar: Occurrence and direct financial losses for Norwegian aquaculture.* 2003. **56**: p. 241-7.
27. Bruno, D. and P. Noguera, *Comparative experimental transmission of cardiomyopathy syndrome (CMS) in Atlantic salmon Salmo salar.* Diseases of Aquatic Organisms, 2009. **87**(3): p. 235-242.
28. Larsson, T., et al., *Fillet quality and gene transcriptome profiling of heart tissue of Atlantic salmon with pancreas disease (PD).* Aquaculture, 2012. **330**: p. 82-91.
29. Nilsen, H.K., *The Health Situation in Norwegian Aquaculture.* Norwegian Veterinary Institute, 2017: p. 90.
30. Petterson, E., et al., *Natural infection of Atlantic salmon (Salmo salar L.) with salmonid alphavirus 3 generates numerous viral deletion mutants.* Journal of General Virology, 2013. **94**(9): p. 1945-1954.
31. Aunsmo, A., et al., *Association of spinal deformity and vaccine-induced abdominal lesions in harvest-sized Atlantic salmon, Salmo salar L.* 2008. **31**: p. 515-24.
32. B, O.A., et al., *A novel systemic granulomatous inflammatory disease in farmed Atlantic cod, Gadus morhua L., associated with a bacterium belonging to the genus Francisella.* Journal of Fish Diseases, 2006. **29**(5): p. 307-311.
33. Aunsmo, A., et al., *Methods for investigating patterns of mortality and quantifying cause-specific mortality in sea-farmed Atlantic salmon Salmo salar.* 2008. **81**: p. 99-107.
34. Ingunn, S., et al., *Vaccines for fish in aquaculture.* Expert Review of Vaccines. **4**(1): p. 89-101.
35. Midtlyng, P.J., K. Grave, and T. Horsberg, *What Has Been Done to Minimize the Use of Antibacterial and Antiparasitic Drugs in Norwegian Aquaculture?* 2011. **42**: p. 28 - 34.
36. Thorud, K. and H. Djupvik, *Infectious anaemia in Atlantic salmon (Salmo salar L.).* Bull Eur Assoc Fish Pathol, 1988. **8**(5): p. 109-111.
37. Jansen, M., et al., *The epidemiology of pancreas disease in salmonid aquaculture: A summary of the current state of knowledge.* 2016. **40**.
38. Aunsmo, A., et al., *Stochastic modelling of direct costs of pancreas disease (PD) in Norwegian farmed Atlantic salmon (Salmo salar L.).* 2009. **93**: p. 233-41.
39. Pettersen, J.M., et al., *The economic benefits of disease triggered early harvest: A case study of pancreas disease in farmed Atlantic salmon from Norway.* 2015. **121**.
40. T, K.R., et al., *Heart and skeletal muscle inflammation in Atlantic salmon, Salmo salar L.: a new infectious disease.* Journal of Fish Diseases, 2004. **27**(6): p. 351-358.
41. Richard, B. and I. Jos, *Updated Estimates of the Costs Associated with Thirty Four Endemic Livestock Diseases in Great Britain: A Note.* Journal of Agricultural Economics, 2005. **56**(1): p. 135-144.
42. Bjørgen, H., et al., *Piscine orthoreovirus (PRV) in red and melanised foci in white muscle of Atlantic salmon (Salmo salar).* Vet Res, 2015. **46**(1).
43. Medeiros, S.D.V., et al., *Effects of Purified Saccharomyces cerevisiae (1→3)-β-Glucan on Venous Ulcer Healing.* Int J Mol Sci, 2012. **13**(7): p. 8142-58.
44. D. Akramiene, A.K., J. Didziapetriene, E. Kevelaitis, *Effects of beta-glucans on the immune system* Med. Kaunas Lith, 2007. **43**(8): p. 597-606.
45. Novak, M. and V. Vetvicka, *β-Glucans, History, and the Present: Immunomodulatory Aspects and Mechanisms of Action.* Journal of Immunotoxicology, 2008. **5**(1): p. 47-57.
46. Dalmo, R.A. and J. Bøgwald, *ß-glucans as conductors of immune symphonies.* Fish & Shellfish Immunology, 2008. **25**(4): p. 384-396.

47. Robertsen, B., et al., *Enhancement of non-specific disease resistance in Atlantic salmon, Salmo salar L., by a glucan from Saccharomyces cerevisiae cell walls.* Journal of Fish Diseases, 1990. **13**(5): p. 391-400.
48. Raa J, R.G., Engstad RE, Robertsen B. Asian Fisheries Society; 1992. pp. , *The use of immunostimulants to increase resistance of aquatic organism to microbial infections. In: Shariff IM, Subasinghe RP, Arthur JR, editors.* . Diseases in Asian Aquaculture. Manila: Health Fish Section, 1992: p. 39–50.
49. Stier, H., V. Ebbeskotte, and J. Gruenwald, *Immune-modulatory effects of dietary Yeast Beta-1,3/1,6-D-glucan.* Nutr J, 2014. **13**: p. 38.
50. J, D., *Carbohydrate- Sugars and Polysaccharides, part 1, Biochemistry of metabolism, Rensselaer.* 2008.
51. Goodridge, H.S., A.J. Wolf, and D.M. Underhill, *β-glucan recognition by the innate immune system.* Immunological Reviews, 2009. **230**(1): p. 38-50.
52. Meena, D.K., et al., *Beta-glucan: an ideal immunostimulant in aquaculture (a review).* Fish Physiology and Biochemistry, 2013. **39**(3): p. 431-457.
53. Soltanian, S., et al., *Beta-glucans as immunostimulant in vertebrates and invertebrates.* Critical Reviews in Microbiology, 2009. **35**(2): p. 109-138.
54. Thompson, I.J., P.C.F. Oyston, and D.E. Williamson, *Potential of the β-glucans to enhance innate resistance to biological agents.* Expert Review of Anti-infective Therapy, 2010. **8**(3): p. 339-352.
55. Chen, J. and R. Seviour, *Medicinal importance of fungal β-(1→3), (1→6)-glucans.* Mycological Research, 2007. **111**(6): p. 635-652.
56. Bagni, M., et al., *Short-and long-term effects of a dietary yeast β-glucan (Macrogard) and alginic acid (Ergosan) preparation on immune response in sea bass (Dicentrarchus labrax).* Fish & Shellfish Immunology, 2005. **18**(4): p. 311-325.
57. Guselle, N.J., et al., *Efficacy of Intraperitoneally and Orally Administered ProVale, a Yeast β-(1,3)/(1,6)-D-glucan Product, in Inhibiting Xenoma Formation by the Microsporidian Loma salmonae on Rainbow Trout Gills.* North American Journal of Aquaculture, 2010. **72**(1): p. 65-72.
58. Fredriksen, B.N., et al., *Early immune responses in Atlantic salmon (Salmo salar L.) after immunization with PLGA nanoparticles loaded with a model antigen and β-glucan.* Vaccine, 2011. **29**(46): p. 8338-8349.
59. Paredes, M., et al., *Immunomodulatory effect of prolactin on Atlantic salmon (Salmo salar) macrophage function.* Fish Physiology and Biochemistry, 2013. **39**(5): p. 1215-1221.
60. Raa, J., *Immune modulation by non-digestible and non-absorbable beta-1,3/1,6-glucan.* Microbial Ecology in Health and Disease, 2015. **26**: p. 10.3402/mehd.v26.27824.
61. Rodríguez, I., et al., *β-Glucan administration enhances disease resistance and some innate immune responses in zebrafish (Danio rerio).* Fish & Shellfish Immunology, 2009. **27**(2): p. 369-373.
62. Djordjevic, B., et al., *Modulation of splenic immune responses to bacterial lipopolysaccharide in rainbow trout (Oncorhynchus mykiss) fed lentinan, a beta-glucan from mushroom Lentinula edodes.* Fish & Shellfish Immunology, 2009. **26**(2): p. 201-209.
63. Schmitt, P., et al., *Immunomodulatory effect of cathelicidins in response to a β-glucan in intestinal epithelial cells from rainbow trout.* Developmental & Comparative Immunology, 2015. **51**(1): p. 160-169.
64. El-Boshy, M.E., et al., *Immunomodulatory effect of dietary Saccharomyces cerevisiae, β-glucan and laminaran in mercuric chloride treated Nile tilapia (Oreochromis niloticus) and experimentally infected with Aeromonas hydrophila.* Fish & Shellfish Immunology, 2010. **28**(5): p. 802-808.

65. Chang, C.-S., et al., *Innate immune responses and efficacy of using mushroom beta-glucan mixture (MBG) on orange-spotted grouper, Epinephelus coioides, aquaculture.* Fish & Shellfish Immunology, 2013. **35**(1): p. 115-125.
66. Guzmán-Villanueva, L.T., et al., *Dietary administration of β-1,3/1,6-glucan and probiotic strain Shewanella putrefaciens, single or combined, on gilthead seabream growth, immune responses and gene expression.* Fish & Shellfish Immunology, 2014. **39**(1): p. 34-41.
67. Dawood, M.A.O., et al., *Interaction effects of dietary supplementation of heat-killed Lactobacillus plantarum and β-glucan on growth performance, digestibility and immune response of juvenile red sea bream, Pagrus major.* Fish & Shellfish Immunology, 2015. **45**(1): p. 33-42.
68. A. Gopalakannan, V.A., *Enhancement of the innate immune system and disease-resistant activity in Cyprinus carpio by oral administration of beta-glucan and whole cell yeast.* Aquacult. Res, 2010. **41**: p. 884-892.
69. Lin, S., et al., *Effects of dietary β-1,3-glucan, chitosan or raffinose on the growth, innate immunity and resistance of koi (Cyprinus carpio koi).* Fish & Shellfish Immunology, 2011. **31**(6): p. 788-794.
70. Falco, A., et al., *Reduced inflammatory response to Aeromonas salmonicida infection in common carp (Cyprinus carpio L.) fed with β-glucan supplements.* Fish & Shellfish Immunology, 2012. **32**(6): p. 1051-1057.
71. J.J. Miest, A.F., N.P.M. Pionnier, P. Frost, I. Irnazarow, G.T. Williams, D. Hoole, *The influence of dietary beta-glucan, PAMP exposure and Aeromonas salmonicida on apoptosis modulation in common carp (Cyprinus carpio).* Fish Shellfish Immunol., , 2012. **33**: p. 846-856.
72. van der Marel, M., et al., *Molecular cloning and expression of two β-defensin and two mucin genes in common carp (Cyprinus carpio L.) and their up-regulation after β-glucan feeding.* Fish & Shellfish Immunology, 2012. **32**(3): p. 494-501.
73. Pionnier, N., et al., *Dietary β-glucan stimulate complement and C-reactive protein acute phase responses in common carp (Cyprinus carpio) during an Aeromonas salmonicida infection.* Fish & Shellfish Immunology, 2013. **34**(3): p. 819-831.
74. Falco, A., et al., *β-Glucan-supplemented diets increase poly(I:C)-induced gene expression of Mx, possibly via Tlr3-mediated recognition mechanism in common carp (Cyprinus carpio).* Fish & Shellfish Immunology, 2014. **36**(2): p. 494-502.
75. Pionnier, N., et al., *Feeding common carp Cyprinus carpio with β-glucan supplemented diet stimulates C-reactive protein and complement immune acute phase responses following PAMPs injection.* Fish & Shellfish Immunology, 2014. **39**(2): p. 285-295.
76. H. Lauridsen, J. and K. Buchmann, *Effects of Short- and Long-Term Glucan Feeding of Rainbow Trout (Salmonidae) on the Susceptibility to Ichthyophthirius Multifiliis Infections.* 2010. **40**: p. 61-66.
77. Refstie, S., et al., *Effects of dietary yeast cell wall ??-glucans and MOS on performance, gut health, and salmon lice resistance in Atlantic salmon (Salmo salar) fed sunflower and soybean meal.* 2010. **305**: p. 109-116.
78. Covello, J.M.F., S.E. Purcell, S.L. Burka, J.F., *Effects of orally administered immunostimulants on inflammatory gene expression and sea lice (Lepeophtheirus salmonis) burdens on Atlantic salmon (Salmo salar).* Aquaculture, 2012. **366-367**: p. 9 - 16.
79. Wei, W., et al., *Application of immunostimulants in aquaculture: current knowledge and future perspectives.* Aquaculture Research, 2017. **48**(1): p. 1-23.
80. Roy Dalmo, J.B.a.C.T., *Fish vaccine: Adjuvants and delivery methods: current and noval.* Springer: p. 75-91.

81. Midtlyng PJ, R.L., Speilberg L. , *Experimental studies on the efficacy and side-effects of intraperitoneal vaccination of Atlantic salmon (Salmo salar L.) against furunculosis.* Fish Shellfish Immunol. , 1996. **6**: p. 335-50.
82. De Smet, R., et al., *Beta-Glucan microparticles are good candidates for mucosal antigen delivery in oral vaccination.* 2013. **172**.
83. Refstie, S., et al., *Effects of dietary yeast cell wall β-glucans and MOS on performance, gut health, and salmon lice resistance in Atlantic salmon (Salmo salar) fed sunflower and soybean meal.* Aquaculture, 2010. **305**(1): p. 109-116.
84. Covello, J.M., et al., *Effects of orally administered immunostimulants on inflammatory gene expression and sea lice (Lepeophtheirus salmonis) burdens on Atlantic salmon (Salmo salar).* Aquaculture, 2012. **366-367**: p. 9-16.
85. Swennen, K., C.M. Courtin, and J.A. Delcour, *Non-digestible Oligosaccharides with Prebiotic Properties.* Critical Reviews in Food Science and Nutrition, 2006. **46**(6): p. 459-471.
86. Dalmo, R.A., et al., *Intestinal absorption of immunomodulatory laminaran and derivatives in Atlantic salmon, Salmo salar L.* Journal of Fish Diseases, 1994. **17**(6): p. 579-589.
87. Løkka, G., et al., *Uptake of yeast cells in the Atlantic salmon (Salmo salar L.) intestine.* Developmental & Comparative Immunology, 2014. **47**(1): p. 77-80.
88. Chen, D. and A.J. Ainsworth, *Glucan administration potentiates immune defence mechanisms of channel catfish, Ictalurus punctatus Rafinesque.* Journal of Fish Diseases, 1992. **15**(4): p. 295-304.
89. T. Yano, R.E.M., H. Matsuyama, *Enhancement of the resistance of carp Cyprinus carpio to experimental Edwardsiella tarda infection, by some beta-1, 3-glucans [derived from fungi]*. Bull. Jpn. Soc. Sci. Fish 1989: p. 55.
90. Duncan, P.L. and P.H. Klesius, *Effects of Feeding Spirulina on Specific and Nonspecific Immune Responses of Channel Catfish.* Journal of Aquatic Animal Health, 1996. **8**(4): p. 308-313.
91. Whittington, R., C. Lim, and P.H. Klesius, *Effect of dietary β-glucan levels on the growth response and efficacy of Streptococcus iniae vaccine in Nile tilapia, Oreochromis niloticus.* Aquaculture, 2005. **248**(1): p. 217-225.
92. Magnadottir, B., *Immunological Control of Fish Diseases.* Marine Biotechnology, 2010. **12**(4): p. 361-379.
93. Edwin, L.C., *Comparative Immunology.* Current Pharmaceutical Design, 2003. **9**(2): p. 119-131.
94. Rauta, P.R., B. Nayak, and S. Das, *Immune system and immune responses in fish and their role in comparative immunity study: A model for higher organisms.* Immunology Letters, 2012. **148**(1): p. 23-33.
95. V. Kumar, A.K.A., N. Fausto, *Robbins and Cotran Pathologic Basis of Disease.* Elsevier Saunders, Philadelphia, PA, 2004: p. 20.
96. Orallo, R.C.J.L.P.M.M.L.S.J.L.F., *Resveratrol modulates innate and inflammatory responses in fish leucocytes.* Vet. Immuno. and Immunopathology. **126**(1): p. 10.
97. Martinez-Rubio L, M.S., Evensen Ø, et al. . Nurminsky DI, ed. PLoS ONE. 2012;7(11):e40266. doi:10.1371/journal.pone.0040266., *Functional Feeds Reduce Heart Inflammation and Pathology in Atlantic Salmon (Salmo salar L.) following Experimental Challenge with Atlantic Salmon Reovirus (ASRV).* PLOS one, 2012. **7**: p. 11.
98. Wessel Ø, B.S., Alarcon M, Haatveit H, Roos N, Markussen T, . , *Infection with purified Piscine orthoreovirus demonstrates a causal relationship with heart and skeletal muscle inflammation in Atlantic salmon.* PLoS ONE 2017. **12**: p. 8.
99. Carroll, M.C., *The complement system in B cell regulation.* Molecular Immunology, 2004. **41**(2): p. 141-146.

100. Hawlisch, H. and J. Köhl, *Complement and Toll-like receptors: Key regulators of adaptive immune responses.* Molecular Immunology, 2006. **43**(1): p. 13-21.
101. ELLIS, A.F., *Optimizing factors in fish vaccination. In: Ellis AE, editor. Fish vaccination. London: Academic Press.* 1988: p. 32–46
102. Dranoff, G., *Cytokines in cancer pathogenesis and cancer therapy.* Nature Reviews Cancer, 2004. **4**: p. 11.
103. Litman, G.W., J.P. Cannon, and L.J. Dishaw, *RECONSTRUCTING IMMUNE PHYLOGENY: NEW PERSPECTIVES.* Nature reviews. Immunology, 2005. **5**(11): p. 866-879.
104. Buchmann, K., *Lectins in fish skin: do they play a role in host–monogenean interactions?* Journal of Helminthology, 2001. **75**(3): p. 227-231.
105. Suzuki, Y., et al., *Molecular diversity of skin mucus lectins in fish.* Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 2003. **136**(4): p. 723-730.
106. Ellis, A.E., *Innate host defense mechanisms of fish against viruses and bacteria.* Developmental & Comparative Immunology, 2001. **25**(8): p. 827-839.
107. Shephard, K.L., *Functions for Fish Mucus.* Reviews in Fish Biology and Fisheries, 1994. **4**: p. 401-429.
108. Bullock, A.M. and R.J. Roberts, *Inhibition of epidermal migration in the skin of rainbow trout Salmo gairdneri Richardson in the presence of achromogenic Aeromonas salmonicida.* Journal of Fish Diseases, 1980. **3**(6): p. 517-524.
109. Fletcher, T.C., R. Jones, and L. Reid, *Identification of glycoproteins in goblet cells of epidermis and gill of plaice (Pleuronectes platessa L.), flounder (Platichthys flesus (L.)) and rainbow trout (Salmo gairdneri Richardson).* The Histochemical Journal, 1976. **8**(6): p. 597-608.
110. Harrell, L.W., H.M. Etlinger, and H.O. Hodgins, *Humoral factors important in resistance of salmonid fish to bacterial disease. II. Anti-Vibrio anguillarum activity in mucus and observations on complement.* Aquaculture, 1976. **7**(4): p. 363-370.
111. Hjelmeland, K., M. Christie, and J. Raa, *Skin mucus protease from rainbow trout, Salmo gairdneri Richardson, and its biological significance.* Journal of Fish Biology, 1983. **23**(1): p. 13-22.
112. Austin, B. and D. McIntosh, *Natural antibacterial compounds on the surface of rainbow trout, Salmo gairdneri Richardson.* Journal of Fish Diseases, 1988. **11**(3): p. 275-277.
113. Noya, M., B. Magarinos, A. E. Toranzo, and J. Lamas. 1995. S. , *equential Pathology of Experimental Pasteurellosis in Gilthead Seabream Sparus-Aurata - a Light- Microscopic and Electron-Microscopic Study.* Diseases of Aquatic Organisms. **21**: p. 177-186.
114. Yano, T., H. Matsuyama, and R.E.P. Mangindaan, *Polysaccharide-induced protection of carp, Cyprinus carpio L., against bacterial infection.* Journal of Fish Diseases, 1991. **14**(5): p. 577-582.
115. Brogden, G., et al., *β-Glucan protects neutrophil extracellular traps against degradation by Aeromonas hydrophila in carp (Cyprinus carpio).* Fish & Shellfish Immunology, 2012. **33**(4): p. 1060-1064.
116. Selvaraj, V., K. Sampath, and V. Sekar, *Administration of yeast glucan enhances survival and some non-specific and specific immune parameters in carp (Cyprinus carpio) infected with Aeromonas hydrophila.* Fish & Shellfish Immunology, 2005. **19**(4): p. 293-306.
117. Olivier, G., C.A. Eaton, and N. Campbell, *Interaction betweenAeromonas salmonicida and peritoneal macrophages of brook trout (Salvelinus fontinalis).* Veterinary Immunology and Immunopathology, 1986. **12**(1): p. 223-234.

118. Burrells, C., P.D. Williams, and P.F. Forno, *Dietary nucleotides: a novel supplement in fish feeds: 1. Effects on resistance to disease in salmonids.* Aquaculture, 2001. **199**(1): p. 159-169.
119. Aakre, R., et al., *Enhanced antibody response in Atlantic salmon (Salmo salar L.) to Aeromonas salmonicida cell wall antigens using a bacterin containing β-1,3-M-Glucan as adjuvant.* Fish & Shellfish Immunology, 1994. **4**(1): p. 47-61.
120. Jeney, G., et al., *Prevention of stress in rainbow trout (Oncorhynchus mykiss) fed diets containing different doses of glucan.* Aquaculture, 1997. **154**(1): p. 1-15.
121. Ashida T, O.E., Ui M, Heguri M, Oyama Y, Amemura A. , *Protection of Japanese flounder Paralichthys olivaceus against experimental Edwardsiellosis by formalin-killed Edwardsiella tarda in combination with oral administration of immunostimulants.* . Fish Sci. , 1999. **65**: p. 527–30.
122. Lokesh, J., et al., *Transcriptional regulation of cytokines in the intestine of Atlantic cod fed yeast derived mannan oligosaccharide or β-Glucan and challenged with Vibrio anguillarum.* Fish & Shellfish Immunology, 2012. **33**(3): p. 626-631.
123. Beaulaurier, J., et al., *Susceptibility of Pacific Herring to Viral Hemorrhagic Septicemia Is Influenced by Diet.* Journal of Aquatic Animal Health, 2012. **24**(1): p. 43-48.
124. Şahan, A. and S. Duman, *Effect of β Glucan on Haematology of Common Carp (Cyprinus Carpio) Infected by Ectoparasites.* Mediterranean Aquaculture Journal, 2010. **3**(1): p. 1-7.
125. Kirchhoff NT, D.A.T., Leef MJ, Hayward CJ, Wilkinson RJ, Nowak BF. , *Effects of immunostimulans on ranched southern bluefin tuna Thunnus maccoyii: Immune response, health and performance.* J Fish Biol., 2011. **79**: p. 331-55.
126. Jaafar RM, S.J., Kania PW, Buchmann K. , *Dose dependent effects of dietary immunostimulants on rainbow trout immune parameters and susceptibility to the parasite Ichthyophthirius multifillis.* J Aquacult Res Dev, 2011.
127. Munang'andu, H.M., et al., *Antigen dose and humoral immune response correspond with protection for inactivated infectious pancreatic necrosis virus vaccines in Atlantic salmon (Salmo salar L).* Vet Res, 2013. **44**(1): p. 7.
128. Rao, X., et al., *An improvement of the 2ˆ(–delta delta CT) method for quantitative real-time polymerase chain reaction data analysis.* Biostat Bioinforma Biomath, 2013. **3**(3): p. 71-85.
129. Kokoshis, P., et al., *Increased resistance to Staphylococcus aureus infection and enhancement in serum lysozyme activity by glucan.* Science, 1978. **199**(4335): p. 1340-1342.
130. Siwicki, A. and M. Studnicka, *The phagocytic ability of neutrophils and serum lysozyme activity in experimentally infected carp, Cyprinus carpio L.* Journal of fish biology, 1987. **31**(sA): p. 57-60.
131. Grinde, B., *Lysozyme from rainbow trout, Salmo gairdneri Richardson, as an antibacterial agent against fish pathogens.* Journal of Fish Diseases, 1989. **12**(2): p. 95-104.
132. Rainger, G. and A. Rowley, *Antibacterial activity in the serum and mucus of rainbow trout, Oncorhynchus mykiss, following immunisation with Aeromonas salmonicida.* Fish & Shellfish Immunology, 1993. **3**(6): p. 475-482.
133. Engstad, R.E., B. Robertsen, and E. Frivold, *Yeast glucan induces increase in lysozyme and complement-mediated haemolytic activity in Atlantic salmon blood.* Fish & Shellfish Immunology, 1992. **2**(4): p. 287-297.
134. Gopalakannan, A. and V. Arul, *Enhancement of the innate immune system and disease‐resistant activity in Cyprinus carpio by oral administration of β‐glucan and whole cell yeast.* Aquaculture research, 2010. **41**(6): p. 884-892.

135. Misra, C.K., et al., *Effect of long term administration of dietary β-glucan on immunity, growth and survival of Labeo rohita fingerlings.* Aquaculture, 2006. **255**(1-4): p. 82-94.
136. Soltys, J. and M.T. Quinn, *Modulation of endotoxin-and enterotoxin-induced cytokine release by in vivo treatment with β-(1, 6)-branched β-(1, 3)-glucan.* Infection and immunity, 1999. **67**(1): p. 244-252.
137. Williams, D.L., et al., *Modulation of the phosphoinositide 3-kinase pathway alters innate resistance to polymicrobial sepsis.* The Journal of Immunology, 2004. **172**(1): p. 449-456.
138. Guzmán-Villanueva, L.T., et al., *Dietary administration of β-1, 3/1, 6-glucan and probiotic strain Shewanella putrefaciens, single or combined, on gilthead seabream growth, immune responses and gene expression.* Fish & shellfish immunology, 2014. **39**(1): p. 34-41.
139. Bogdan, C., J. Mattner, and U. Schleicher, *The role of type I interferons in non‐viral infections.* Immunological reviews, 2004. **202**(1): p. 33-48.
140. Siwicki, A.K., et al., *Influence of immunostimulants on the effectiveness of vaccines in fish: in vitro and in vivo study.* Journal of Applied Ichthyology, 1998. **14**(3-4): p. 225-227.
141. Raa, J., *The use of immunostimulatory substances in fish and shellfish farming.* Reviews in Fisheries Science, 1996. **4**(3): p. 229-288.
142. ., R.J., *The use of immune-stimulants in fish and shellfish feeds.* In: Cruz–Suárez LE, Ricque-Marie D, Tapia-Salazar M, Olvera-Novoa MA, Civera-Cerecedo R, editors. Advances en Nutrición Acuícola V. Memorias del V Simposium Internacional de Nutrición Acuícola .Mexico, 2000: p. 47–56.
143. Welker, T.L., et al., *Immune Response and Resistance to Stress and Edwardsiella ictaluri Challenge in Channel Catfish, Ictalurus punctatus, Fed Diets Containing Commercial Whole-Cell Yeast or Yeast Subcomponents.* Journal of the World Aquaculture Society, 2007. **38**(1): p. 24-35.
144. Sealey, W.M., et al., *Evaluation of the ability of barley genotypes containing different amounts of β-glucan to alter growth and disease resistance of rainbow trout Oncorhynchus mykiss.* Animal Feed Science and Technology, 2008. **141**(1): p. 115-128.
145. Cook, M.T., et al., *The efficacy of a commercial β-glucan preparation, EcoActiva™, on stimulating respiratory burst activity of head-kidney macrophages from pink snapper (Pagrus auratus), Sparidae.* Fish & Shellfish Immunology, 2001. **11**(8): p. 661-672.
146. Jørgensen, J.B., et al., *Effect of a yeast-cell-wall glucan on the bactericidal activity of rainbow trout macrophages.* Fish & Shellfish Immunology, 1993. **3**(4): p. 267-277.
147. Yoshida, T., R. Kruger, and V. Inglis, *Augmentation of non-specific protection in African catfish, Clarias gariepinus (Burchell), by the long-term oral administration of immunostimulants.* Journal of Fish Diseases, 1995. **18**(2): p. 195-198.
148. Rørstad, G., P.M. Aasjord, and B. Robertsen, *Adjuvant effect of a yeast glucan in vaccines against furunculosis in Atlantic salmon (Salmo salar L.).* Fish & Shellfish Immunology, 1993. **3**(3): p. 179-190.
149. Sahoo, P.K. and S.C. Mukherjee, *The effect of dietary immunomodulation upon Edwardsiella tarda vaccination in healthy and immunocompromised Indian major carp (Labeo rohita).* Fish & Shellfish Immunology, 2002. **12**(1): p. 1-16.
150. Figueras, A., M. M. Santarém, and B. Novoa, *Influence of the sequence of administration of β-glucans and a Vibrio damsela vaccine on the immune response of turbot (Scophthalmus maximus L.).* Veterinary Immunology and Immunopathology, 1998. **64**(1): p. 59-68.
151. Cook, M.T., et al., *Administration of a commercial immunostimulant preparation, EcoActiva™ as a feed supplement enhances macrophage respiratory burst and the*

growth rate of snapper (Pagrus auratus, Sparidae (Bloch and Schneider)) in winter. Fish & Shellfish Immunology, 2003. **14**(4): p. 333-345.
152. Bagni, M., et al., *Effect of Long-term Oral Administration of an Immunostimulant Diet on Innate Immunity in Sea Bass (Dicentrarchus labrax).* Journal of Veterinary Medicine, Series B, 2000. **47**(10): p. 745-751.
153. Skjermo J, S.T., Hansen K, Handa A, Oie G. Aquaculture. , *Evaluation of beta-(1-3, 1-6)-glucans and high-M alginate used as immunostimulatory dietary supplement during first feeding and weaning of Atlantic code (Gahus morhua L.).* 2006. **261**: p. 1088–101.
154. Cuesta, A., et al., *Early local and systemic innate immune responses in the teleost gilthead seabream after intraperitoneal injection of whole yeast cells.* Fish & Shellfish Immunology, 2007. **22**(3): p. 242-251.
155. Ai, Q., et al., *Effects of dietary β-1, 3 glucan on innate immune response of large yellow croaker, Pseudosciaena crocea.* Fish & Shellfish Immunology, 2007. **22**(4): p. 394-402.
156. Shelby, R.A., et al., *Effects of Yeast Oligosaccharide Diet Supplements on Growth and Disease Resistance in Juvenile Nile Tilapia, Oreochromis niloticus.* Journal of Applied Aquaculture, 2009. **21**(1): p. 61-71.
157. Przybylska-Diaz, D.A., et al., *β-glucan enriched bath directly stimulates the wound healing process in common carp (Cyprinus carpio L.).* Fish & Shellfish Immunology, 2013. **35**(3): p. 998-1006.
158. Sveinbjørnsson, B., et al., *Intestinal uptake and organ distribution ofimmunomodulatory aminated β-1,3-d-polyglucose in Atlantic salmon (Salmo salar L.).* Fish & Shellfish Immunology, 1995. **5**(1): p. 39-50.
159. Løvoll, M., et al., *The C3 subtypes are differentially regulated after immunostimulation in rainbow trout, but head kidney macrophages do not contribute to C3 transcription.* Veterinary Immunology and Immunopathology, 2007. **117**(3): p. 284-295.
160. Chansue N, E.M., Kono T, Sakai M. , *The stimulation of cytokine-like protein in tilapia (Oreochromis niloticus) orally treated with β-1,3 glucan.* Asian Fish Sci. , 2000. **13**: p. 271-8.
161. Saurabh, S. and P. Sahoo, *Lysozyme: an important defence molecule of fish innate immune system.* Aquaculture research, 2008. **39**(3): p. 223-239.
162. Suzuki, I., et al., *Effect of orally administered β-glucan on macrophage function in mice.* International journal of immunopharmacology, 1990. **12**(6): p. 675-684.
163. Vetvicka, V. and J. Vetvickova, *A comparison of injected and orally administered β-glucans.* JANA, 2008. **11**(1): p. 42-48.
164. Jaafar, R.M., et al., *Dose dependent effects of dietary immunostimulants on rainbow trout immune parameters and susceptibility to the parasite Ichthyophthirius multifiliis.* Journal of Aquaculture Research and Development S, 2011. **3**: p. S3-001.

8.0 Appendix list

Appendix 01: First weight registration of the experimental fish

22 September Ås

#	Fish Nr.	Anaesthesia	Tank 19/grams	Fish Nr.	Anaesthesia	Tank 20
1	1	√	116	26	√	112
2	2	√	148	27	√	94
3	3	√	122	28	√	164
4	4	√	121	29	√	140
5	5	√	117	30	√	145
6	6	√	112	31	√	112
7	7	√	149	32	√	104
8	8	√	116	33	√	151
9	9	√	134	34	√	122
10	10	√	108	35	√	98
11	11	√	123	36	√	94
12	12	√	111	37	√	115
13	13	√	91	38	√	130
14	14	√	164	39	√	126
15	15	√	90	40	√	99
16	16	√	141	41	√	101
17	17	√	103	42	√	106
18	18	√	137	43	√	149
19	19	√	152	44	√	133
20	20	√	132	45	√	151
21	21	√	98	46	√	100
22	22	√	86	47	√	126
23	23	√	102	48	√	119
24	24	√	101	49	√	86
25	25	√	141	50	√	122
Weight			**3015**			**2999**
Average			120,6			119,96

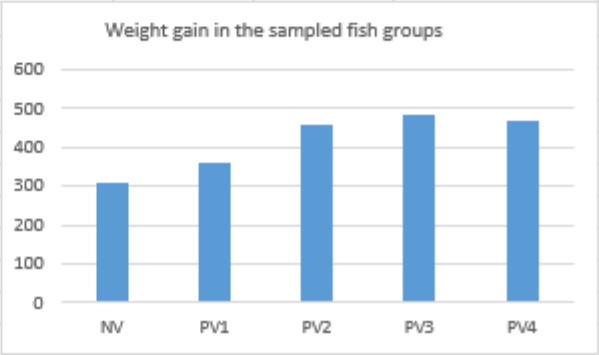
Appendix 02: First weight registration of the experimental fish (continuation)

Fish Nr.	Anaesthesia	Tank 21	Fish Nr.	Anaesthesia	Tank 22	
51	√	118	77	√	104	
52	√	120	78	√	95	
53	√	104	79	√	122	
54	√	117	80	√	140	
55	√	129	81	√	115	
56	√	81	82	√	75	
57	√	86	83	√	88	
58	√	102	84	√	104	
59	√	116	85	√	116	
60	√	134	86	√	128	
61	√	93	87	√	143	
62	√	128	88	√	107	
63	√	109	89	√	105	
64	√	149	90	√	99	
65	√	127	91	√	121	
66	√	126	92	√	156	
67	√	110	93	√	143	
68	√	159	94	√	118	
69	√	121	95	√	140	
70	√	93	96	√	142	
71	√	128	97	√	94	
72	√	96	98	√	97	
73	√	126	99	√	127	
74	√	117	100	√	111	
75	√	98	101	√	106	
76	√	127	102	√	132	
Weight		**3014**			**3028**	
Average		115,9231			116,4615	**118,2362**

Appendix 03: Experimental fish weight from each sampling date

		Control diet	Experim. die	Control diet	Experim. diet	
Fish number	Euthanizec	Tank 18	Tank 19	Tank 20	Tank 21	Total weight
1	prest	-210	-363	-218	-382	NV
2	prest	-212	-389	-268	-397	
3	prest	-307	-379	-316	-253	
4	prest	-258	-396	-274	-305	
1	prest	-399	-292	-473	-371	PV1
2	prest	-262	-440	-411	-461	
3	prest	-303	-512	-381	-444	
4	prest	-318	-383	-262	-224	
5	prest	-326	-451	-208	-196	
1	prest	-442	-431	-280	-514	PV2
2	prest	-579	-485	-381	-559	
3	prest	-602	-524	-317	-519	
4	prest	-438	-277	-461	-515	
1	prest	-441	-507	-643	-515	PV3
2	prest	-515	-463	-609	-587	
3	prest	-572	-437	-463	-215	
4	prest	-534	-373	-459	-430	
1	prest	-564	-479	-523	-518	PV4
2	prest	-491	-425	-522	-405	
3	prest	-479	-403	-508	-440	
4	prest	-445	-460	-387	-496	
						Average w.
01 des.	NV	-987	-1527	-1076	-1337	-307,9375
08. des.	PV1	-1608	-2078	-1735	-1696	-355,85
27.des	PV2	-2061	-1717	-1439	-2107	-457,75
15.jan	PV3	-2062	-1780	-2174	-1747	-485,1875
29.jan	PV4	-1979	-1767	-1940	-1859	-471,5625
	avlivet og tatt prøver av					

Days	Group	Average w.
0	NV	308
7	PV1	358
26	PV2	458
45	PV3	485
60	PV4	471



Appendix 04: Protocol for RNA extraction

(Conventional with Qiagen RNA kit method)

1: Cut 30 mg tissue and keep in tube with cap (also have 3 steel weed to crush the tissue)

2: Add 1 ml TRIzol and homogenize using homogenizer until tissue are crush for 5 minutes not more than **10 minutes**

3: Remove the weed using magnet rod

4: Centrifuge the tube with the tissue and TRIzol mix at for **10 minutes** at 12000 x g at 4^0C

5: keep supernatant in fresh Eppendorf tube and add 200 µl chloroform, mix well (Vortex for 15s) and incubate at room temperature for **5 minutes**

6: Centrifuge Eppendorf tube for **15 min**. at 12000 x g at 4^0C

7: Carefully, transfer aqueous phase to a fresh tube and add 600 µl 70% ethanol and mix well

8: Now use Column from Qiagen RNA kit and solution from line number 7 (600 µl at once) twice and centrifuge for **30 s** at 8000 x g at 4^0C.

9: Add 700 µl RW1 buffer to column and centrifuge for **30 s** at 8000 x g at 4^0C

10: Add buffer 500 µl buffer RPE and centrifuge for 30 s at 8000 x g at 4^0C

11: Add buffer 500 µl buffer RPE and centrifuge for **2 minutes** at 8000 x g at 4^0C

12: Add the column in new fresh Eppendorf tube and add 30-40 µl RNAs free water and centrifuge for **1minute** at 8000 x g at 4^0C.

13: Remove the column and keep RNA at -20^0C for further use.

Appendix 05: RNA concentration used for the study

Name	260/280	260/230	ng/µL	Sample	dH2O	Sample x4	dH2O x4
PV2-1	2,172	2,103	1966,291	0,51	9,49	2,0	38,0
PV2-2	2,15	2,091	2094,581	0,48	9,52	1,9	38,1
PV2-3	1,441	1,445	2998,482	0,33	9,67	1,3	38,7
PV2-4	1,936	2,001	2856,407	0,35	9,65	1,4	38,6
PV2-5	2,184	2,212	1765,921	0,57	9,43	2,3	37,7
PV2-6	1,882	2,011	2923,377	0,34	9,66	1,4	38,6
PV2-7	2,003	2,047	2860,749	0,35	9,65	1,4	38,6
PV2-8	2,161	2,302	1763,128	0,57	9,43	2,3	37,7
PV2-9	1,794	1,86	3022,07	0,33	9,67	1,3	38,7
PV2-10	2,236	2,094	1113,099	0,90	9,10	3,6	36,4
PV2-11	2,164	2,363	2337,412	0,43	9,57	1,7	38,3
PV2-12	2,141	2,305	2534,781	0,39	9,61	1,6	38,4
PV2-13	2,206	2,411	2057,609	0,49	9,51	1,9	38,1
PV2-14	2,22	2,423	1719,436	0,58	9,42	2,3	37,7
PV2-15	2,221	2,301	1061,649	0,94	9,06	3,8	36,2
PV2-16	2,2	2,391	1585,87	0,63	9,37	2,5	37,5
PV2-17	2,181	2,266	2063,471	0,48	9,52	1,9	38,1
PV2-18	2,194	2,336	1882,882	0,53	9,47	2,1	37,9
PV2-19	2,196	2,299	1035,011	0,97	9,03	3,9	36,1
PV2-20	2,199	2,385	1997,965	0,50	9,50	2,0	38,0
PV2-21	1,512	1,555	3037,237	0,33	9,67	1,3	38,7
PV2-22	2,207	2,367	1892,149	0,53	9,47	2,1	37,9
PV2-23	2,239	2,222	976,608	1,02	8,98	4,1	35,9
PV2-24	2,232	1,482	1188,855	0,84	9,16	3,4	36,6
PV2-25	2,167	1,737	2509,471	0,40	9,60	1,6	38,4
PV2-26	2,239	2,124	1962,681	0,51	9,49	2,0	38,0
PV2-27	2,202	2,098	2130,482	0,47	9,53	1,9	38,1
PV2-28	2,046	2,137	2845,482	0,35	9,65	1,4	38,6
PV2-29	2,2	2,241	2226,304	0,45	9,55	1,8	38,2
PV2-30	2,058	2,125	2813,839	0,36	9,64	1,4	38,6
PV2-31	2,097	2,181	2782,343	0,36	9,64	1,4	38,6
PV2-32	2,22	2,351	2165,87	0,46	9,54	1,8	38,2
PV2-33	2,101	2,115	2532,711	0,39	9,61	1,6	38,4
PV2-34	1,873	1,899	2822,667	0,35	9,65	1,4	38,6
PV2-35	2,166	2,18	2324,642	0,43	9,57	1,7	38,3
PV2-36	2,121	1,877	2310,087	0,43	9,57	1,7	38,3
PV2-37	2,167	2,216	2432,675	0,41	9,59	1,6	38,4
PV2-38	1,902	1,998	2924,956	0,34	9,66	1,4	38,6
PV2-39	2,141	1,951	2492,819	0,40	9,60	1,6	38,4
PV2-40	2,184	2,119	2491,233	0,40	9,60	1,6	38,4
PV2-41	2,21	2,075	189,471	5,28	4,72	21,1	18,9
PV2-42	2,193	2,308	2392,571	0,42	9,58	1,7	38,3
PV2-43	2,232	2,263	1349,254	0,74	9,26	3,0	37,0
PV2-44	2,211	2,14	1655,57	0,60	9,40	2,4	37,6
PV2-45	2,14	2,161	2644,043	0,38	9,62	1,5	38,5
PV2-46	2,261	2,099	1422,777	0,70	9,30	2,8	37,2
PV2-47	2,223	2,403	1685,076	0,59	9,41	2,4	37,6

Appendix 06: Vaccine producer's description

Aquavac PD7 vet.

MSD Animal Health
Vaksine til fisk.
ATCvet-nr.: QI10A L05

Egenskaper | Indikasjoner | Bivirkninger | Forsiktighetsregler | Interaksjoner | Drektighet / Laktasjon | Dosering | Overdosering / Forgiftning | Tilbakeholdelsestider | Oppbevaring og holdbarhet | Pakninger

INJEKSJONSVÆSKE, emulsjon til atlantisk laks: *1 dose (0,1 ml) inneh.:*Inaktivert SPDV (Salmon pancreas disease virus) stamme F93-125 ≥75% RPP, inaktivert, infeksiøs pankreasnekrosevirus (IPNV) serotype Sp ≥1,5 ELISA-enheter, inaktivert Aeromonas salmonicida subsp. salmonicida ≥10,7 log_2ELISA-enheter, inaktivert Vibrio salmonicida ≥90 RPS, inaktivert Vibrio anguillarum serotype O1 ≥75 RPS, inaktivert Vibrio anguillarum serotype O2a ≥75 RPS, inaktivert Moritella viscosa ≥5,8 log_2 ELISA-enheter. Adjuvans: Parafin, lett flytende.

Egenskaper

Klassifisering: Inaktivert virus- og bakterievaksine.
Virkningsmekanisme: Stimulerer til aktiv immunitet mot pankreassykdom, infeksiøs pankreasnekrose, furunkulose, kaldtvannsvibriose, vibriose og vintersår. Begynnende immunitet: 500 døgngrader etter vaksinering for de bakterielle antigenene og SPDV, og 608 døgngrader etter vaksinering med IPNV. Varighet av immunitet: Minst 18 måneder for de bakterielle antigenene. Minst 16 måneder for SPDV. Ikke dokumentert for IPNV.

Indikasjoner

Aktiv immunisering av atlantisk laks for å redusere kliniske symptomer, virusutskillelse og dødelighet forårsaket av infeksjoner med SPDV (pankreassykdom), og for å redusere kliniske symptomer og dødelighet forårsaket av infeksjoner med IPNV (infeksiøs pankreasnekrose), Aeromonas salmonicida subsp. salmonicida (furunkulose), Vibrio salmonicida (kaldtvannsvibriose), Vibrio anguillarum serotype O1 og O2a (vibriose) og Moritella viscosa (vintersår).

Bivirkninger

Oljeadjuvanser øker risikoen for bivirkninger etter vaksinering i form av sammenvoksinger og pigmenteringer i bukhulen. Vanligvis sees moderate forandringer som kan fjernes manuelt, og som normalt ikke medfører nedklassing ved slakt. Forandringene har typisk et gjennomsnitt på Speilberg score ≤2, med en spredning i enkeltfisk fra 1-3. Mer omfattende forandringer (Speilberg score 4) kan forekomme, normalt hos <3% av vaksinert populasjon. Vaksinasjon kan gi redusert appetitt den første tiden etter vaksinering.
Rapportering av bivirkninger

Forsiktighetsregler

Skal ikke brukes til syk eller svekket fisk, fisk som får medisinsk behandling eller på fisk som er under smoltifisering. Vaksinering skal ikke skje ved temperaturer <2,5°C eller >17°C. Vaksinering ved høye vanntemperaturer (≥17°C) eller av fisk under anbefalt vekt kan gi flere lokale reaksjoner. Feil vaksinering, stress eller dårlig hygiene kan gi flere bivirkninger. Personlig beskyttelsesutstyr som kanylebøyle bør brukes ved håndtering av preparatet. Ved utilsiktet egeninjeksjon, søk straks legehjelp og vis legen pakningsvedlegget eller etiketten.

Interaksjoner

Sikkerhet og effekt er ikke undersøkt hos fisk som får fôrtilskudd som hevdes å stimulere immunsystemet. Det er i enkelte tilfeller sett defekter i og rundt ryggraden i vaksinert fisk som har fått slikt fôrtilskudd, uten at en klar sammenheng er vist.

Drektighet/Laktasjon

Fertilitet: Skal ikke brukes til stamfisk da mulig innvirkning på gyteevnen ikke er undersøkt.

Dosering

0,1 ml pr. fisk >30 g. Fisken skal sultes i minst 2 døgn og bedøves før vaksinering. ***Administrering:*** Rist flasken godt før bruk. Kanylelengde og -diameter skal tilpasses fiskens størrelse. Injiseres intraperitonealt langs midtlinjen, ca. 1 bukfinnelengde foran bukfinnens basis. Påse at dosen er deponert i bukhulen før kanylen trekkes ut. Se pakningsvedlegg.

Overdosering/Forgiftning

Overdose kan gi flere lokale reaksjoner

Appendix 07: Protocol

AQUAMEDIC.NO

PROTOCOL FOR A FRESHWATER SALMON FINGERLING TRIAL

Modulation of inflammatory and immune responses by a beta-glucan (Macrogard®)

AQUAMEDIC.NO

Authors:
Ana Carolina Sulen Tavara (DVM), NMBU School of Veterinary Medicine, Institute of Production Animal Sciences

Dr. Paul J. Midtlyng, NMBU School of Veterinary Medicine, Institute of food Safety and Infection Biology

CONFIDENTIAL

www.aquamedic.no
info@aquamedic.no

Gaustadalléen 21
0349 Oslo, Norway
Tel: +47 97 06 18 60

BACKGROUND

The specificity of the protection induced by vaccines and the possibility of the induction of resistance, to chemotherapy, has recently led immunologists to investigate immunostimulation as an alternative strategy in disease prevention and control.

It has been suggested that the immunostimulant activity of β-glucans, at least in part is mediated by the modulation of the pro-inflammatory cytokines and chemokines gene expression profile. For example, Selvaraj et al, reported that β-glucan injection induced the gene expression of interleukin (IL)1β in carp head kidney macrophages. Also, in rainbow trout (Oncorhynchus mykiss), il1β and il6 transcript levels increased in liver, head kidney and spleen after β-glucan injection (Lovoll et al. 2007). In vitro studies showed that treatment with β-glucan of head kidney leukocytes from trout up-regulated il1β, il6, il10 and tumor necrosis factor α (TNFα) and decreased transforming growth factor β (TGFβ) transcript levels (Chettri et al 2012). Similar results were obtained for il1β and il6 expression levels when treating trout head kidney macrophages.

In previous, β-glucan supplemented diet administered to common carp decreased the transcript levels of several pro-inflammatory cytokines in gut and head kidney tissues. The infection with *A. salmonicida* did not modify this tendency in gut, but levels of tnfα1, tnfα2, il1β and il6fam became significantly higher in fish fed β-glucan supplemented diet at 6 h post-infection. Such differential effects may reflect the complex interactions between the bacterium and the immunostimulant's effect on the inflammatory response of the host. Analysis of the antibody response however revealed at one month after injection antibody levels against *A. salmonicida* were 4-times lower in this group. Further research on the molecular aspects of the different β-glucan forms, dose, duration, administration and experimental models (fish-pathogen) are needed to better understand the immunological activities of this immunostimulant (Falco et al. 2012).

STUDY OBJECTIVE(S)

The objective of this experiment is to evaluate if, and to which degree a well-established functional feed ingredient (β-1,3/1,6 glucan; Macrogard®) is able to modulate selected inflammatory mechanisms in freshwater reared Atlantic (*Salmo salar* L.).

EXPERIMENTAL FISH AND STUDY SITE

The *in vivo* phase of the study (the feeding trial) will be initiated using Atlantic salmon parr at a starting weight of approx. 120 gram. The trial will be carried out at the "Fiskelaboratoriet" at the NMBU Campus Ås.

GROUPS, MARKING, DISTRIBUTION IN TANKS OR NET PENS

The recruitment population has been hatched and raised in the Ås experimental facility. The study fish will be recruited, individually weighed and carefully allocated to 4 tanks each containing a biomass of approximately 3000 grams. Start weights and allocation to tanks will be documented.Water supply will be fresh water at a temperature of 12°C ± 2°C. Water flow, feeding and husbandry will be conducted according to the facility procedures, that are in conformity with Norwegian regulations on fish welfare.

TREATMENT TO BE STUDIED

A standard salmon parr feed, selected not to contain beta-glucan or yeast-derived ingredients, will be used as the base feed for the study. Two tanks will be given the base formulation (without Macrogard®), while the same feed top coated with Macrogard®at the rate recommended by the manufacturer (1g / kg feed). Fish oil will be used as a sealant for top coating. Preparation of test and control feeds will be carried out and documented following standardised Aquamedic procedures. Administration of the test or control diet will commence at 1% of the tank biomass daily, increasing to 1,8% of the tank biomass in accordance with appetite. Feeding of the study diets will be continued for at least 8 weeks before the final sampling, and will be documented according to facility procedures.

RANDOM ALLOCATION OF TEST OR CONTROL FEED

Allocation of test or control feed to duplicate tanks containing study fish will be performed randomly using a

flip-coin procedure, and will be documented through a note in the study file. Each tank will be kept on the same feed until termination of the study and final samplings.

VACCINATION

After at least 3 weeks of feeding the study diets, a fraction of each study group will be sampled, while the remaining fish will be vaccinated using a commercially available, multivalent vaccine for salmon. Vaccination will be carried out under anaesthesia, according to common industry practices, and documented in the study master file. Eight fish per dietary group will, however undergo mock vaccination to serve as controls during the final sampling.

OBSERVATIONS, MEASUREMENTS AND SAMPLING

The fish will be observed daily and removal of dead or moribund fish, feeding, and husbandry routines will be carried out and recorded as per the facility's standard procedures. Any signs of illness or abnormal behaviour will be immediately reported to the Investigator. Husbandry recording sheets will be documented in the study master file.

At immunisation, four fish from each (tank will be sampled to obtain pre-immunisation blood and tissue samples. Individual fish weights will be recorded during each sampling after feeding of the test diets has commenced, as will intraabdominal vaccine reactions using the Speilberg scoring sheet (Midtlyng et al. 1995).

Tissues to be sampled:

Tissue to be sampled	For which assay?	Storage/conservation
Skin mucus	Lysozyme assay	-70°C
Head Kidney	qRT-PCR for	RNA Later
Spleen	qRT-PCR for	RNA Later
Distal intestine	qRT-PCR for	RNA Later

The study will be terminated upon completion with final terminal sampling of blood and tissues for *in vitro* immune and cytokine assays, and measurements of weight.

SAMPLE ANALYSES AND STATISTICAL EVALUATION

The samples collected during the study will be analysed for the following outcomes, following standardized procedures to supplied by the collaborating partners:

1. Lysozyme activity in blood will be compared between study groups using *Micrococcus luteus* assay (collaboration with Dr. Henning Sørum, NMBU School of Veterinary Medicine).
2. Expression of selected inflammatory cytokine genes in tissue from the injection-site will be quantified by real-time RT-PCR and compared between immunised fish from both feeding groups. (Collaboration with Section for Aquamedicine, NMBU School of Veterinary Medicine; Dr.Hetron Muna'gandu).

TRIAL TIMELINE

Week #	Activity
41	Commencement of trial feeding; zero sampling
48	Collecting of fish for pre-vaccination blood and tissue samples (1 sampling
49	4-7 days after vaccination (2nd sampling)
53	28-30 days after vaccination (3rd sampling)
04	45 days after vaccination (4th sampling)
6-7	60 days after vaccination (5th sampling), final sampling of blood and tissues including from the unvaccinated fish

CALCULATION OF ANIMAL NUMBERS:

	β-glucan feed	control feed	total	Date/day
Pre-feeding sample	4	4	8	0
Pre- vaccination sample	8	8	16	28-30
4-7 days post vaccination	10	10	20	32-37
28-30 days post vaccination	8	8	16	56-60
45-50 days post vaccination	8	8	16	73-80
≥60 days post vaccination	16	16	32	88-90
Total	**54**	**54**	**108**	

LITERATURE REFERENCES:

Chettri J.K., Raida M.K., Kania P.W. & Buchmann K. (2012) Differential immune response of rainbow trout (Oncorhynchus mykiss) at early developmental stages (larvae and fry) against the bacterial pathogen Yersinia ruckeri. Developmental and Comparative Immunology 36,463–

Falco A, Frost P, Miest J, Pionnier N, Irnazarow I, Hoole D. Reduced inflammatory response to Aeromonas salmonicida infection in common carp (Cyprinus carpio L.) fed with β-glucan supplements. Fish Shellfish Immunol. 2012 Jun;32(6):1051-7. Epub 2012 Mar 2.

Løvoll M., Johnsen H., Boshra H., Bøgwald J., Sunyer J. O., Dalmo R. A. (2007). The ontogeny and extrahepatic expression of complement factor C3 in Atlantic salmon (*Salmo salar*). Fish Shellfish. Immunol. 23, 542–552 10.1016/j.fsi.2007.01.002

Paulsen SM, Engstad RE, Robertsen B. Enhanced Lysozyme Production in Atlantic Salmon (*Salmo salar* L.) Macrophages Treated with Yeast Beta-Glucan and Bacterial Lipopolysaccharide. *Fish & Shellfish Immunology* 2001, 11(1):23-37.

Raa J, Rørstad G, Engstad R, Robertsen B. The Use of Immunostimulants to Increase Resistance of Aquatic Organisms to Microbial Infections. In: *Diseases in Asian Aquacultur.* pp. 39-50 (I.M. Shariff, R.P.Subasinghe, and J.R. Arthus, Eds.). Manila, Philippines: Fish Health Section, Asian Fisheries Society (1992).

Selvaraj V, et al. Administration of yeast glucan enhances survival and some non-specific and specific immune parameters in carp (Cyprinus carpio) infected with Aeromonas hydrophila. Fish Shellfish Immunol. 2005 Oct;19(4):293-306. Epub 2005 Apr 18.

SIGNATURES:

Ana Carolina Sulen Tavara (DVM)

29/11 - 2017

Dr. Paul J. Midtlyng, PhD

Appendix 08: Links

Buffodine: https://www.pharmaq.no/sfiles/45/5/file/pis_buffodine.pdf
AquaGen: https://aquagen.no/en/

Fishguard: https://fishguard.no/about-fishguard/services/

Chemical:
https://pdfs.semanticscholar.org/3207/fb26ed160ea3cd2424340d1796f9da3f9e15.pdf

Feed formulae (ingredient composition):

http://www.fao.org/fileadmin/user_upload/affris/docs/Atlantic_Salmon/table_3.htm

NOFIMA: https://nofima.no/nyhet/2015/08/kostnadsdrivere-i-oppdrett/

NOFIMA:http://www.kontali.no/public_files/docs/Rapport_nr_41-2015_-Kostnadsdrivere_i_lakseoppdrett.pdf

DN (Dagens Næringsliv)https://www.dn.no/nyheter/2017/08/17/1249/Havbruk/parasitten-ingen-kan-knekke

EUMOFA: http://www.eumofa.eu/

Appendix 09: List of primer used for real time PCR

Gene	Primer	Length(bp)	Tm^{0}C	GC %
TNFα3	FWD-AGA TAT TTA GGC GAA CAT TCA GTT	24	55.9	33.3
	REV-TGA CTC AGA GGA GTG GTA CG	20	59.4	55
TGF β	FWD -AGT TGC CTT GTG ATT GTG GGA	21	57.9	47.6
	REV-CTC TTC AGT AGT GGT TTG TCG	21	57.9	47.6
IL-1β	FWD -CGT CAC ATT GCC AAC CTC AT	20	57.3	50
	REV-ACT GTG ATG TAC TGC TGA AC	20	55.2	45
B-actin	FWD -CCA GTC CTG CTC ACT GAG GC	20	63.4	65
	REV-GGT CTC AAA CAT GAT CTG GGT CA	23	60.6	47.8
FOXP3	FWD -AGC TGG CAC AGC AGG AGT AT	20	59.4	55
	REV-CGG GAC AAG ATC TGG GAG TA	20	59.4	55
IL-6	FWD -GGA GGA GTT TCA GAA GCC CG	20	61.4	60
	REV-TGG TGG TGG AGC AAA GAG TCT	21	59.8	52.4
IFNγ	FWD- CTA AAG AAG GAC AAC CGC AG	20	57.3	50
	REV-CAC CGT TAG AGG GAG AAA TG	20	57.3	50

Appendix 10: Coating procedure

Mixer containing the base feed, ready to be coated with Macrogard

Study:	BGD
Sponsor:	Aquamedic AS
Date:	aug.17

Base Feed	
Name: Nutra Olympic 2,0	Manufacturer: Skretting AS
Pellet size: 2mm	Expiry date: 16.08.18
Feed batches to be made: 02	Batch weight: 4Kg

Coating ingredient	
Name: Macrogard	Manufacturer: Biorigin

Inclusion rate (%)	Ingredient required (g)	Ingredient/Base feed(g/Kg)
0,10 %	*4*	1g/1Kg

Coating oil & Sprayer	
Amount of oil required(ml): 40 ml	Duration of spraying(s):
1% of base feed weight	*08 seconds*
Spray nozzle opening:	Spray rate (ml/min)
full	300 ml min^{-1} 40 ml in 08 seconds

Drum mixer	
Duration of mixing after adding coating ingredient:	Duration of mixing after spraying oil:
60 seconds	60 seconds

Inclussion form, filled during coating procedure.

Coating protocol:

MACROGARD

Standard Operating Procedure
SOP no:
Date issued:
Date last revision:
Pages:

Name: Feed Pellet Coating protocol

1. – Objective

The Coating of feed formulations with different additives is going to be described in detail, through this practical and easy description is expected to avoid variation in the procedure that may affect the quality of the final product.

2. - Materials & Equipment

Core materials: Fish feed pellets Nutra Olympic 2mm

Coating ingredient: Macrogard®

Sealant: Fish oil

Scale for Kg and for grams

Tablespoon

Plastic containers and labels for the weighed products

Oil aspersion device

Sieve to evenly distribute the coating ingredient

Drum mixer electric, 50 Kg capacity

Plastic foil and elastic rope to cover the mixer opening while operating

Buckets with soap and water to clean the mixer after used.

3. – Procedure

Get an open location with access to electric contact, enough light and ventilation, the room temperature must be over 15 degrees.

Coating process

Air was sprayed from the aspersion device to be sure is delivering the amount needed to cover the core materials.

Check the drum mixer, eventually remove some dust that could be contained and make sure the drum is perfectly clean before purring down the weighed core material, turn on the drum mixer and start the aspersion evenly while the drum is rotating, after the oil was delivered stop the drum mixer and close it with the plastic foil and the elastic rope, keep mixing for another 3 minutes, stop the machine remove the plastic foil and spray the rest of the sealant, close the drum and mix for the last 3 minutes until the control feed is ready, remove the content and place it on the labeled bucket. Once the drum is empty, clean the surface and make it ready for the next round, pur the weighed core material and spray it with the fish oil turn on the drum mixer during 3 minutes, after that straw the coating ingredient, cover the drum opening with the plastic foil and the elastic rope and turn it on for 3 minutes, after the even distribution of the product the second fish oil spraying is performed and manually rotate the drum until all the pellets are exposes d to the sealant,

Close the drum opening and turn it on during 3 minutes until the process is finished.

This condition gave more uniform pellets with smaller variation in the finished product, collect the treatment feed into the labeled bucket.

At the end of the procedure make sure everything is properly cleaned specially the drum, using warm water and soap to remove any particle of feed or fish oil, place back in its original location.

Norges miljø- og biovitenskapelige universitet
Noregs miljø- og biovitskapelege universitet
Norwegian University of Life Sciences

Postboks 5003
NO-1432 Ås
Norway