An Roinn Talmhaíochta,
Bia agus Mara
Department of Agriculture,
Food and the Marine

15S745–
Amoebic disease of salmon

Final Report

This project was funded under the Department of Agriculture, Food and the Marine Competitive Funding Programme.

SUMMARY

As with most intensive aquaculture systems, economic viability is continually challenged by disease and cage-reared mortality rates of up to 20% have been estimated to occur within the Irish Atlantic salmon (Salmo salar) industry base. Of greater concern to industry in Ireland are the estimated loss (12%) incurred specifically to gill disease and is now considered to constitute one of the major health challenges in salmonid farming regions. In terms of economic impact, the most significant disease caused by gill parasites is amoebic gill disease (AGD). The main objectives of the ADIOS project were to:

1. Increase knowledge of Amoebic Gill Disease and pathogenic organisms associated.
2. Increase knowledge of host immune response to N. perurans.
3. Developing preventative measures to minimise disease.
4. Effective dissemination and exploitation:

Gill microbiome analysis was found to differ depending on the geographical location of the farm, with the prokaryotic community on salmon gills shifting toward lower diversity and less balance with the onset of disease. The outcomes are relevant to practitioners applying freshwater bathing strategies and monitoring AGD outbreaks. Host immune analysis determined that N. perurans appears to suppress the salmon's immune response on infection, inhibiting the development of either an innate or adaptive immune response. The initial discovery and description of immune regulation provides the basis for future studies to elucidate the mechanisms behind this potential immune evasion strategy. Several pathogen virulence factors were identified demonstrating cytoskeletal, oxidative and immunomodulatory roles. The discovery of these novel virulence factors hold great potential for the development of targeted therapies which are currently under development. An off-the-shelf feeding solution was demonstrated to show the potential to reduce the disease progression in salmon and holds great potential as an immediate solution available to industry.

KEYWORDS

Amoebic Gill Disease

ACRONYM	ADIOS
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PUBLICATION DATE	29/03/2023

Section 1 - Research Approach & Results

Start Date

01 March 2017

End Date

31 December 2020

Research Programme

Research Stimulus Fund

TRL Scale

TRL 5: Technology validated in relevant environment

NRPE Priority area

Sustainable Food Production and Processing

Total DAFM Award

€601,346.88

Total Project Expenditure

€545,918.13

Rationale for undertaking the Research

Aquaculture presents Europe with an opportunity to reduce dependence on imported seafood and provides Ireland with an opportunity to enhance our seafood exports both within the EU and to developing markets worldwide. This potential is reflected in both Irish government (Food Wise report 2025 (DAFM); Food Harvest 2020 (DAFM); NRPE (Forfás)) and EU Maritime and Fisheries Fund policy documents. Various aetiological agents are associated with mortalities in salmon; however, in terms of economic impact the most significant is Neoparamoeba perurans, also called Paramoeba perurans, (causative agent of amoebic gill disease (AGD)). First documented in Ireland in 1995 AGD is now estimated to potentially wipe out up to 25% worth of European production in 2016/2017 with the prospective of increasing its impact over the coming years. AGD prevalence and pathogenicity continues to increase significantly and is now regarded as the most serious problem affecting the future of the salmon industry in Ireland and Europe.

In conjunction with producers, state agencies, veterinary community and other HEIs this project set out a programme of research activities to address the knowledge gaps and enhance our understanding of: the host pathogen pathways and other causative agents associated with the disease; the host response (acute phase response) to initial N. perurans infections and re-infections (adaptive immune response); and the variation in tolerance/resistance to the disease observed on-site between two strains/families.

.**Methodology**

The programme of research undertaken was broken into 7 tasks. The first task focused on gill microbial communities in farmed salmon and their association with disease. Sample gill samples were collected from a fish farm. 16S rRNA gene sequencing and N. perurans 18S rRNA gene quantification was conducted. Task 2 focused on Gill microbiome response of AGD susceptible and resistant Atlantic salmon to challenge by N. perurans. Six fish farms along the western coast of Ireland were sampled from June to December 2018.

Task 3 investigated the acute phase response in salmon infected (sub-clinical) with N. perurans. An experiment was conducted and differential transcriptomic and proteomic expression analysis was performed using NextGen Sequencing and gel free approach, with mass spectrometry. Task 4 was unfortunately could not be completed due to Covid mitigation policies.

Task 5 investigated the host immune response in salmon re-infected with N. perurnas. Fish were challenged with N. perurans (500 amoebae/L) and 28 days after freshwater treatment the remaining fish were re-inoculated with N. perurans. Gill histology, disease progression and gene expression analysis was conducted on the samples obtained.

Task 6 Investigated the use of dietary supplements and evaluate their performance in mitigating AGD. Negative and positive control fish were maintained on Feed A throughout the duration of the trial with experimental fish maintained on Feed B and maintained for 21 days prior to infection challenge with N.perurans, and sampled over a 3 week period. Gill histology, disease progression and gene expression analysis was conducted on the samples obtained. The primary aim of task 7 was to identify and characterise the virulence factors of N. perurans (both amoebic and bacterial). The virulence of clonal-cultures was established using an in vivo experiment prior to conducting proteomic techniques to investigate differentially proteins exhibiting virulent like properties.

Project Results

From the gill microbiome analysis (task 1) it was determined that after the onset of a gill disease, the prokaryotic community on salmon gills shifted toward lower diversity and less balance. The data from Task 2 found that the gill microbiome was different depending on the geographical location where the gills were sampled. The outcomes are relevant to practitioners applying freshwater bathing strategies and monitoring AGD outbreaks. The results potentially underpin the future development of assays targeting indicator microbial species associated with AGD episodes.

The gill transcriptomic profile of pre-clinical AGD using RNA-sequencing technology was characterised (task 3). A host-immune response through the activation of complement and the acute phase genes was evident with a concurrent immune suppression involving cytokine signaling, notably in interleukins, interferon regulatory factors and tumour necrosis factor-alpha genes. The results support the theory that N. perurans can evade immune surveillance during the initial stages of gill colonisation through interference of signal transduction pathways. The initial discovery and description of cytokine down-regulation during the amoeba attachment phase of early-stage AGD in the gill of salmon provides the basis for future studies to elucidate the mechanisms behind this potential immune evasion strategy.

Investigation of AGD in salmon gill and serum, at a proteomic level, across various sub-clinical and clinical time points has provided a novel insight into protein expression in very early disease stages (task 3). The results demonstrated a lack of a robust classical immune response, and many of the differentially expressed proteins identified have suppressive roles in the immune response. The pro-immune trend observed in serum and the diversity of down-regulated proteins in the gill may indicate a local pathogen derived suppression of host immune response. This study has identified many proteins which may have significant roles in the host response to AGD contributing to our understanding of the underlying pathology of the disease.

The immune response of Atlantic salmon (task 5) re-infected with N. perurans found proteins of the innate immune system were expressed in response to gill re-colonization by N. perurans, while no features of adaptive immunity were found to be differentially expressed.

The effect of a novel feed formulation (task 6) on the rate of AGD progression, as assessed by gill score, was significant, with Feed B slowing disease progression and reducing the number of positive fish when compared to standard feed. More extensive studies in an industry setting are required to validate the findings of this study and to enhance our understanding of the mechanisms underlying the protective action observed in this study.

An in vivo experimental confirmed the loss of N. perurans virulence in attenuated clones validating clones for differential proteomic analysis, revealing that hydrophilic virulence proteins are differentially expressed between a virulent-and attenuated culture. Further analysis indicated elevated levels of proteins involved in cytoskeletal, oxidative and immunomodulatory roles. Proteins involved in potential cell-mediated adhesion roles such as as GAPDH and GTPase signaling proteins were also found and may represent putative targets of treatment.

Section 2 - Research Outputs

Summary of Project Findings

A multidisciplinary approach was undertaken to address key gaps in our understanding of the host response to the parasite and the dynamic between the host-pathogen and its environments. Gill microbial communities analysis has led to the identification of bacteria species associated with the onset of AGD and provides targets for the development of molecular diagnostic assays and the monitoring of susceptible cohorts of fish. Deep sequencing also revealed the variation of the gill microbiome between geographical located sites, further underpinning the potential of assays targeting indicator microbial species with AGD episodes.

Various scientific approaches were undertaken to elucidate the salmon host immune response prior to the on-set of clinical symptoms associated with AGD and salmon re-infected N. perurans. Both transcriptomic and proteomic analysis of naïve and re-infected fish provides sufficient evidence to suggest a potential immune evasion strategy employed N. perurans. Indeed, both the transcriptomic and proteomic analysis revealed novel molecules associated with disease susceptibility offering the potential to further develop immune diagnostic assays and informing future breeding programmes. A potential option to mitigate the disease was the prospect of developing fish fed with dietary supplements to manipulate the gill microbial community and their potential to enhance the host immune response. In collaboration with an industry partner, an off-the-shelf feeding solution was shown to delay the on-set of clinical symptoms. For the industry, this would have significant impact in mitigating the disease and reducing the associated costs of freshwater treatments.

Further host-parasite analysis revealed a number of virulence factors associated with N. perurans. These findings are very novel and provides input to a number of potential membrane proteins and their association with immunomodulation. Further research is currently developing vaccines for each of the virulence factors identified.

Summary of Staff Outputs

Research Output	Male	Female	Total Number
PhD student	1	1	2
Post Doctorates		2	2
Research Technicians/ Assistants	1	1	2

Summary of Academic Outputs

Research Output	Total Number	Details
Publications in Peer Reviewed Scientific Journals	7	Birlanga, V. B., McCormack, G., Ijaz, U. Z., MacCarthy, E.,Smith, C. & Collins, G. (2021), Dynamic Gill and mucus microbiomes track an amoebic gill disease episode in farmed Atlantic salmon. Scientific Reports (Accepted) Ní Dhufaigh, K., Botwright, N., Dillon, E., O'Connor, I., MacCarthy, E. & Slattery, O. Slattery (2021), Differential exoproteome and biochemical characterisation of Neoparamoeba perurans. Microorganisms 9(6), 1258; DOI: 10.3390/microorganisms9061258 Ní Dhufaigh, K., Dillon, E., Botwright, N., Talbot, A., O'Connor,I., MacCarthy, E. & Slattery, O. (2021), Comparative proteomic profling of newly acquired, virulent and attenuated Neoparamoeba perurans proteins associated with amoebic gill disease. Scientific Reports 11, Article number: 6830; doi.org/10.1038/s41598-021-85988-8 Talbot, A. T., Gargan, L., Moran, G., O'Connor, I., Mirimin, L.Carlsson, J., & MacCarthy, E. (2021) Transcriptomic profiling of Atlantic salmon (Salmo salar L.) gill with pre-clinical Amoebic Gill Disease: Immune suppression via nuclear factor–kappa beta (NF-κβ)? Scientific Reports doi.org/10.1038/s41598-021-99996-1 McCormack, M., Talbot, A., Dillon, E., O Connor, I.,MacCarthy, E. (2021), Investigation of the host response of naïve Atlantic salmon gill to repeat inoculation with Paramoeba perurans Microorganisms https://doi.org /10.3390/microorganisms9050993 McCormack, M., Dillon, E., O Connor, I., MacCarthy, E. (2021),Investigation of the host response of naïve Atlantic salmon (Salmo salar) inoculated with Paramoeba perurans prior to the onset of clinical symptoms. Microorganisms https://doi.org/10.3390/microorganisms9040746. Ní Dhufaigh, K., Dillon, E., Botwright, N., Talbot, A., O'Connor,I., MacCarthy, E. & Slattery, O. (2021), Comparative proteomic profiling of newly acquired, virulent and attenuated Neoparamoeba perurans proteins associated with amoebic gill disease. Scientific Reports DOI https://doi.org/10.1038/s41598-021-85988-8
PhD Theses	2	Kerrie NiDhufaigh Phd Thesis Virulence factors associated with amoebic gill disease from Neoparamoeba perurans, GMIT Victor B. Birlanga May 2022 PhD thesis Microbial Community Dynamics of farmed Atlantic Salmon Gill Microbiome During Amoebic Gill Disease Episodes, NUIG
Other	0	Technical Article: Fish farmer Magazine: ADIOS to AGD https://issuu.com/fishfarmermagazine/docs/fish_farmer_magazine_december_2018/1?ff
Training Courses	0	EAFP Porto 2019 histopathology workshop Gill Health initiative 2018 AGD workshop Sure Network 2018 Aquaculture Europe 2019, Berlin. International Society for Microbial Ecology Conference (2018) International Workshop of PhDs and Post-doctoral Fellows on Anadromous Salmonidsmeeting (March 2021) LAST Training GMIT Lab safety training course GMIT Project management course GMIT Research Cycle Foundation Level 9 (ongoing) ELDAed- Next Generation Sequencing Theory and Practice Professional Cert in Bioinformatics Bioinformatics (Glasgow University) Digital PCR

Intellectual Property

A number of host immune molecules identified have the potential for the development of reliable, sensitive, specific and widely available diagnostic tests that will have commercial value and provide decision making tools for industry. To date the research team have focused on the development of novel vaccines for virulence factors associated with the pathogen and other microbial targets associated with the on-set of the disease. To date, the team has secured €1.05M from BIM's Knowledge Gateway fund to develop vaccines for a range of targets identified through the ADIOS project.

Summary of other Project Outputs

Project Outputs	Details	Total No.
New Processes	Novel DNA extraction procedure using entire gill arches was developed. The procedure means a standardised and optimised methodology for future gill microbiome studies.	1
New Industry Collaborations Developed	The programme allowed the team engage with an global supplier of farmed salmon and also a major fish feed supplier Biomar. The expertise developed with the programme has attracted companies to collaborate with GMIT. Example of this collaboration is the direct funding for investigating nutraceuticals to mitigate AGD with Prewivo (Norway). Led to development of an MOU between Bimedia and ATU. Enterprise Ireland Innovation partnership submitted in January 2023 for the development of vaccines for AGD.	1
New Industry Collaborations Developed	Industry collaboration between Biomar and ATU has been extended. MOU with Bimeda	1
Licenses	GMIT licensed IP from CSIRO Australia to develop foreground IP.	0

Potential Impact related to Policy, Practice and Other Impacts

Impact	Details
Industry	The proposed research has built significant capacity and knowledge in Ireland to support industry that was lacking in the areas of fish proteomics, disease resistance, diagnostic technologies and treatment. Data generated from the host immune response to the pathogen provides industry an insight into the genetic traits associated with AGD susceptibility. The outputs of the grant have also led to growth in commercial activities between the university and industry. The majority of that growth has focused on the development therapeutics products such as vaccines, including clinical trials assessing novel dietary supplements. A customised seawater re-circulation experimental unit was developed by the team and installed at GMIT. This system allows industry to conduct trials in a highly controlled environment and is a unique offering to industry in Ireland and in Europe., which has been key to securing a number of industry focused projects. This commercial activity is reflected by the 3 knowledge gateway grants (€1.05M) secured by the team to develop vaccine targeting a number of bacterial strains and virulence factors associated with AGD. An MOU was also put in place with Bimedia for the development of targeted therapies for AGD.

Dissemination Activities

Activity	Details
Workshops at which results were presented	EAFP Porto 2019: 2 paper presentations Gill Health initiative 2018: 3 papers EAFP Irish and UK branch 2018: 3 papers Sure Network 2018 Aquaculture Europe 2019, Berlin

Knowledge Transfer Activities

Identify knowledge outputs generated during this project.	Novel DNA extraction procedure using entire gill arches was developed. The procedure means a standardised and optimised methodology for future gill microbiome studies. - The gill microbiome was different depending on the geographical location and 3 different genera found to be maximally relatively abundant on gills and mucus samples before the gross signs of disease appeared. - Initial discovery and description of cytokine down-regulation during the amoeba attachment phase of early-stage AGD in the gill of Atlantic salmon, and provides the basis for future, more in-depth studies to elucidate the mechanisms behind this potential immune evasion strategy. - This research demonstrated that an off-the-shelf feeding solution has the potential to delay the on-set of clinical symptoms of AGD. For the industry, this would have significant impact in reducing the number of treatments and hence the associated costs. - This tasks generated new knowledge on the virulence factors associated with the pathogen. This work is very novel and provides input to a number of potential membrane proteins and their association with immunomodulation. Indeed, the outputs of this research may be applicable to other amoeba-like pathogens.
Identify any knowledge transfer activities executed within the project.	Publication in industry focused journal Aquaculture on the impacts of nutraceuticals on the progression of disease. - Clinical trial carried out for European based company on the impact of novel product on the progression of disease. - Gill Health Initiative industry and academia workshops in 2016, 2017 and 2018. - Secured 3 ESIF and EMFF Funded Knowledge Gateways for developing and delivering vaccine therapies for the treatment of disease (Value €1.05M) - CSIRO, Australia - licence of IP and co-authored peer reviewed publications - Norwegian Veterinary Institute - development of cell line - Marine Scotland - Material Transfer Agreement - Glasgow University - Microbiome analysis - Industry/academic steering group meeting to discuss outputs of project. - MOU with Bimedia for the development of targeted therapies for AGD
List any impacts resulting from the knowledge transferred during the project.	Commercial review of ADIOS carried out by Intrigo. The feasibility investigated the to transfer commercially-relevant aquaculture research and development outputs from the research community to the Irish aquaculture sector and other target users. - Development of autogenous vaccine. - BioMar are conducting an extensive trial on the dietary supplement which delayed the on-set of clinical symptoms of AGD. For the industry, this would have significant impact in reducing the number of treatments and hence the associated costs.

Section 3 - Leveraging, Future Strategies & Reference

Leveraging Metrics

Type of Funding Resource	Funding €	Summary
EU R&I programmes	€1,050,000.00	BIM (ESIF and EMFF Fund) Knowledge Gateway €350,000, Vaccines for Irish Aqauculture sector 20/KGS/001 BIM (ESIF and EMFF Fund Knowledge Gateway €350,000, Development of an AGD vaccine for salmon based on immunisation with the membrane fraction of Neoparamoeba perurans BIM Knowledge Gateway €350,000, Vaccines for Irish Aquaculture sector 18/KGS/001 20/KGS/002
Other	€100,000.00	BIM Capital Equipment Grant for Digital PCR
Exchequer National Funding	€960,000.00	SFI Research Partnership Fund. Gill Disease of Atlantic Salmon. Partners: Leads - ATU and UCD; Collaborators - Vet Aqua International, MOWI, Biomar (Project Code 21/FFP-A/9170)

Future Strategies

The ADIOS project enabled the team to develop strong links with researcher groups based in Norway, Scotland and Australia. Due to the applied focus of the work undertaken, this has allowed the team to develop strong links with industry partners both in Ireland and across Europe. In addition to completed direct funded work with companies, the research team will continue to target national funding and European funding. To date the team has secured €2.1M from various funding bodies which leverages on the outputs from the ADIOS project. A feasibility analysis was also conducted by Intrigo at the request of BIM with the view to commercializing a range of the vaccine technologies. The plan is to either license this technology or to seek Enterprise Ireland Commercialisation Funding to start a new company to bring these technologies to the market place.

Project Publications

1) Birlanga, V. B., McCormack, G., Ijaz, U. Z., MacCarthy, E., Smith, C. & Collins, G. (2021), Dynamic Gill and mucus microbiomes track an amoebic gill disease episode in farmed Atlantic salmon. Scientific Reports (Accepted)

2) Ní Dhufaigh, K., Botwright, N., Dillon, E., O'Connor, I., MacCarthy, E. & Slattery, O. Slattery (2021), Differential exoproteome and biochemical characterisation of Neoparamoeba perurans. Microorganisms 9(6), 1258; DOI: 10.3390/microorganisms9061258

3) Ní Dhufaigh, K., Dillon, E., Botwright, N., Talbot, A., O'Connor, I., MacCarthy, E. & Slattery, O. (2021), Comparative proteomic profling of newly acquired, virulent and attenuated Neoparamoeba perurans proteins associated with amoebic gill disease. Scientific Reports 11, Article number: 6830; doi.org/10.1038/s41598-021-85988-8

4) Talbot, A. T., Gargan, L., Moran, G., O'Connor, I., Mirimin, L. Carlsson, J., & MacCarthy, E. (2021) Transcriptomic profiling of Atlantic salmon (Salmo salar L.) gill with pre-clinical Amoebic Gill Disease: Immune suppression via nuclear factor–kappa beta (NF-κβ)? Scientific Reports doi.org/10.1038/s41598-021-99996-1

5) McCormack, M., Talbot, A., Dillon, E., O Connor, I., MacCarthy, E. (2021), Investigation of the host response of naïve Atlantic salmon gill to repeat inoculation with Paramoeba perurans Microorganisms https://doi.org/10.3390/microorganisms9050993

6) McCormack, M., Dillon, E., O Connor, I., MacCarthy, E. (2021), Investigation of the host response of naïve Atlantic salmon (Salmo salar) inoculated with Paramoeba perurans prior to the onset of clinical symptoms. Microorganisms https://doi.org/10.3390/microorganisms9040746.

7) Ní Dhufaigh, K., Dillon, E., Botwright, N., Talbot, A., O'Connor, I., MacCarthy, E. & Slattery, O. (2021), Comparative proteomic profiling of newly acquired virulent and attenuated Neoparamoeba perurans proteins associated with amoebic gill disease. Scientific Reports DOI https://doi.org/10.1038/s41598-021-85988-8