Virulence factors associated with amoebic gill disease from *Neoparamoeba perurans*

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Doctor of Philosophy

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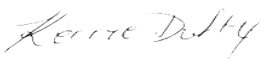
Declaration

I hereby declare that the results presented are to the best of my knowledge correct, and that this thesis represents my own original work, carried out during the designated research project period, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed:  , the candidate

ID Number: G00282066

Date: 5th October 2020

Two roads diverged in a yellow wood,
And sorry I could not travel both
And be one traveller, long I stood
And looked down one as far as I could
To where it bent in the undergrowth;

Then took the other, as just as fair,
And having perhaps the better claim,
Because it was grassy and wanted wear;
Though as for that the passing there
Had worn them really about the same,

And both that morning equally lay
In leaves no step had trodden black.
Oh, I kept the first for another day!
Yet knowing how way leads on to way,
I doubted if I should ever come back.

I shall be telling this with a sigh
Somewhere ages and ages hence:
Two roads diverged in a wood, and I—
I took the one less travelled by,
And that has made all the difference.

Robert Frost

TABLE OF CONTENTS

TABLE OF CONTENTS vi

GLOSSARY xi

LIST OF FIGURES xiii

LIST OF TABLES xv

ABSTRACT xvii

ACKNOWLEDGEMENTS xx

CHAPTER 1 22

INTRODUCTION 22

1.1. Background 23

1.2. Amoebic gill disease 24

1.2.3. Host response 31

1.3. Virulence factors 35

1.4. Proteomics 37

1.4.1. Omic technology in defining pathogenesis 38

1.4.2. Membrane proteins 39

1.4.3. Extracellular proteins 40

1.5 Aims and Objectives 42

1.6. References 46

CHAPTER 2 59

Comparative proteomic profiling of attenuated and virulent *Neoparamoeba perurans* proteins associated with amoebic gill disease....59

2.1. Abstract....60

2.2. Introduction....61

2.3. Materials and Methods....64

2.3.1. *Neoparamoeba perurans* isolation and culture....64

2.3.2. *In vivo* challenge trial....65

2.3.3. Validation of virulence....66

2.3.4. DNA sequence analysis of the *Neoparamoeba perurans* microbiome....66

2.3.4.1. DNA extraction from *Neoparamoeba perurans* cultures....66

2.3.4.2. 16S rRNA gene sequencing in Illumina....67

2.3.4.3. Sequencing analyses....68

2.3.5. Protein extraction from *Neoparamoeba perurans* cultures....68

2.3.6. Two-dimensional electrophoresis....69

2.3.7. Gel image analysis....70

2.3.8. Spot preparation and mass spectrometry....70

2.4. Results....73

2.4.1 *In vivo* challenge....73

2.4.2. 16S rRNA sequencing of attenuated and virulent-1 *Neoparamoeba perurans* microbiome....74

2.4.3 Two-dimensional gel electrophoresis and protein identification....77

2.5. Discussion....81

2.6. References 90

CHAPTER 3 97

Differential exoproteome and biochemical characterisation of *Neoparamoeba perurans* 97

3.1. Abstract 98

3.2. Introduction 99

3.3. Materials and Methods 101

3.3.1. *Neoparamoeba perurans* culture 101

3.3.2. Antibiotic treatment of *Neoparamoeba perurans* cultures 101

3.3.3. Extraction of extracellular proteome 102

3.3.4. In-solution trypsin digestion 102

3.3.5. Liquid chromatography tandem mass spectrometry 103

3.3.6. Raw data processing and label free quantification (LFQ) 103

3.3.7. Enzymatic characterisation 105

3.3.8. Enzyme inhibition assay 105

3.3.9. RTgill W1 culture and assay preparation 106

3.3.10. Cytotoxicity alamar blue preparation 107

3.4. Results 108

3.4.1. Exoproteome identification using differential proteomics 108

3.4.2. Antibiotic treated exoproteome identification 109

3.4.3. Enzymatic characterisation and inhibition assay 114

3.4.4. Cytotoxicity assay 115

3.4.5. Cytotoxicity of exoproteome from antibiotic-treated *Neoparamoeba perurans* cultures 115

3.5. Discussion 117

3.6. References 127

CHAPTER 4 131

The cell surface and membrane proteome of *Neoparamoeba perurans*, the causative agent of amoebic gill disease 131

4.1. Abstract 132

4.2. Introduction 133

4.3. Materials and Methods 135

4.3.1. *Neoparamoeba perurans* culture 135

4.3.2. Isolation of membrane bound proteins 135

4.3.3. Isolation of cell surface proteins 137

4.3.4. Protein quantification and one-dimensional (1D) gel 138

4.3.4. In gel digestion 139

4.3.5. Liquid chromatography tandem mass spectrometry 140

4.3.6. Raw data processing and label free quantification (LFQ) 140

4.3.7. In silico analysis 141

4.4. Results 142

4.4.1. One dimensional gel 142

4.4.2. Membrane protein identification 142

4.4.3. Cell-surface protein identification 153

4.5. Discussion 163

4.6. References 172

CHAPTER 5 177

General discussion 177

5. General discussion 178

5.1 Virulence factors 179

5.3 Uncharacterised proteins 186

5.4 Microbiome characterisation 188

5.5 Limitations of the study 190

5.6 Future research 192

References 195

APPENDICES 200

GLOSSARY

Term	Definition
Attenuated culture/parasite	An axenic (both amoeba and bacteria in co-culture) culture of *N. perurans* that was maintained for 3- years or more
Virulent-1 culture/parasite	An axenic culture that was maintained for 1 year
Virulent-2 culture/parasite	An axenic culture that was maintained for 70 days in culture
Virulent-3 culture/parasite	An axenic culture that was maintained for over 70 days in culture
Cyclic hypoxia	Oxygen partial pressure fluctuation from super saturated to a hypoxic condition
Digitate pseudopodia	Protrusion of amoeboid cell for movement and feeding
Hyaline ectoplasm	The translucent and dense part of cell's cytoplasm
Interlamellar vesicles	A small, intracellular, membrane-enclosed sac between the gill lamellae
Juxanuclear	Near the nucleus of a cell
Lectin	Carbohydrate-binding proteins that are highly specific for sugar groups
Glycoconjugate	Are both glycolipids and glycoproteins and are posttranslational modifications of lipids and proteins, respectively, mediated by specific enzymes
Micelles	Spherical amphiphilic structures that have a hydrophobic core and a hydrophilic shell
Exosomes	A type of extracellular vesicle that contain constituents (protein, DNA, and RNA) of the cells that secrete them

Term	Definition
Moonlighting protein	A phenomenon by which a protein can perform more than one function.
Nosocomical pathogen	An infectious agent that is contracted in a certain location, such as a hospital.
Ligand attachment	A molecule or atom which binds reversibly to a protein

LIST OF FIGURES

Chapter 1

Figure 1: AGD macroscopic gill score as outlined in Taylor et al. (2009). The images in each panel represent different levels of AGD infection. Panel A = score zero (no infection); panel B = score 1; panel C = score 2; panel D = score 3; panel E = score 4; panel F = score 5 (severe infection). Images were adapted from Taylor et al. (2015).

Figure 2: *Neoparamoeba perurans* trophozoites imaged under phase contrast microscopy (x200)**.** Image shows the attached amoebae with visible digestive vacuoles. Amoeboae in suspension are free-floating with long pseudopodia.

Chapter 2

Figure 1: An example of H&E stained gill samples taken from non-AGD and AGD infected fish from the challenge trial. Scale bar = 100 μm. A) gill sample from the attenuated cohort. B) gill sample from the virulent cohort.

Figure 2: Relative abundance of the prokaryotic community in the attenuated and virulent-1 cultures by 16S rRNA gene sequencing.

Figure 3: Rarefaction curve of the pooled virulent-1 and attenuated cultures shows a trend of consistent richness. Y axis shows amplicon sequencing variants or operational taxonomic unit against the library on the x axis.

Figure 4: 2D gel of the reference gel from the virulent-2 culture of *Neoparamoeba perurans*. 11 spots displayed differential expression and were excised for LC MS/MS.

Chapter 3

Figure 1: Hierarchical clustering of statistically significant proteins between the attenuated and virulent-3 exoproteome replicate samples. LFQ intensity values were normalised by z- scoring with red colours showing increased abundance and green displaying a decreased expression of proteins.

Figure 2: Protease activity and inhibition assay of exoproteome samples from the attenuated, virulent-3 and antibiotic treated attenuated and virulent-3 cultures using azocasein as a substrate. Trypsin (2mg/mL) was used as positive control and sterile seawater as a negative. Results are expressed as a percent of the positive control. All data sets are represented as ±SEM from biological replicates. Two-way ANOVA

followed by a Tukey post hoc test for multiple comparisons: Attenuated vs PMSF and EDTA, ****(p <0.0001); Virulent vs PMSF,*(p <0.0284), EDTA non-significant (ns); Attenuated vs Virulent, #### (p <0.0001); Attenuated Pen Strep vs PMSF ****(p <0.0001); EDTA ns; Virulent Pen Strep vs PMSF, ** (p <0.0044), EDTA ns; Attenuated Pen Strep vs Virulent Pen Strep, #### (p <0.0001).

Figure 3: Cytotoxicity assay using RTgill W1. A) 40 µg/mL of attenuated and virulent-3 exoproteome. B) 40 µg/mL of pen-strep treated attenuated and virulent-3 exoproteome. RTgill W1 cells in sterile PBS were used as positive (+) control and sterile seawater as a negative. Results are expressed as a percent of the positive control and are representative of one independent experiment. All data sets are represented as ±SEM from biological replicates. One-way ANOVA followed by Tukey post hoc test for multiple comparisons: A) Attenuated vs + control, ****(p <0.0001); Virulent vs + control, ns; Virulent vs +control, ns; Attenuated vs virulent, #### (p <0.0001). B) Attenuated Pen Strep vs + control, * (p <0.0278); Virulent Pen Strep vs + control; ns; Attenuated Pen Strep vs Virulent Pen Strep, ns.

Chapter 4

Figure 1: SDS-PAGE of A) membrane proteins and B) biotin-labelled, cell surface proteins from attenuated and virulent *Neoparamoeba perurans* cultures. Att. = protein sample from attenuated *N. perurans*. Vir. = protein sample from virulent-3 *N. perurans*. Note: 25 µg of protein loaded in each lane. Bands were excised and digested overnight with 0.5 µg trypsin.

Figure 2: Normalised cell membrane LFQ values achieved using Z- scoring of the 302 significantly expressed proteins. Hierarchical clustering was performed using Euclidean distance. Protein intensity is represented as a colour chart with red colours showing increased abundance and green displaying a decreased expression of proteins.

Figure 3: Gene ontology (GO) enrichment analysis of the 56 significantly elevated *Neoparamoeba perurans* membrane proteins from the virulent-3 culture plus two of the Amoebozoa identifications; histone 4 and cytochrome c oxidase subunit 2 of *A. castellanii str, Neff* and *P. aparasomata*, respectively.

Figure 4: Gene ontology (GO) enrichment analysis of the 133 significantly elevated proteins from the microbiome of the *N. perurans* virulent-3 culture.

Figure 5: Normalised cell- surface proteins LFQ values achieved using Z score of the 118 significantly expressed proteins of the attenuated and virulent-3 parasites. Hierarchical clustering was performed using Euclidean distance.

Figure 6: Gene ontology (GO) enrichment analysis of *Neoparamoeba perurans* significantly expressed cell- surface proteins. Proteins described are significantly elevated in the virulent-3 parasite.

Figure 7: Gene ontology (GO) enrichment analysis of *Neoparamoeba perurans* microbiome significantly expressed cell- surface proteins. Proteins described are elevated in the virulent-3 culture.

LIST OF TABLES

Chapter 2

Table 1: 2D spots with significant ($p \leq 0.05$) fold changes from the virulent-2 *Neoparamoeba perurans* culture identified by LC-MS/MS from *N. perurans*. Several proteins that are shared between *N. perurans* and species found in the bacteria and Amoebozoa database are distinguished in the organism header of Appendix IV.

Chapter 3

Table 1: All 11 *Neoparamoeba perurans* protein identifications and 1 putative homologous protein associated with *Planoprotostelium fungivorum* from MaxQuant analysis of the attenuated and virulent-3 exoproteomes, characterised by a two-way Students *t*- test in Perseus.

Table 2: Top 6 non –*Neoparamoeba perurans* proteins of bacterial origin, identified by Max Quant analysis of the attenuated and virulent-3 *N. perurans* exoproteomes. Proteins are ranked based on relevance to extracellular function. The complete set of identifications are provided in Appendix V.

Table 3: All 6 *Neoparamoeba perurans* proteins and 1 putative homologous protein associated with *Dictyostelium discoideum* identified using Max Quant analysis of the exoproteomes of pen-strep treated attenuated and virulent-3 cultures.

Chapter 4

Table 1: Top 10 significantly elevated membrane proteins based on fold enrichment in the virulent-3 *N. perurans* culture (including the associated microbiome).

Table 2: *Neoparamoeba perurans* top 10 significantly elevated membrane proteins based on fold enrichment in the virulent-3 parasite.

Table 3: Top 10 significantly reduced membrane proteins based on fold enrichment in the virulent-3 *Neoparamoeba perurans* culture (including the associated microbiome). These proteins reflect significantly elevated proteins in an attenuated culture.

Table 4: *Neoparamoeba perurans* top 10 significantly reduced membrane proteins based on fold enrichment in the virulent-3 parasite. These proteins reflect significantly elevated proteins in an attenuated culture.

Table 5: Top 10 significantly elevated cell-surface membrane proteins based on fold enrichment in the virulent-3 *Neoparamoeba perurans* culture (including the associated microbiome).

Table 6: *Neoparamoeba perurans* top 10 cell-surface membrane proteins based on fold enrichment significantly elevated in the virulent-3 parasite relative to the attenuated parasite.

Table 7: Top 10 significantly reduced cell- surface membrane proteins based on fold enrichment in the virulent-3 *Neoparamoeba perurans* culture (including the associated microbiome) relative the attenuated culture.

Table 8: *Neoparamoeba perurans* top 10 significantly reduced cell- surface membrane proteins based on fold enrichment in the virulent-3 parasite.

Chapter 5

Table 1: Summary table of all virulence associated *Neoparamoeba perurans* and microbiome associated proteins identified across the proteomic fractions and their potential functions.

Table 2: Summary table of the uncharacterised proteins, their fold change and total razor and unique peptide count found in *Neoparamoeba perurans* from all experimental chapters.

ABSTRACT

Determining the virulence factors associated with pathogenic organisms is imperative in comprehending disease pathogenesis and ultimately paves the way for methods of treatment and disease management. *Neoparamoeba perurans* is an ectoparasitic amoeba responsible for the hyperplastic gill infection of marine cultured finfish referred to as amoebic gill disease (AGD). *Neoparamoeba perurans* is suspected to have a repertoire of putative virulence factors that enable the attachment and colonisation of the host, as well as resistance to host immune response.

The primary aim of this study was to identify the virulence factors of *N. perurans* (both amoebic and bacterial) using a proteomic approach, and to increase understanding of the parasite's mechanisms as an infectious agent in AGD of farmed fish. This thesis is divided into five chapters comprising: an introduction to AGD, *N. perurans* and virulence factors, three experimental chapters written as manuscripts for publication and finally, a general discussion that reviews the findings and future outcomes of this research.

The first experimental chapter (chapter two) confirmed the reported loss of *N. perurans* virulence in long-term culture, as determined by an *in vivo* challenge trial. This work validated AGD infection in the virulent cohort and validated the lack of AGD infection in the attenuated cohort by gill scoring, histopathology, and qPCR of gill tissue. The microbiome of the virulent-1 and attenuated *N. perurans* were characterised by 16S rRNA Illumina MiSeq sequencing for informing the bacterial database protein search. Two-dimensional gel electrophoresis (2D gel) and LC-MS/MS analysis revealed that hydrophilic virulence proteins are differentially expressed between a virulent-1, virulent-2 and attenuated culture. Differentially expressed proteins were actin-associated proteins, lipoxygenase, an ABC membrane transporter and profilin. Overall,

these proteins suggest that the virulent-2 culture of *N. perurans* maintains elevated levels of proteins involved in cytoskeletal, oxidative and immunomodulatory roles.

The second experimental chapter (chapter three) assessed the extracellular secretions, or exoproteome, of a virulent-3 and attenuated parasite for virulence factors. Using label–free LC-MS/MS, proteins that were differentially expressed were found to be elevated in the attenuated parasite. Additionally, the extracellular secretions of the attenuated parasite had a greater cytotoxic effect on the salmonid RTgill W1 cell line compared with that of the virulent-3 isolate. This effect was believed to be associated with the increased growth of bacteria in the attenuated culture of *N. perurans*. Antibiotics were employed to reduce the presence of bacteria in the culture, which lessened the cytotoxicity observed in RTgill W1. *Neoparamoeba perurans* specific proteins were found in both exoproteomes that have serine protease activity which may facilitate AGD infection, but are not the main drivers of cytotoxicity. Additionally unknown and hypothetical proteins of *N. perurans* were identified that warrant further investigation.

The third experimental chapter (chapter four) investigated the cell-surface and membrane proteome of a virulent-3 and attenuated *N. perurans*. Proteins involved in potential cell-mediated adhesion roles such as GAPDH and GTPase signalling proteins were found and may represent putative targets of treatment. Overall, the proteins identified reveal moonlighting roles for common proteins such as proteins involved in glycolysis that may aid host ligand attachment.

The outcome of this work is expected to facilitate progress in the design of *N. perurans* targeted therapies for the treatment of AGD in farmed fish. Developing treatments that target and disrupt *N. perurans* will improve gill health in salmonids and will promote aquaculture sustainability and productivity.

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First and foremost, I would like to thank my supervisors Dr Orla Slattery, Dr Eugene MacCarthy, Dr Ian O' Connor and Dr. Hamish Rodger for their never-ending support and guidance from start to finish of this research project. Orla, there are not enough words to describe how grateful I am to have you as my primary supervisor, you were always so patient, kind and undeniably perceptive in both laboratory techniques and scientific writing. You are one of the best, so thank you.

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CHAPTER 1

INTRODUCTION

1.1. Background

World fish production reached 171 million tonnes fish in 2016, with aquaculture accounting for 53% of this total (FAO, 2018). Domestically, the production of farmed salmon in Ireland was the most economically significant aquaculture sector in 2018, with production reaching 12,000 tonnes (BIM Aquaculture Report, 2019). One of the most significant challenges to the salmon aquaculture industry is finfish health and disease management (FAO, 2018; Boerlage *et al.,* 2020). Several diseases plague cultured finfish and present infections in different organs (Johnson *et al.*, 2004; Ariel and Olesen, 2002), however gill disease is one of the main health problems for aquaculture, in particular Atlantic salmon (Mitchell and Rodger, 2011; Oldham *et al.,* 2016; Bloecher *et al.,* 2018). The bacteria *Piscichlamydia salmonis* and *Tenacibaculum maritimum* primarily parasitize the gills, however pathogens can also secondarily appear in several organs, such as infectious salmon anaemia virus (ISAV), presumably by using the gills as an entry point (Aamelfot *et al.*, 2015). Atlantic salmon paramyxovirus (ASPV) is a relatively common infectious agent of the gills, causing proliferative gill inflammation in farmed fish.

Several infectious and non-infectious agents can cause gill disease and more often the causative agent is of multifactorial aetiology and non-specific, leading to what is referred as complex gill disease (Mitchell and Rodger, 2011; Herrero *et al.,* 2018; Gjessing *et al.,* 2019). CGD also encompasses proliferative gill disease, a non-specific term used to describe the gross lesions observed in the gills of salmon (Herrero *et al.,* 2018; Boerlage *et al.,* 2020). Co-infections may occur where the host is severely compromised due to increased parasite activity however, there is also potential for a primary infection to carry and transmit a secondary pathogen to the host (Kotob *et al.*, 2016). A preliminary study conducted by Nowak *et al.* (2010) found *N. perurans* DNA on *Lepeophtheirus salmonis* commonly known as the sea louse, an ectoparasite of salmon. The authors suggested *L. salmonis* could act as a potential carrier and transmitter of AGD to

susceptible hosts however, the findings could also be incidental, and therefore further research is required to determine the potential involvement of the sea louse in AGD transmission. In Chile, *N. perurans* was reported as a co-infectious agent together with *Caligus rogercresseyi*, a sea louse similar to *Lepeophtheirus,* (Bustos *et al.*, 2011). *Neoparamoeba perurans* was also reported to be present during a yersiniosis infection, caused by the gram-negative bacteria *Yersinia ruckeri* resulting in in hemorrhaging on the body (Valdenegro-Vega *et al.*, 2015). Co-infections have been shown to result in reduced resistance to disease in salmon. For example, a significant increase in mortality was observed in an experimental trial where *Salmo salar* L. was challenged with both *Caligus rogercresseyi* and *Piscichlamydia salmonis* compared *P. salmonis* alone (Lhorente *et al.*, 2014). Further awareness of co-infections needs to be considered during AGD outbreaks in order to consider potential involvement of these secondary pathogens in promoting AGD severity.

1.2. Amoebic gill disease

Amoebic gill disease (AGD) is a marine pathogenic disease that is restricted to the gill epithelium of cultured finfish and if left untreated, can result in death (Bridle *et al.*, 2010). The primary agent of the disease is a marine ectoparasite belonging to the *Neoparamoeba* genus, a group of well documented pathogenic amoebae known to cause infection in marine fish and invertebrates (Dyková *et al.*, 2007; Mullen *et al.*, 2004; Jellett *et al.*, 1989; Bustos *et al.*, 2011; Bridle *et al.*, 2010). First reported in Tasmania in the mid 1980's, AGD has resulted in economic losses and time consuming treatment efforts to the mariculture industry to the present day (Oldham et al., 2016). The disease has a variety of susceptible hosts however high prevalence and intensity have been mainly reported on the marine grow-out phase of Atlantic salmon (*Salmo salar L*). farms. The disease spreads in sea farms via release of the causative amoeba,

Neoparamoeba perurans (Young *et al.*, 2007) from infected fish mucus into surface water wherein uninfected fish are exposed to the pathogen.

Outbreaks of AGD on marine salmon farms have occurred in all salmon producing countries; USA (Kent *et al.,* 1988), Australia (Adams and Nowak, 2001), Chile (Bustos *et al.,* 2011), Norway (Steinum *et al.,* 2008), and Ireland (Bermingham *et al.,* 2004), with the recent addition of Canada (ICES, February 2016). AGD was first reported in Europe in 1995 affecting Spain, France, and Ireland (Rodger and McArdle, 1996). Current predictions suggest that AGD outbreaks are expected to increase due to climate change related rainfall and increased sea surface temperature (Johnson-Mackinnon *et al.,* 2016), presenting further challenges to an already well-established disease. Cyclic hypoxia has also been shown to progress the development of AGD in Atlantic salmon, and this is also presumed to increase due its correlation with climate change (Oldham *et al.*, 2020). Bathing the infected fish in freshwater or hydrogen peroxide for a minimum of two hours is presently the only mechanism of treating the disease (Vera and Migaud, 2016). While this is an effective short-term measure (Munday *et al.,* 2001), the procedure causes substantial stress to the fish due to movement and high stocking densities incurred throughout the treatment (Ashley, 2007). Additionally, reinfection may occur through trophozoites still present in the host's environment or remaining lodged in the gill epithelia, avoiding osmotic pressure (Taylor *et al.*, 2009), therefore the treatment is not a long-term solution. Both freshwater and hydrogen peroxide treatments emphasise the issue of fish welfare, a current topic of concern in aquaculture and warrant the need for disease prevention rather than treatment (Mustapha, 2014). Other exploratory methods of treatment such as bithional and bithional sulphoxide (Florent *et al.,* 2010) chlorine dioxide (Powell, 2002) and chloramine-T (Harris *et al.*, 2005) have been tried and deemed ineffective in complete eradication of amoebae from the gills of infected fish.

Clinical criteria for the diagnosis of AGD include infected gill tissue that displays numerous raised white mucoid lesions (Adams *et al.,* 2004) with surplus production of mucus from the host (Peyghan and Powell, 2006), lethargy and reduced growth of moribund fish (Mitchell and Rodger, 2011) as well as laboured respiration and movement towards the water surface layer (Rodger, 2014; Munday, 1986; Bustos *et al.*, 2011). The multifocal lesions of AGD are used as an initial diagnostic for disease characterisation and can be used as a guidance towards treatment through the use of gill scoring (Adams *et al.,* 2004). Taylor *et al.* (2009) defined a gill scoring system that assigns numbers based on rates of disease advancement: 0 for clear healthy gill tissue to 5 being the most severely infected tissue (Fig 1). Gill scoring benefits fish husbandry units in decision making of scheduled baths for disease mitigation but cannot provide confirmation of the causative species.

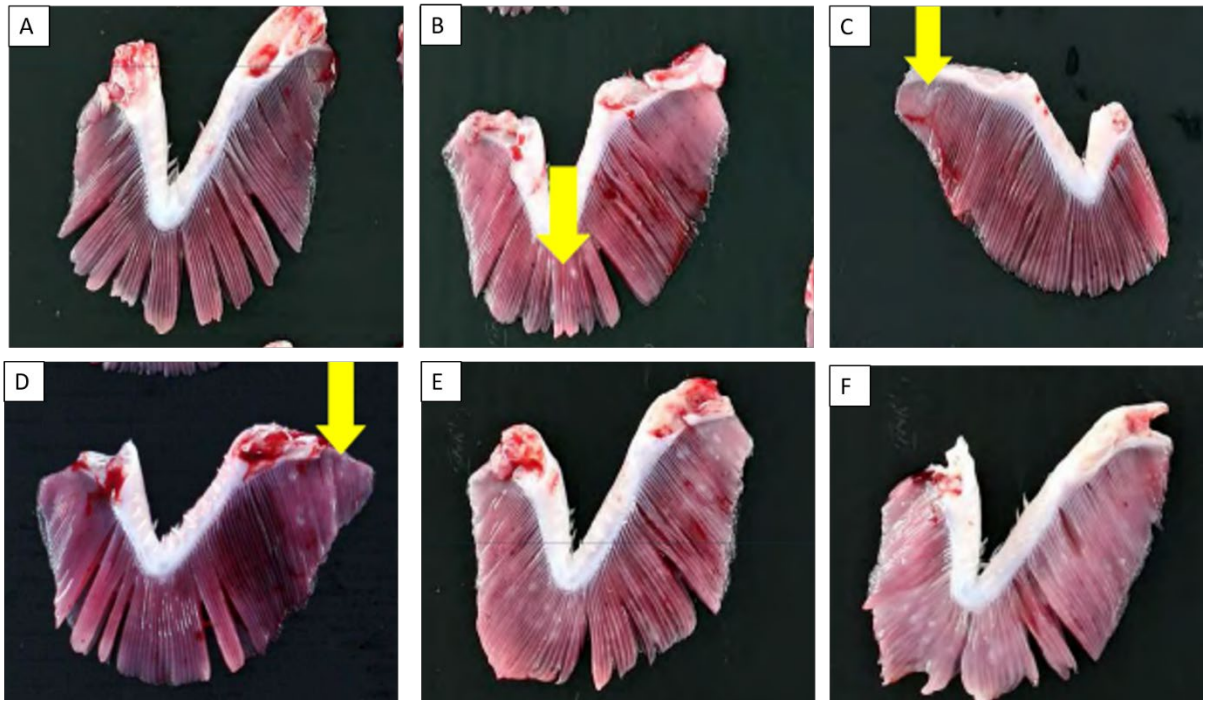

Figure 1: Amoebic gill disease (AGD) macroscopic gill score as outlined in Taylor *et al.* (2009). The images in each panel represent different levels of AGD infection. Panel A = score zero (no infection); panel B = score 1; panel C = score 2; panel D = score 3; panel E = score 4; panel F = score 5 (severe infection). Yellow arrows indicate mucoid patches. Images were adapted from Taylor *et al.* (2015).

1.1.2. *Neoparamoeba perurans*

Neoparamoeba spp. are single celled protozoan parasites (Figure 2) that are lobose in their locomotion, may display up to 50 digitate pseudopodia and have a significantly hyaline ectoplasm that enables their diversely shaped locomotive form (Woo and Buchmann, 2012). *N. perurans* is a free living and opportunistic protozoan that localises its infection in fish resulting in hyperplasia and hypertrophy on the secondary gill lamellae (Adams and Nowak, 2003), inducing desquamation (Valdenegro-Vega *et al.*, 2014) and the production of excess chloride and mucous cells (Morrison *et al.*, 2006b). AGD can initiate interlamellar vesicles wherein amoebae can reside and proliferate extensively as a result of hypertrophy and edema (Karlsbakk *et al.,* 2013; Zilberg and Munday, 2000).

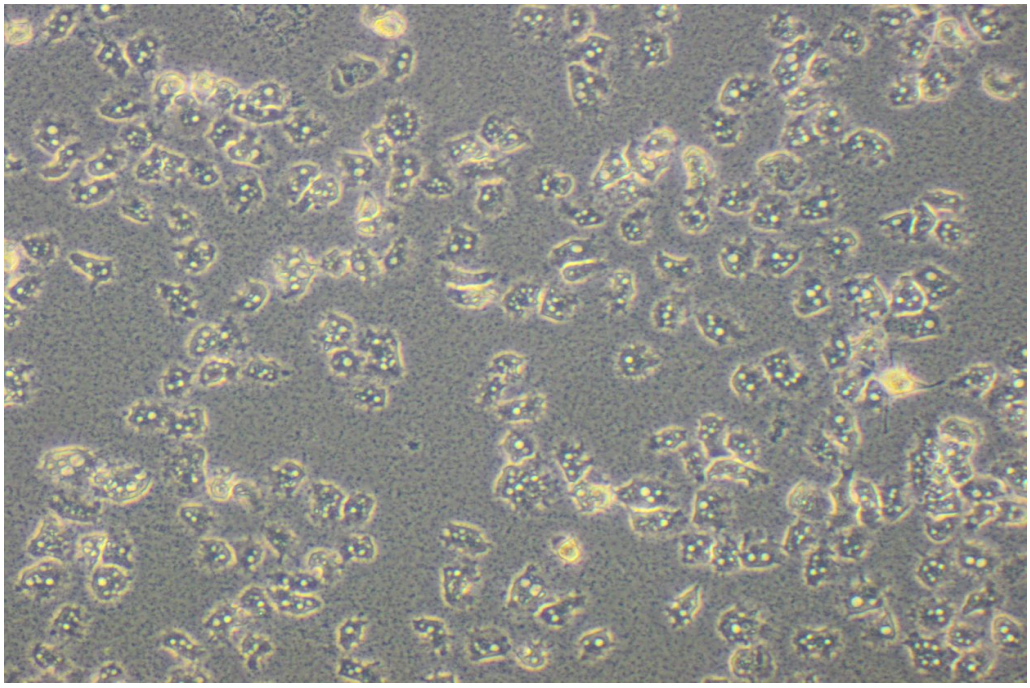

Figure 2: *Neoparamoeba perurans* trophozoites imaged under phase contrast microscopy (x200)**.** Image shows the attached amoebae with visible digestive vacuoles. Amoeboae in suspension are free-floating with long pseudopodia.

Detection of the parasite for disease management is facilitated by molecular diagnostics such as the PCR assay described by Downes *et al.* (2015). The smaller 70 bp segment of *N. perurans* 18S rRNA gene is amplified and the assay can detect as little as 2.68 copy numbers of *N. perurans* DNA μl^{-1} , offering a robust monitoring tool for AGD management (Downes *et al.,* 2015).

Confirmation of AGD comes from histopathological analysis of the infected gill epithelia (Adams and Nowak, 2003) that show the typical symptoms of the disease, namely, hyperplasia and the presence of amoebae containing one or more endosymbionts or parasomes in a juxanuclear position in the cell. These *Perkinsella amoeba*-like organisms (PLOs) in the cells of *Neoparamoeba* spp., are a defining characteristic of the species and can clearly be seen

under a microscope, providing a visual aid in identification of *N. perurans* present on the gills (Karlsbakk *et al.*, 2013; Young *et al.*, 2014).

The endosymbiont may also be regarded as a parasome, "Nebenkörper" (Schaudinn, 1896) or secondary body. Hollande (1980) defined the eukaryotic endosymbiont as a kinetoplastid protozoan, after viewing their prominent disk of DNA and provided the name *Perkinsinella amoeba* to the cytoplasmic-bound organism. Their identification as a separate organism was confirmed by Dyková *et al.* (2003) through phylogenetic analyses of their small subunit ribosomal ribonucleic acid (SSU rRNA) gene. The name *Perkinsinella* was already in use as a separate taxon so Dyková *et al.,* (2008) coined the term *Perkinsela amoeba* to avoid misinterpretation.

The molecular work conducted by Dyková *et al.* (2003) revealed the parasome was closely related to the species *Ichthyobodo necator*, a flagellate protist that primarily parasitises juvenile freshwater and marine cultured fish (Chettri *et al.*, 2014). *Ichthyobodo necator* is an ectoparasite that attaches to the host's skin and gills, resulting in excessive mucus production and proliferative gill disease. Interestingly, a reduction in these PLOs number was noted by Dyková *et al.* (2003) throughout *Neoparamoeba perurans* cell passage over time, but one PLO is always retained in a juxanuclear position in the amoeba cell.

A study by Tanifuji *et al.* (2017) concluded an interdependence between a *Neoparaomeba* spp., *Neoparamoeba pemaquidenesis* and its *Perkinsela* sp. through biochemical and metabolic pathways. *Perkinsela* sp. appears capable of making trypanothione, but the endosymbiont relies on glutathione and spermidine synthesized by the amoeba host (Tanifuji *et al.,* 2017). Trypanothione biosynthesis was originally only described for trypanosomes and were considered unique to this genus of protozoans (Krauth-Siegel *et al.,* 2003). The trypanothione biosynthesis has now been characterised in amoebic parasites,

namely *Naegleria fowleri* (Ondarza *et al.*, 2006) and PCR amplification revealed the trypanothione reductase gene in *Entamoeba histolytica* (Tamayo *et al.*, 2005). This biochemical pathway enables the parasites to maintain a high thiol environment by synthesising glutathione and spermidine to form trypanothione which offers protection from oxidation toxicity. This interesting find may offer novel therapeutic targets in treating infectious disease, including AGD, by exploiting the endosymbionts metabolic pathways that could result in a detrimental fate for the amoeba.

The parasitic amoeba, *Acanthamoeba castellanii* Neff strain, harbours endosymbionts of clinical importance and the protozoan can therefore act as a Trojan horse and directly carry additional opportunistic infectious agents to susceptible hosts (Siddiqui and Khan, 2012). *Neoparamoeba perurans* may also have this potential to transmit pathogenic microbes that aid proliferation of the amoeba on the gill and exacerbate AGD. Elucidation of the microbiome of *N. perurans* isolates identified *Pseudomonas* spp.*, Marinomonas* sp. and *Flavobacterium* sp. as co- inhabitants of the parasite (McCarthy *et al.*, 2015). Furthering this microbiome characterisation, Benedicenti *et al.,* (2019) identified both gram positive and gram negative bacteria with differing relative abundances of communities between one polyclonal and two clonal *N. perurans* isolates. The ability to define clonal cultures of the parasite that differ in virulence provides additional means, besides comparative means using attenuated isolates, in defining the precise virulence factors of AGD (Collins *et al.*, 2017).

Young *et al.,* (2007) discovered the true etiological agent of AGD through delineating the discrepancies between the 18s rRNA and the 28S rRNA using *in situ* hybridisation (ISH) probes that bound to the newly described phylogenetic lineage, *N. perurans*. Further progress in AGD research occurred when Crosbie *et al.* (2012) fulfilled Koch's postulates by maintaining cultures of *N. perurans in vitro* that caused AGD in experimental challenge trials with *Salmo*

salar L. Following this advancement, Bridle *et al.* (2015) discovered that loss of virulence occurs in *N. perurans* cultures over time. This paper addressed the need to understand the virulence factors of the parasite and suggested a dependence on host-derived material to implement pathogenicity. Numerous other pathogenic agents of fish and mammals have been recorded to lose virulence through long culturing periods but by passaging several times through the host organisms the necessary virulence factors are reacquired (Ali *et al.*, 2013). Cano *et al.* (2019) also reported a loss of virulence of *N. perurans* in culture and interestingly noted that both virulent and attenuated virulent cultures of *N. perurans* extracellular secretions could induce a host immune response in a gill epithelial cell line (RTgill W1).

Encystment formation is recognised as a strategy imposed by free-living amoebae when adverse conditions prevail (Fouque *et al.*, 2012). *N. perurans* employs a resemblance to a mature true cyst when experiencing osmotic exposure from freshwater but lacks a double-wall cyst also known as the structure pseudocyst (Lima *et al*., 2017). This finding has implications for amoebicidal agents in treating AGD, where such antimicrobials may not be able to penetrate the wall. Additionally, cellular and biochemical changes occurring during encystment could lead to therapeutic agents that target such pathways and proteases that are involved in this process.

1.2.3. Host response

Physical and chemical barriers comprising of the skin and gills are principal mucosal defenders of both the innate and adaptive immune systems (Marcos-Lopez *et al.*, 2017a). This defence aims to protect teleost fish against a potential infection (Ellis, 2001) with response implemented through cellular and humoral immunity (Tort *et al.,* 2003). The mucus of fish comprises carbohydrates, lipids (Brinchmann, 2016) and several biochemical defensive proteins

including lectins, mucins, cytokines, protease inhibitors and immunoglobulins (Ellis, 2001; Kitani *et al.,* 2007) forming a complex viscous, nutrient rich mixture that attracts microbial adhesion and colonization (Benhamed *et al.*, 2014). Mucins are the glycoproteins that construct the mucus matrix and house important anti-microbial peptides (AMPs) including defensins, piscidins (van der Marel *et al.*, 2012) and histone-derived salmon antimicrobial peptide [SAMP H1] (Lüders *et al.*, 2005). Cellular components of the innate immune response play a pivotal role in pathogen recognition (Gomez *et al.,* 2013) belonging to eosinophils, dendritic cells and macrophages (Haugland *et al.,* 2012). Mucosal immunity is influenced by stress and direct pathogenic interactions (Benhamed *et al.*, 2014), which show the local host response is activated throughout a host-pathogen interaction.

The complex interaction of host, parasite and gill-derived bacteria have been suggested as key contributing factors in the pathogenicity of AGD. The anadromous lifestyle of *Salmo salar L.* challenges the physiology of the fish and immune system throughout the fishes' movement from freshwater to seawater and as aging proceeds from birth up to smoltification (Zwollo, 2017). Significant alterations occur in hormone levels and a reduction in expression of genes that are associated with transmitting and receiving immune signals once *Salmo salar* L. entered seawater were reported from a study by Johansson *et al.* (2016). Approximately 300 immune genes were downregulated after seawater transfer with T-cells displaying the highest downregulation. This study highlights the implications of host immune suppression during freshwater to seawater transfer, resulting in increased host vulnerability to infection.

Previous reports highlight certain bacterial genera such as *Psychroserpens* (Bowman and Nowak, 2004) and *Winogradskyella* (Embar-Gopinath *et al.*, 2008) prevail in AGD infected gill tissue. However, the general consensus is a gill microbiome dysbiosis occurs during the onset of AGD infection (Slinger *et al.,* 2020; Birlanga *et al.*, 2020). The clinical symptoms of

AGD are speculated to be the host cells responding to the amoeba's attachment on the gill surface (Adams and Nowak, 2003; Morrison *et al.*, 2006a). As mentioned previously, AGD clinically develops on the gills resulting in hypertrophy and hyperplasia. Other pathological features of AGD are host inflammatory responses including cavitations or 'cysts' formed due to the hosts white blood cells infiltrating in bulk (Adams and Nowak, 2003). During AGD outbreaks, neutrophils and macrophages penetrate lesions (Morrison *et al.*, 2006b) whilst lesion recovery follows an intrusion from lymphocytes with large numbers of eosinophilic granule cells present (Lovy *et al.,* 2007). The infection results in the expression of the inflammatory cytokines interleukin-1 (IL-1) beta isoform 1 (Bridle *et al.*, 2006) and down regulation of the major histocompatibility complex (MHC) class 1 (Young *et al.*, 2008).

AGD infected rainbow trout also upregulate IL-1 beta in the gill epithelium in response to the disease and it is speculated by Bridle *et al.* (2006) that the excessive mucus production is a by-product of this cytokine protein. IL-1 beta activation presumably impairs the parasite but could involuntarily causes acute injury to the host's tissue, a secondary result of its activation (Lopez-Castejon and Brough, 2011). Variations in expression of anterior gradient -2 (AG-2) and p53 tumour suppressor protein in gill infected tissue were observed; tumor suppressor protein p53 mRNA was downregulated, whilst AG-2 was upregulated (Benedicenti *et al.*, 2015). The presence of p53 induces expression of the highly conserved cellular protein, proliferating cell nuclear antigen (PCNA), in AGD lesions but at high detection levels of p53, repression of PCNA was observed (Morrison *et al.*, 2006a). Cano *et al.* (2019) also found increased expression of PCNA and AG-2 in the host cell line, RTgill W1, that was infected with AGD. The involvement of p53, AG-2 and PCNA gene expression suggest a potential role in AGD pathogenesis, as these gene products have been associated with higher vertebrate diseases and infections (Pinheiro *et al.*, 2007; Branca *et al.*, 2007).

Immunoglobulins are present in teleosts in the form of antibodies or B-cell receptors (Salinas *et al.,* 2011). Immunoglobulin M (IgM) tetramer operates as part of the humoral immune system of fish, playing a role in the adaptive immune response (Magnadóttir *et al.*, 2005). Immune related proteins of humoral release consist of acute phase proteins such as lysozyme, peroxidases and antiproteases (Alvarez-Pellitero, 2008). Signature cytokines of the host adaptive immune response; Treg, Th17 and Th1 cells are downregulated in the interbranchial lymphoid tissue of late stage AGD infection (Benedicenti *et al.*, 2015). Furthermore, IL-4/13a expression was elevated, suggesting either an allergic response or a parasitic immune evasion response is occurring in the gills of AGD infected fish (Benedicenti *et al.*, 2015). Cano *et al.* (2019) also found that the Th2 pathway was up-regulated in the gill epithelial cell line RTgill W1, with cytokines such as IL10, IL6 and IL-4/13a showing increased gene expression in response to AGD infection. A similar report by Boison *et al.* (2019) found downregulation of some immune-related genes such as genes that encode chemokine ligands and reproposed the role of a parasitic immune evasion. Marcos-Lopez *et al.* (2017a) determined the involvement of the humoral response proteins esterase, lysozyme and antiprotease in the gills and serum during AGD pathogenesis. Key conclusions drawn from the study were variations of humoral response between the gills and serum, highlighting the capacity of the gills to respond to a pathogen and that the local humoral response in the gills decreased as severity of the disease increased. Peroxidase activity was reduced in the gills but was higher in serum. The reduced activity could be an indicator of reduced immunity or on-going oxidative stress. Oxidative stress has been shown to affect the performance of these humoral components (Yarahmadi *et al.*, 2016) when the production of reactive oxygen species (ROS) is triggered by stress (Livingstone, 2003).

1.3. Virulence factors

The literature is limited in describing the virulence factors of free-living amoeba that cause infection in marine and aquatic hosts, despite the economic loss associated with these infections. In order to understand what putative virulence factors these marine pathogenic amoebae may possess, one can scope the literature of well-described virulence factors from human based infections.

Scientific reports on pathogenic amoebae have addressed the identification of the parasite's virulence factors such as toxins, proteases, factors involved in host entry and host immune evasion (Serrano-Luna *et al.*, 2013). Such studies have isolated and characterized secretion proteinases and suggested that these and other imperative metabolic enzymes involved in glycolysis, would be suitable drug targets (Jacobs *et al.*, 1998; Ondarza, 2007).

Pathogenic amoebae have the ability to produce hydrolytic enzymes that cause detrimental damage to a host's membrane (Siddiqui and Khan, 2014), and it has been speculated that *N. perurans* secretes cytolytic enzymes that result in host cellular degradation (Butler and Nowak, 2004; Bridle *et al.*, 2015). The adherence of *E. histolytica* to host cells is directed by the galactose or N- acetyl-D-galactosamine (Gal/GalNac) lectin that attaches to the host cells glycoproteins (Gilchrist and Petri, 1999) and enable intestinal colonization of the host. The host cell glycoproteins are of mucin origin and it has been shown that mucin 2 (MUC2) has a high binding affinity for the parasite Gal/GalNac lectin (Begum *et al.,* 2015). A transmembrane protein, the mannose binding protein, mediates *Acanthamoeba* attachment to the cornea during *Acanthamoeba* keratitis (Panjwani, 2010). Research examining the role of lectin involvement in *N. fowleri* host cell attachment is limited but a study by Carrasco-Yepez

et al. (2013) suggested the role of glycoconjugates with mannose residues were involved in *N. fowleri* attachment to a mouse epithelium.

Further forms of virulence factors in pathogenic amoebae are pore forming peptides, or amoebapores, that degrade host tissue and cytolytic proteases that also cause tissue degradation and assist in host invasion (Leippe, 2014; Serrano-Luna *et al.*, 2013). Major pore forming peptides, of the amoebapore A, B and C families, have been isolated and their cysteine pattern structure shares homology with saposin-like proteins (Bruhn and Leippe, 2001). An acanthaporin isolated from *Acanthamoeba* had a different tertiary structure to traditional amoebapores of *E. histolytica* and naegleriapores of *N. fowleri* and its structure was of a new protein fold revealed by DALI server (a network server for comparing protein structures in 3D) (Michalek *et al.*, 2012). Degradation of host extracellular matrix is a key virulence factor arbitrated by proteases of parasitic amoebae. *Entamoeba* and *Naegleria* proteases are members of the cysteine protease family and their structure resembles cathepsin-L enzymes. Conversely, *Acanthamoeba* releases numerous other forms of proteases of the serine, cysteine and metalloprotease family (Ondarza, 2007).

Successful cultivation of numerous parasites has aided the characterisation of genomes, transcriptomes and proteomes of these infectious agents. However, it has been documented that continued long term maintenance of parasite cultures can inadvertently reduce or ultimately cause the complete loss of virulence of the parasitic organism being studied (Bridle *et al.,* 2015; Wong *et al.,* 1977). The solution to this is to collect newly acquired parasites however, the loss of virulence in the organism can be a beneficial outcome. It can provide novel insights to what biomolecules are present and absent and responsible for the virulent phenotype observed in newly acquired or ‘wild’ strains of amoeba compared with ‘attenuated’ strains of amoebae.

1.4. Proteomics

Structural and functional analysis of the complete systemic set of proteins from an organism or cell at a particular point in time is referred to as proteomics (Peng, 2013; Chandramouli and Qian, 2009). The datasets obtained have implications for biomarker discovery, health and, disease monitoring (Patterson and Aebersold, 2003) of many species including marine fish (Rodrigues *et al.*, 2012). A diversity of techniques are available in studying the proteome that can exceed information provided by other "omic" technologies (Sallam, 2015). Shotgun proteomics and protein microarrays have been successful in identifying post-translational modifications (Chen and Snyder, 2010). High throughput technologies such as mass spectrometry have dominated many studies due to capabilities in analysing complex samples (Blonder *et al.*, 2006; Marcos-Lopez *et al.*, 2017b) and are defined by bottom up (digested peptides) or top down (whole proteome) mass spectrometry approaches (Feist and Hummon, 2015).

Electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) have significantly advanced in recent years (Feist and Hummon, 2015; Aebersold and Mann, 2003). The high sensitivity of liquid chromatography coupled with tandem mass spectrometry have allowed for accurate assay development (Shaik *et al.*, 2017), with many studies still employing gel-based techniques such as one and two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) (Braceland *et al.*, 2013) due to the visual assessment of differential expression. 2D gel electrophoresis separates proteins based on isoelectric point (pI), typically through immobilized pH gradient strips (IPG strips) followed by separation based on molecular mass using denaturing polyacrylamide gels containing sodium dodecyl sulfate (SDS) (Rabilloud and Lelong, 2011). Visualization of spots ensues by using a sensitive staining method, followed by protein identification through mass spectrometry. 2D gel electrophoresis

offers rapid characterization of proteins making this methodology extremely informative (Chevalier, 2010).

1.4.1. Omic technology in defining pathogenesis

The emergence of integrative omic technologies: genomics, epigenomics, transcriptomics, proteomics, and metabolomics, offers high throughput monitoring of both normal and patho-physiological processes. As such these technologies have applications in clinical research of infectious diseases and biomarker identification (Wanichthanarak *et al.,* 2015; Beltran *et al.,* 2017). Omic technologies result in the generation of large datasets containing a wealth of biological information that is analysed through bioinformatics. The field of omic technologies has revolutionised our understanding of diseases, in particular virulence factors. Each omic technique offers invaluable information about the organism studied but all have advantages and disadvantages. Genomic research can reveal associated pathogenic genes and some antigenic proteins (Lluch-Senar *et al.*, 2015) but lacks information of the complex activities of the proteins identified, such as post-translational modifications, protein-protein interactions and protein trafficking (Chandramouli and Qian, 2009; Rang *et al.*, 2015). Nevertheless, techniques in genomics such as microarray-based comparative genomic hybridization (CGH) have assisted in the identification of virulence factors (Sarkar *et al.*, 2006; Tagini, and Greub, 2017).

Integrations of transcriptomic or expression profiling (through microarrays, cDNA amplified fragment length polymorphism [cDNA-AFLP] and RNA-seq) and proteomic data (generated from gel-based and gel-free analysis) are two characterisation approaches (Prasad *et al.*, 2017) but significant computational challenges prevail when interpreting the biological data (Huang *et al.,* 2017). Noteworthy differences in results obtained from samples of mRNA

and protein expression indicate the more robust 'omic' approach is proteomics. Eukaryotic mRNA average half-life ranges from a few minutes to 24 hours (Tourriére et al., 2002), in contrast to the eukaryotic protein half-life of 48-72 hours (Hargrove *et al.,* 1989). Gene expression regulates mRNA transcription, with many mRNA molecules not transcribed due to targeted mRNA decay (Schonberg and Macquat., 2012). Furthermore, proteomic alterations (including post-translational modifications) are not captured by transcriptomic techniques (Heijne *et al.*, 2005). Taken together, these advantages favour the choice of a proteomic approach to characterise target proteins involved in AGD for this project.

1.4.2. Membrane proteins

Membrane proteins and other membrane bound organelles are actively involved in pathophysiological processes within the cell (Blonder *et al.*, 2006). These proteins are of pivotal importance, constituting 30% of proteins that are encased in cellular membranes and that one third of genes code for membrane proteins (Braun *et al.*, 2007; Tan *et al.,* 2008). Classification of membrane proteins is based on the protein-membrane interaction which can be integral (transmembrane) or peripheral membrane proteins. Integral membrane proteins transverse the phospholipid bilayer as alpha helices in contrast to the hydrophilic nature of peripheral membrane proteins which do not transverse the membrane but interact by localisation on the membranes surface (Tan *et al.,* 2008). Extraction of membrane proteins is a complex process as integral membrane proteins have non-polar hydrophobic amino acids, requiring strong solubilisation and reconstitution into detergent micelles (Braun *et al.*, 2007). Nevertheless, studies of infectious diseases have targeted membrane proteomics and have found novel pathways that have assisted towards treatment and vaccine candidates (von Specht *et al.,* 1996; Loukas *et al.,* 2007). Most of these drug targets acting on membrane proteins have

been reported from fungi (Sant *et al.*, 2016) and bacteria (Alexander *et al.*, 2018) with less reports documented for protozoan parasites, thus highlighting the need to investigate what potential roles *N. perurans* membrane proteins may have in AGD. Few reports have however documented the antiprotozoal potential of bioactive antimicrobial peptides on the parasites' plasma membrane (Giovati *et al.*, 2018). The plasma membrane offers candidate drug protein targets of protozoan parasites but these proteins localised in the membrane can develop drug resistance over time. An ATP-binding cassette (ABC) transporter protein LABCG2, that is partially localised on *Leishmania major* plasma membrane, was suspected to be a virulence factor of leishmaniasis and suspected of developing drug resistance (Perea *et al.*, 2016). LABCG2 was overexpressed in *Leishmania* parasites to determine the transporter proteins' potential in blocking a common therapeutic for the disease, antimony (Perea *et al.*, 2016). *Leishmania* parasites that over-expressed LABCG2 were resistant to antimony's therapeutic effects, highlighting the complexity of a parasite's membrane protein response to drug therapies.

1.4.3. Extracellular proteins

The secretome or exoproteome of pathogenic organisms has gained increasing attention in recent years, as the secreted proteins are known to be involved in host-pathogen associations such as: adhesion, morphogenesis and a toxic response to the host's immune system (Ranganathan and Garg, 2009; Hiery *et al.*, 2015). The secreted proteins can be regarded as a virulence factor of infectious diseases as they play a role in disease progression (Serrano-Luna *et al.*, 2013). Protozoan parasites secrete glycoproteins, proteinases such as elastases as well asmetalloproteases such as; serine and cysteine proteases that facilitate in host tissue invasion (Gonçalves *et al.*, 2018), therefore warranting the investigation of the exoproteome of *N. perurans*. Extracellular vesicles (EVs) are often used for the successful release of proteases by parasites such as *Plasmodium falciparum, Leishmania major* and *Trypanosoma cruzi* (Marcilla

et al., 2014). EVs are composed of exosomes that fuse and shed with multivesicular bodies from the plasma membrane. EVs are also composed of microparticles (MVs) or ectosomes that release from the plasma membrane (Marcilla *et al.*, 2014). EVs protect the virulent components they carry (kinases, proteases, lipids and glycans) from the host immune response (Twu and Johnson, 2014) and in the case *of Acanthamoeba*, can direct tropism to target tissues (Gonçalves *et al.*, 2018). Therefore, secretory proteins of protozoan parsites are a desired target for therapuetic intervention, due to the their ability to carry potent virulent factors and their role in cell-cell communciation (Tzelos *et al.*, 2016).

1.5 Aims and Objectives

The primary aim of this study is to identify virulence factors of *Neoparmaoeba perurans* (i.e. amoebic and bacterial) using a proteomic approach to increase understanding of the parasite's mechanisms as an infectious agent in AGD of farmed fish. To validate *N. perurans* specific proteins, a custom protein database consisting of *N. perurans, Paramoeba*, Amoebozoa and bacteria identified from the 16S rRNA gene sequencing described in Chapter 2, was searched against to map peptides to proteins in each experimental chapter. Several proteomic and biochemical techniques are used in determining the *N. perurans* virulent proteome. The objectives of the study were to:

1. **Identify key virulence factors of *Neoparamoeba perurans* (both amoebic and bacterial) to analyse the hydrophilic protein fraction of an attenuated and two virulent (virulent-1,virulent-2) parasites. Characterise the microbiome of the virulent-1 and attenuated *N. perurans* for delineating parasite and bacteria specific proteins.**

   A difference in virulent phenotypes between long-term and newly acquired cultivated strains of parasites has been demonstrated in the literature for soluble protein expression (Cuervo *et al.*, 2008). Similarly, *N. perurans* has been reported to lose virulence over time in culture (Bridle *et al.*, 2015) but the causative virulence factors remain undescribed. To define virulence factors of hydrophilic origin, chapter two (presented in manuscript style) aims to assess the hydrophilic profile of a virulent-1 (1-year in culture), virulent-2 (70 days in culture) and attenuated (3 years in culture) *N. perurans* culture using 2D gel electrophoresis and LC- MS/MS. This chapter also aims to confirm the loss of virulence in the attenuated isolate and investigates the microbial communities living within a virulent-1 and attenuated *N. perurans* culture using 16S rRNA gene Illumina MiSeq sequencing. The microbial community characterisation will facilitate what bacterial derived protein databases to search against in the LC-MS/MS analysis.

2. **Characterise the exoproteome profile of *Neoparamoeba perurans* using biochemical, proteomic and *in vitro* cell culture techniques.**

The parasite *N. perurans* is suspected to have a set of extracellular virulent proteins (Bridle et al, 2015; Butler and Nowak, 2004) that result in the clinical manifestation of AGD in susceptible farmed fish. In a study by Bridle *et al.* (2015), extracellular *N. perurans* proteins cultivated with a host cell line, of a long-term cultured isolate failed to induce a cytotoxic effect on a Chinook salmon embryo (CHSE -214) cell line. While the study demonstrated a loss in virulence of a long-term cultured *N. perurans* strain compared with a virulent 'wild type', the authors did not identify potential virulence factors. Chapter three (presented in manuscript style) investigates the proteins in the soluble extracellular fraction of a virulent-3 and attenuated *N. perurans* culture (amoebic and bacterial) cultivated on marine yeast agar using gel-free LC-MS/MS, protease assays and *in vitro* cell culture cytotoxicity assays with the gill cell line, RTgill W1 (Bols *et al.*, 1994).

3. **Isolate and identify the membrane bound and membrane surface proteome of a virulent and attenuated strain of *Neoparamoeba perurans.***

The main theory of how AGD is initiated is that enigmatic attachment receptors on the surface of the parasite enable membrane fusion and host-parasite interaction with the epithelium of *Salmo salar L*. gills (Lovy *et al.*, 2007; Nowak and Archibald, 2018 ; Butler and Nowak, 2004; Adams and Nowak, 2004; Villavedra *et al.*, 2010). Characterising the membrane proteins and cell-surface proteins may uncover the putative attachment proteins of AGD and therefore facilitate future therapeutic based studies targeting these proteins. Chapter four (presented in manuscript style) aims to identify both the membrane and cell-surface proteome of a virulent-3 and attenuated *N. perurans* (amoebic and bacterial) using LC-MS/MS and streptavidin-affinity chromatography, respectively.

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CHAPTER 2

Comparative proteomic profiling of attenuated and virulent *Neoparamoeba perurans* proteins associated with amoebic gill disease

2.1. Abstract

The parasitic amoeba, *Neoparamoeba perurans* is the causative agent of amoebic gill disease in salmonids. The parasite has previously been reported to lose virulence during prolonged *in vitro* maintenance. In this study, the impact of prolonged culture on *N. perurans* virulence and its proteome was investigated. Three isolates of *N. perurans* maintained in culture for varying durations were compared. Two isolates, attenuated and virulent-1, had their virulence assessed in an experimental trial using Atlantic salmon smolts. To facilitate protein identifications from the commensal bacteria within *N. perurans,* the attenuated and virulent-1 isolates bacterial community composition was evaluated by 16S rRNA Illumina MiSeq sequencing. Soluble proteins were isolated from two virulent (virulent-1, virulent-2) and attenuated *N. perurans* culture and were analysed using two-dimensional electrophoresis (2D PAGE) coupled with liquid chromatography tandem mass spectrometry (LC-MS/MS). An experimental challenge trial using Atlantic salmon smolts confirmed a loss in virulence in an *N. perurans* culture that was maintained *in vitro* for three years. A greater diversity of bacterial communities was found in the microbiome of the virulent-1 isolate harbouring predominant genera belonging to *Pseudoaltermonas spp*, *Vibrio spp* and *Fluviicola spp*. Microbial community richness was reduced in the attenuated microbiome, with a singular species, *Thalassopira xiamenensis,* representing a large proportion of its microbiome. A collated proteome database of *N. perurans*, Amoebozoa and four bacterial genera resulted in 24 proteins differentially expressed between the three cultures. The present LC-MS/MS results indicate that protein synthesis, oxidative stress and the plausible occurrence of immunomodulation are ultimately upregulated in the virulent-2 *N. perurans* culture and future studies may exploit these protein identifications for therapeutic purposes in infected farmed fish.

2.2. Introduction

Neoparamoeba perurans is an ectoparasitic protozoan responsible for the hyperplastic gill infection of marine cultured finfish referred to as amoebic gill disease (AGD) (Young *et al.*, 2007). Originally described in Tasmania in the 1980s (Munday, 1986), AGD now has a global impact on susceptible finfish farms (Bridle, Morrison and Nowak, 2006; Dykova *et al.*, 1998) and is regarded as one of the most economically damaging, parasitic diseases of these farms (Mitchell and Rodger, 2011). The disease has a variety of hosts such as Chinook salmon, turbot and rainbow trout (Karlsbakk *et al.*, 2013; Munday *et al.,* 2001); however increased susceptibility has been reported in the marine grow-out phase of *Salmo salar L.* farms (Munday *et al.,* 2001; Oldham *et al.,* 2016). Curative measures rely on the use of freshwater or hydrogen peroxide baths as persistent chronic infections may lead to fatalities; however, reoccurrence of the disease is common (Adams and Nowak, 2004a). Clinical criteria for the diagnosis of AGD include infected gill tissue that displays numerous raised white mucoid lesions (Adams *et al.,* 2004a) with over production of mucus from the host (Peyghan and Powell, 2006), as well as lethargy and reduced growth of moribund fish (Mitchell and Rodger, 2011). Gill histopathology of AGD infected fish display hyperplasia and lamellar fusion (Adams and Nowak, 2003; Adams *et al.,* 2004b).

The *Neoparamoeba* genus are a group of well documented pathogenic amoebae known to cause infection in marine fish and invertebrates (Bridle *et al.*, 2010; Bustos *et al.*, 2011; Dyková *et al.*, 2007; Jellett *et al.*, 1989; Mullen *et al.*, 2004). The virulence factors of *N. perurans* have not been defined; however the presence of an extracellular protein (Bridle *et al.*, 2015) and molecules involved in host attachment (Lovy *et al..,* 2007; Villavedra *et al.*, 2007; Villavedra *et al.*, 2010; Vincent *et al.*, 2009) are suspected to be key virulence factors associated with the disease. Long term cultivation of *N. perurans* results in the loss of parasitic virulence

in vivo (Bridle *et al.*, 2015), offering a platform for comparative research of virulent and attenuated cultures of the parasite. Development of attenuated cultures may occur as the parasite adapts to long term cultivation *in vitro,* where gene expression may be altered to aid proliferation in the new environment (Sutherland *et al.*, 1996).

Free living amoebae are home to numerous amoeba-resisting microorganisms, comprising bacteria, viruses and fungi (Greub and Raoult, 2004). Initial evaluations of the bacterial community structure of *N. perurans* were derived from AGD positive gill samples (Bowman and Nowak, 2004; Embar-Gopinath *et al.*, 2008) with a common family of bacteria successfully identified, namely *Flavobacteriaceae,* however the discriminating genus differed in the two studies. The presence of *Pyschroserpens* spp. was found by Bowman and Nowak (2004), in contrast to the finding of Embar-Gopinath *et al.* (2008) where *Winogradskyella* spp. was identified. Interestingly, *Winogradskyella* spp. nearest phylogenetic neighbour is *Psychroserpens burtonensis* (Nedashkovskaya *et al.*, 2005) however, Embar-Gopinath *et al.* (2008) failure to identify *Psychroserpens* spp. in their study may be due to its fastidious behaviour in culture. *Neoparamoeba perurans* isolate based microbiome characterisation was recently achieved by McCarthy *et al.* (2015) and Benedicenti *et al.,* (2019). Several genera coupled to *Pseudomonas* spp.*, Marinomonas* sp and *Flavobacterium* sp were identified (McCarthy *et al.*, 2015). Polygonal (a population of amoeba derived from gills) and clonal (individual amoebae) isolates of *N. perurans* shared several genera of both gram positive and gram negative bacteria with significant differences in relative abundances between the three isolates (Benedicenti *et al.,* 2019). Despite the speculation surrounding the role of *N. perurans* microbiome in AGD, no study exists that directly compares the microbiome of a long-term ('attenuated') cultured *N. perurans* isolate to a virulent isolate.

In the present study, the microbiome of an attenuated and virulent-1 isolate of *N. perurans*, both maintained at 16°C, was characterised by 16S rRNA Illumina MiSeq sequencing to identify similarities and divergences between isolates. Furthermore, this collated information was used to inform the 'bacterial protein database' that was incorporated with an amoebozoa database and *N. perurans* specific database, into the Andromeda search engine of MaxQuant (Cox *et al.*, 2011) for protein identification from 2D PAGE. In addition, two phenotypes of *N. perurans* were investigated for differential virulence against naïve smolts to validate the attenuation of *N. perurans* virulence over time. These phenotypes were compared by characterising their soluble proteome using 2D PAGE, alongside a recently acquired culture (virulent-2) of *N. perurans*, providing a proteomic timeline of differential virulence.

2.3. Materials and Methods

2.3.1. *Neoparamoeba perurans* isolation and culture

All *N. perurans* trophozoites (feeding stage of protozoa) were isolated from AGD infected Atlantic salmon located on a commercial farm in the west of Ireland. Trophozoites were collected by swabbing AGD infected gills and placing swabs in 0.2 µm filtered sterile seawater for 4 hours. This combined mixture was subsequently plated and maintained xenically at 16°C on marine yeast agar plates (MYA; 0.01% malt, 0.01% yeast, 2% Bacto Agar, sterile sea water at 30 ppt salinity) overlaid with 7 mL of 0.2 µm filtered seawater (Crosbie *et al.*, 2012).

Inoculated plates were washed weekly with 7 mL of sterile seawater to control bacterial growth. The amoebae were sub-cultured weekly by transferring free-floating cells to fresh MYA plates. Confirmation of *N. perurans* identity was performed using real time PCR as previously described by Downes *et al.* (2015). Parasites from cultures established at 3- years, 1- year and 70 days are referred to as attenuated, virulent-1 and virulent-2, respectively. The attenuated culture of amoeba was established in October 2015, while the virulent-1 culture was propagated for one year prior to inoculation of naïve smolts. These two cultures were used in the *in vivo* challenge trial. The virulent-2 culture was propagated for 70 days prior to harvesting for proteomic analysis. The virulent-2 culture was deemed virulent in a separate feeding trial experiment with naïve smolts (data not shown).

2.3.2. *In vivo* challenge trial

Naïve Atlantic salmon smolts (n=120) weighing approximately 120g were transported from a commercial hatchery to the animal housing unit in Galway-Mayo Institute of Technology. Fish were divided into three cohorts, namely the control cohort (n=40), the attenuated cohort (n=40) and virulent cohort (n=40). These cohorts were distributed into six circular 1000 L (n=20) circular tanks and maintained for a two-week acclimation period prior to inoculation. Tanks were monitored for dissolved oxygen, salinity and water quality. Tanks were maintained at 16°C and salinity of 30 ppt. All fish used in this study were fed daily to satiation using a commercial food pellet and were approved for experimentation by the Health Products Regulatory Authority (HPRA) in Ireland under project authorisation number AE 19137/P002 following the Animals Scientific Procedures Act 1986 (Directive 2010/63/EU transposed into Irish law by S.I. No 543 of 2012).

Fish were transferred to a 300 L saltwater bath at 30 ppt and temperature maintained at 16°C for inoculation with the parasite for 4 hours. The control group were placed in a 300 L saltwater bath, minus the addition of the parasite, serving as the negative control. The attenuated cohort was challenged with 2000 cells/L, achieved by cell count using a Sedgewick rafter counting chamber (API Supplies), of a 3-year old *N. perurans* culture that was maintained since October 2015. The virulent cohort was challenged with 2000 cells/L of a 1-year old *N. perurans* culture that was maintained since October 2018. After the bathing period, fish were placed back into their respective 1000 L tanks for the duration of the trial.

2.3.3. Validation of virulence

Six fish from each cohort (n=18) were removed at 0, 7, 14 and 21-days post infection (dpi) and euthanized in an overdose of anaesthetic containing 400 mg/L of tricaine methane sulfonate (MS -222) for lethal sample collection. Gross macroscopic gill scores were assessed according to Taylor *et al.* (2009). Gill samples from the first gill arch were excised for histology and quantitative polymerase chain reaction (qPCR) detection of *N. perurans.* Samples for histological examination were placed into 10% neutral buffered formalin (Sigma-Aldrich). Samples for qPCR detection of *N. perurans* were excised, flash frozen and analysed as described by Downes *et al.* (2015).

2.3.4. DNA sequence analysis of the *Neoparamoeba perurans* microbiome

2.3.4.1. DNA extraction from *Neoparamoeba perurans* cultures

DNA was extracted using the AllPrep DNA/RNA Mini Kit (QIAGEN, Hilden, Germany) from the attenuated and virulent-1 *N. perurans* cultures in triplicate. Three plates of the attenuated and virulent-1 cultures were individually prepped for DNA extraction by mechanically scraping cells from MYA plates into a 50 mL falcon tube, yielding a suspension of adherent and floating cells in seawater. The suspensions were briefly vortexed to homogenise them. One mL of each *N. perurans* cell suspension was added to 600 µL of RLT lysis buffer followed by vortexing for 10 min. Fresh lysozyme dilution (20 µL of 10 mg/mL) was added to each tube, prior to 30 min incubation at 37 °C on a shaker platform. Subsequently, 10 µL of Proteinase K (> 600 mAU/ml; QIAGEN, Hilden, Germany) was added into each tube followed by 30 min incubation at room temperature.

The rest of the DNA extraction was performed following manufacturer's instructions (AllPrep DNA/RNA Mini Kit [QIAGEN, Hilden, Germany]). All DNA concentrations after the extraction processing were measured using Qubit dsDNA BR Assay Kit (ThermoFisher Scientific, Paisley, United Kingdom), following its instructions.

2.3.4.2. 16S rRNA gene sequencing in Illumina

The sequencing targeted the V4 and ITS1-spanning regions from 16S rRNA genes in the DNA samples using the primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') (Caporaso *et al.*, 2011). All sequencing was carried out in FISABIO's facilities (FISABIO / Avda. de Catalunya, 21 / 46020 Valencia, Spain). PCR reaction tubes contained (25 µL final volume): 12.5 ng (genomic DNA), 0.2 µM (final concentration) 515F primer, 0.2 µM 806R primer, and 12.5 µL 2x KAPA HiFi Hot Start Ready Mix (0.5 U per 25 µL reaction; Roche). All PCR reactions followed the same program: 1) 95°C for 3 min, 2) 25 cycles containing: 95°C for 30 s 55°C for 30 s and 72°C for 30 s; 3) 72°C for 30 s.

After a PCR clean-up on each sample, Illumina sequencing adapters were added using Nextera XT Index Kit (Cambridge, United Kingdom) following instructions from the company. Prior to DNA quantification (Agilent Technologies 2100 Bioanalyzer), a second PCR clean-up was done. A DNA normalisation (to 4 nM) protocol was done, followed by libraries pooling with unique indices. Pools were denatured (using 0.2 N of NaOH), diluted and heat denatured before sequencing. Resulting amplicons were sequenced (Illumina MiSeq platform) (Caporaso et al., 2012) using 5% PhiX as an internal control.

2.3.4.3. Sequencing analyses

All sequenced demultiplexed FASTQ data from samples were analysed using QIIME 2.2019.10 (Quantitative Insight Into Microbial Ecology) following the "Moving Pictures" tutorial workflow from the official QIIME 2 website (https://docs.qiime2.org/2019.10/tutorials/moving-pictures/). Raw sequences were imported using the "Casava 1.8 paired-end demultiplexed fastq" procedure. They were assembled and quality filtered at a phred score limit of Q25. Resulting files were denoised using the Dada2 command. Sequences were aligned and clustered based on a 97% identity, later each cluster was taxonomically classified using the Greengenes database 13-8 (DeSantis *et al.*, 2006). Taxonomic bar plots were visualised and saved from the QIIME 2 View webpage (https:// view.qiime2.org/).

2.3.5. Protein extraction from *Neoparamoeba perurans* cultures

The virulent-2, virulent-1, and attenuated cultures of *N. perurans* were harvested in isolation of each other to avoid cross contamination. Confluent cultures of *N. perurans* were harvested by mechanical scraping followed by centrifugation at 1000 x g for 10 min at 4°C. Cell pellets were resuspended in lysis buffer: 7M urea, 2% w/v 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) and 50 mM Dithiothreitol (DTT) with HALT protease inhibitor cocktail (Thermo Fisher). Samples were incubated on ice for 30 min and sonicated at 35 Hz on ice for three cycles of 30 seconds "pulse on" and 15 seconds "pulse off". Samples were centrifuged to pellet the cell debris at 12, 000 x g at 4°C for 10 min and the supernatant was transferred to a fresh 1.5 mL microcentrifuge tube for further removal of residual seawater.

Each supernatant received 20% trichloroacetic acid (TCA) in acetone and was centrifuged for 30 min at 4°C at 12,000 x g. The pellet, containing precipitated proteins, was washed twice in 100% acetone and left to air dry before solubilisation in rehydration buffer 1 (7M urea, 2M thiourea, 1% ASB-14, 40 mM Tris, 0.001% Bromophenol Blue, 2% Bio-Lyte® [Bio-Rad, Hercules, CA]). Samples were incubated for 60 min at room temperature before determining protein concentration using the RC/DC kit (Bio- Rad).

2.3.6. Two-dimensional electrophoresis

The soluble protein fraction from the virulent-2, virulent-1 and attenuated *N. perurans* cultures were analysed in triplicate using 2D electrophoresis. Triplicate immobilised pH gradient strips (IPG, pH 4-7, 11 cm) were passively rehydrated overnight with 120 µg of protein in a final volume of 200 µL of re-hydration buffer 1. The strips were focused in a PROTEAN® i12™ IEF system (Bio-Rad) at a current limit of 50 Amp/IPG strip using a step voltage gradient (250 V for 20 min, stepped up to 8000 V maximum for 1 h; 26,000 V-h total) at 20°C. Prior to the second dimension IPG strips were equilibrated in a reducing buffer (6M urea, 0.375 M Tris-HCl pH 8.8, 2% (w/v) SDS, 20% (v/v) glycerol, 2% (w/v) DTT) for 20 min and subsequently placed in an alkylating buffer (6 M urea, 0.375 M Tris-HCL pH 8.8, 2% (w/v) SDS, 20% (v/v) glycerol, 2.5 % iodoacetamide) for 20 min. The second dimension was performed using AnykD™ Criterion™ TGX™ Precast gel in the Criterion™ Dodeca™ cell (Bio-Rad, CA) where IPG strips were electrophoresed for 40 min at 200 V. Gels were stained using QC Colloidal Coomassie (Bio-Rad) overnight and destained by washing in deionised water in triplicate, followed by incubation in wash buffer (10% ethanol [v/v], 7.5 % acetic acid [v/v]).

2.3.7. Gel image analysis

Gel images were acquired using the Gel Doc™ EZ Gel Documentation System (Bio-Rad, CA) and images analysed using the SameSpots 5.1.0.0 software (TotalLab, Non-linear Dynamics, UK). Triplicate gels used to analyse the proteins extracted from the virulent-2, virulent-1 and attenuated cultures were automatically aligned, and normalisation of spots was performed using gels from the virulent-2 culture as a reference to detect differentially expressed proteins. The software normalises volumes of each spot and detects differential proteins based on a difference in spot intensity using the 'between subject' experimental design. Differential spots were chosen for further analysis based on maximum fold change of ≥ 2 and ANOVA statistical significance ($p < 0.05$) using the software's statistical analysis function.

2.3.8. Spot preparation and mass spectrometry

Enzymatic in-gel digestion was performed as described by Shevchenko *et al.* (2006). Gels were rinsed with deionised water and spots of interest were excised and cut into pieces using a sterile scalpel. Gel pieces were microcentrifuged to collect excess water, destained with 100 µL of 100 mM ammonium bicarbonate/acetonitrile (1:1, v/v) and incubated for 30 min with vortexing every 10 min. Following the addition of ammonium bicarbonate, 500 µL of neat acetonitrile (ACN) was added and incubated at room temperature until gel pieces were destained and decreased in size. The gel pieces were dried and rehydrated with trypsin buffer overnight at 37°C (13 ng/µl trypsin) in 10 mM ammonium bicarbonate containing 10% [v/v] ACN on a thermomixer at 350 rpm.

To terminate enzymatic digestion, 1% acetic acid (AA) was added to the samples. Peptides were desalted using C18 stage tips with Equilibration Buffer (0.1% trifluoroacetic acid [TFA] in MS grade water) and Elution Buffer (50% ACN 0.1% TFA in MS grade water). After drying by vacuum centrifugation, peptides were acidified by AA, desalted with C18 STAGE tips (Rappsilber, Mann and Ishihama, 2007), and resuspended in 2.5% ACN, 0.5% AA. Peptide fractions were analysed on a quadrupole Orbitrap (Q-Exactive, Thermo Scientific) mass spectrometer equipped with a reversed-phase NanoLC UltiMate 3000 HPLC system (Dionex LC Packings, now Thermo Scientific). Peptide samples were loaded onto C18 reverse phase columns (10 cm length, 75 μm inner diameter) and eluted with a linear gradient from 1 to 27% buffer B containing 0.5% AA and 97.5% ACN in 58 min at a flow rate of 250 nL/min. The injection volume was 5 μL.

Raw data from the Orbitrap Q-Exactive was processed using MaxQuant version 1.6.6.0 for identification of proteins (Cox and Mann, 2008), incorporating the Andromeda search engine and MaxQuants contaminants fasta file (Cox *et al.*, 2011). To identify peptides and proteins, MS/MS spectra were matched to a combined custom database comprised of *N. perurans* (20,887 proteins [v2, 07/08/2019, CSIRO]); UniProt reference proteome databases of *Amoebozoa* (109,415 proteins) and bacteria selected from the microbiome analysis (148,582 proteins) as well as a database of *Paramoeba* proteins from a taxonomy search on UniProtKB (5,001 proteins) were included. All databases except for *N. perurans* were downloaded on May 8th 2020 from UniProt and further information on individual species protein counts are in Appendices I, II and III.

All searches were performed with tryptic specificity allowing two missed cleavages. The database searches were performed with carbamidomethyl (C) as fixed modification and acetylation (protein N terminus) and oxidation (M) as variable modifications. Mass spectra were searched using the default setting of MaxQuant with a false discovery rate of 1% at the peptide and protein level.

2.4. Results

2.4.1 *In vivo* challenge

The control cohort remained negative for *N. perurans* for the duration of the trial, as confirmed by gill scoring, qPCR and histology. During gill scoring, both the control and attenuated cohort had no visible gross macroscopic lesions at 7 dpi and were assigned a gill score of 0, while two fish from the virulent cohort were gill score 1. One fish from the attenuated cohort and all fish from the virulent cohort sampled at 7 dpi were qPCR positive for *N. perurans*. Histological analysis revealed no pathology for fish sampled at 7 dpi. This observation is consistent with the early stage of the disease where gross gill pathology is not evident (Downes *et al.*, 2015; Ruane *et al.*, 2013), further strengthening the use of qPCR in identifying the presence of the parasite (Ruane *et al.*, 2013; Downes *et al.*, 2015; Downes *et al.*, 2017). Gill scores >1 were observed for each fish at 14 dpi for the virulent cohort, with qPCR analysis confirming the presence of *N. perurans* for each fish sampled. qPCR detected the presence of *N. perurans* in one fish sampled 14 dpi from the attenuated cohort, however no corresponding gill score or pathology was observed with this fish. Negative qPCR results were observed for each fish sampled in the attenuated cohort at 21 dpi. At 21 dpi, half the fish sampled from the virulent cohort were observed to have a gill score of 2 and the experiment was terminated. No AGD-like pathology was detected in the attenuated cohort (Fig. 1*a*). Histopathology for the virulent cohort revealed lamellar epithelium hyperplasia and fusion, with occasional formation of interlamellar lacunae (Fig. 1*b*).

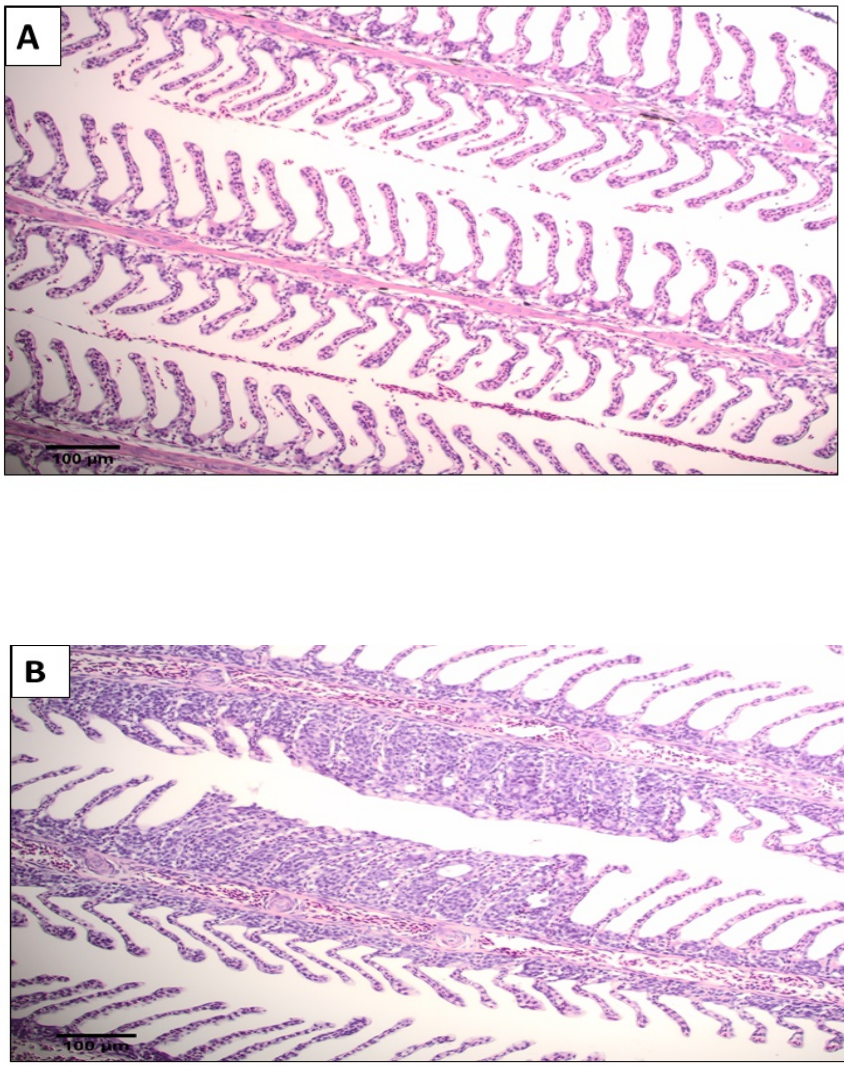

Figure 1: An example of H&E stained gill samples taken from non-AGD and AGD infected fish from the challenge trial. Scale bar = 100 µm. A) gill sample from the attenuated cohort. B) gill sample from the virulent cohort.

2.4.2. 16S rRNA sequencing of attenuated and virulent-1 *Neoparamoeba perurans* microbiome

Relative abundances of operational taxonomic units (OTUS) (Fig. 2) were used to generate the resulting list of prominent taxa in both the attenuated and virulent-1 *N. perurans* cultures. Gram negative bacteria were identified with Proteobacteria and Bacteroidetes being the

dominant phyla in both cultures. A loss of microbial diversity and relative abundance is evident in the attenuated culture, presumably reflecting the lack of adaptation to prolonged culturing of these particular genera. This was most true for several identifications of Gammaproteobacteria in the virulent-1 culture.

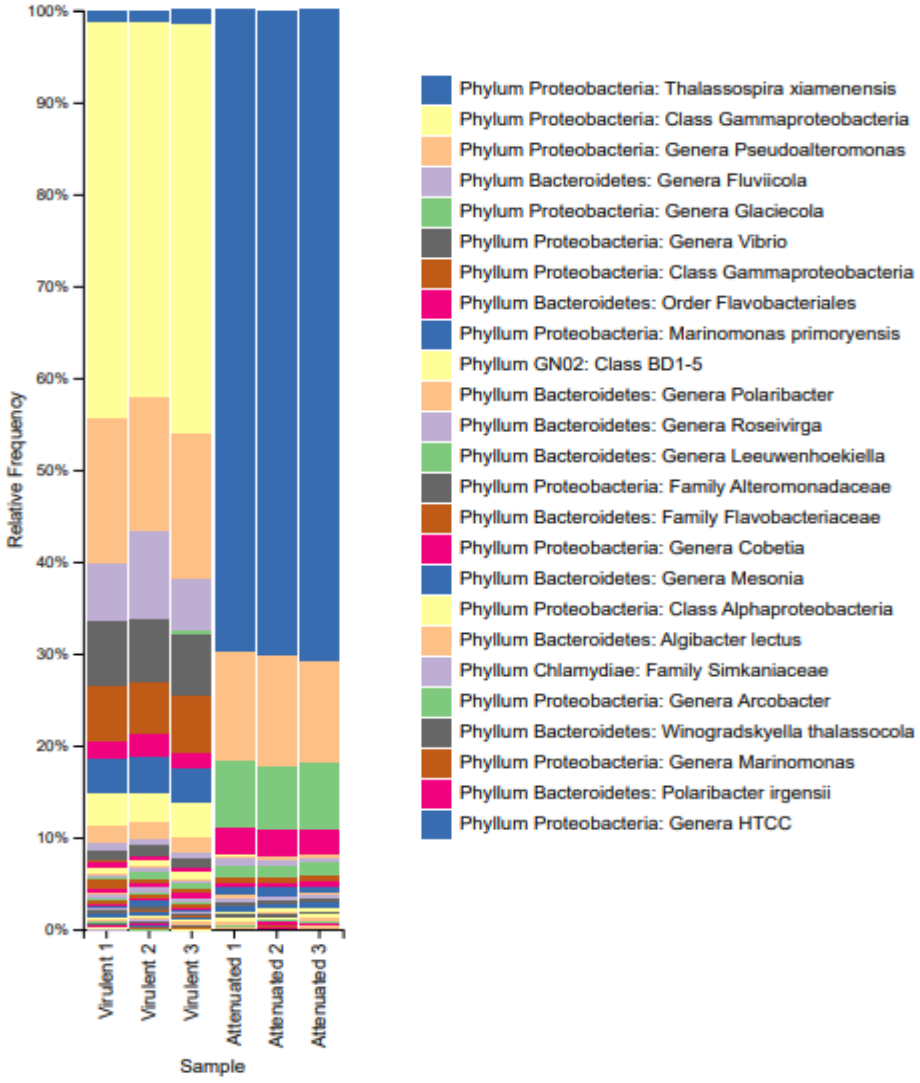

Figure 2: Relative abundance of the prokaryotic community in the attenuated and virulent-1 cultures by 16S rRNA gene sequencing.

Three independent identifications appeared only in the virulent-1 culture suggesting the presence of this class warrants further investigation. An unknown Gammaproteobacteria had a higher relative abundance in the virulent-1 culture and could potentially be implicated in AGD virulence. Differences in community structure were found in shifts of relative abundance of the species *Thalassospira xiamenensis* belonging to the class Proteobacteria from ~1% coverage in the virulent-1 culture and ~70% coverage in the attenuated culture. The genus *Vibrio* was only identified in the virulent-1 microbiome as was the phyla *Chlamydiae*, an interesting find due to both this genus and phylum's role causing marine finfish disease. *Winogradskyella* was also identified in both microbiomes, however in much lower relative abundance than other genera. The UniProt proteomes of three genera from this sequencing analysis were selected for creation of the 'bacterial' protein database used in the proteomic characterisation of the *N. perurans* cultures in this study. These genera were: *Pseudoaltermonas, Fluviicola* and *Vibrio*. Additionally, due to the large coverage of *T. xiamenensis* in the attenuated isolate, this species was also included in the database. The complete richness of the sequencing was evaluated using a rarefaction curve (Fig. 3). The rarefaction curve for each sample reached the plateau state at a high number of reads, indicating that no new OTU would be sequenced.

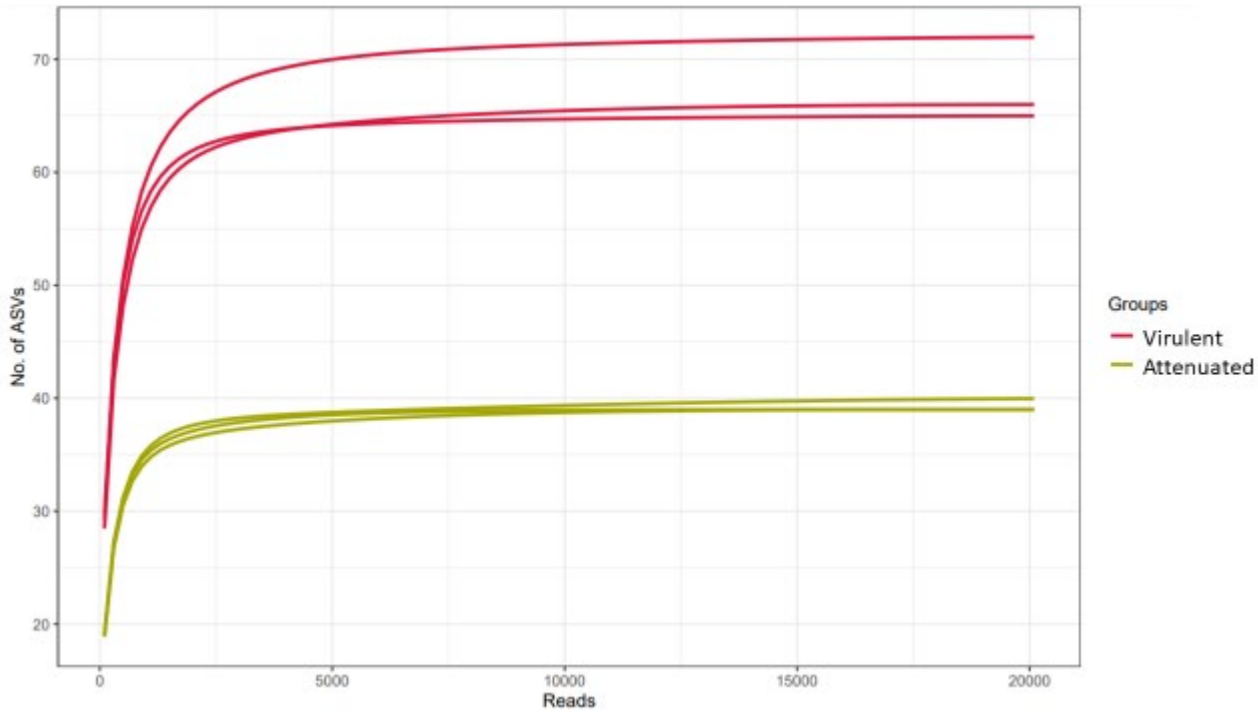

Figure 3: Rarefaction curve of the pooled virulent-1 and attenuated cultures shows a trend of consistent richness. Y axis shows amplicon sequencing variants or operational taxonomic unit against the library on the x axis.

2.4.3 Two-dimensional gel electrophoresis and protein identification

A total number of 98 spots identified by the Same Spots software were found to be statistically different in intensity, ($p \leq 0.05$) across the three sets of replicate gels from the virulent-2, virulent-1 and attenuated protein extracts. Eleven spots with a maximum fold change of ≥ 2 were chosen for further analysis using LC-MS (Fig. 4), resulting in the identification of 24 proteins (Appendix IV), the majority derived from the *N. perurans* database (Table 1), four *N. perurans* proteins of which shared homology to proteins of other Amoebozoa species (*Acanthamoeba spp*, *Planoprotostelium spp*) and 1 protein shared homology with *Thalassospira xiamenensis,* the gram negative bacterium largely evident in the attenuated

microbiome analysis. Six proteins were not *N. perurans* associated, 3 proteins of which were bacterial and the remaining 3 proteins were from the Amoebozoa group. Thirteen proteins were exclusively assigned to *N. perurans* only, therefore the proteins identified in this study are predominantly amoebic in nature and furthermore, are from *N. perurans*.

Ten of the 11 spots were found to be elevated in the virulent-2 culture compared to the virulent and attenuated culture, except for spot 69 which was more upregulated in the attenuated culture in comparison to both the virulent-1 and virulent-2 cultures. Protein expression was highest in the virulent-2 culture, followed by the virulent-1 and attenuated cultures from 10 of the 11 spots. Four of the protein spots were elevated ($p < 0.05$) in the virulent-2 culture followed by low expression in the attenuated culture-3 and virulent-1 cultures, however the change between the attenuated and virulent-1 cultures was not statistically significant ($p > 0.05$). The identity of differentially expressed proteins is listed in Table 1, along with the corresponding intensity fold change and molecular weights. The function of these proteins can be broadly attributed to cellular proliferation, metabolism and immunomodulation. Some of the described proteins are involved in oxidative defence, plausibly protecting the parasite from the hostile host response.

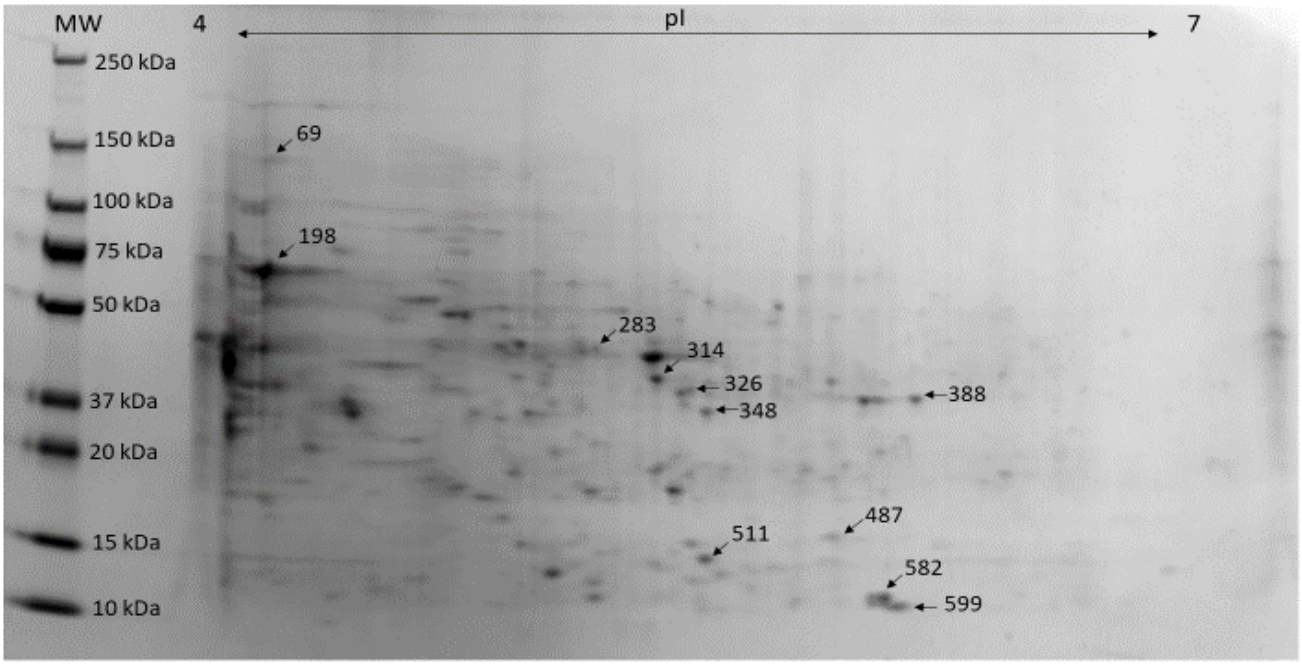

Figure 4: 2D gel of the reference gel from the virulent-2 culture of *Neoparamoeba perurans*. 11 spots displayed differential expression and were excised for LC MS/MS.

Table 1: 2D spots with significant (p ≤0.05) fold changes from the virulent-2 *Neoparamoeba perurans* culture identified by LC-MS/MS. Several proteins that are shared between *N. perurans* and species found in the bacteria and Amoebozoa database are distinguished in the organism header of Appendix IV.

Estimated MW	**Estimated pI**	**Fold change**	***P*- value ≤0.05**	**Protein identification**	**No Peptides Matched**	**Coverage (%)**	**Biological function**
20	5.61	4.6	0.005	ATP synthase subunit mitochondrial-like	2	6	Metabolism
20	5.61	4.6	0.005	Histone H2B	2	18.3	Protein synthesis
20	5.61	4.6	0.005	ADP-ribosylation factor 4 and 1	2	25.8	Cellular signalling
40	5.55	3	0.042	Heat shock protein 8	3	7.1	Stress response
70	4.45	2.5	9.55E-05	Peptidase C53 family protein	4	9	Immune evasion
70	4.45	2.5	9.55E-05	Carbohydrate ABC transporter substrate-binding protein	3	2.5	Metabolism
16	6.12	4.4	1.10E-04	Actin, cytoplasmic A3a isoform	8	22.6	Cytoskeleton
21	5.95	2.1	0.003	Cu-Zn Superoxide dismutase	2	16.3	Oxidative response
21	5.95	2.1	0.003	ADF-like domain-containing protein	2	15.5	Cytoskeleton
21	5.95	2.1	0.003	Lipoxygenase (lox) homology domain-containing protein 1	2	26.4	Cellular signalling
17	6.08	7.2	6.25E-04	Profilin conserved site domain-containing protein	6	64.3	Cytoskeleton, immunomodulation
17	6.08	7.2	6.25E-04	Profilin allergen	6	44.4	Cytoskeleton, immunomodulation
36	5.61	5.2	0.007	Malate dehydrogenase	4	13.8	Metabolism
45	5.36	1.9	0.033	Fragmin A	3	12.8	Cytoskeleton
45	5.36	1.9	0.033	Elongation factor 1	2	17.4	Cytoskeleton
45	5.36	1.9	0.033	Component of cytosolic 80S ribosome and 60S large subunit	2	7.7	Translation
45	5.36	1.9	0.033	Citrate synthase active	2	5.6	Metabolism
142	4.5	3.3	0.018	Aconitate hydratase	2	2.4	Oxidative stress

2.5. Discussion

Attenuation of parasitic virulence has been attributed to long term cultivation *in vitro* that negatively impacts the organism's capacity to establish infection in the host model (Olivos *et al.*, 2005; Veríssimo *et al.*, 2013; Wong, Karr and Chow, 1977). A complete loss of virulence has been previously reported during experimental *in vivo* trials using a clonal culture of *N. perurans* that was maintained *in vitro* for 3 years (Bridle *et al.*, 2015; Cano *et al.*, 2019). In the present study, a 3-year culture of *N. perurans* had a markedly reduced ability to establish AGD in a challenge trial using naïve salmon smolts, confirming attenuation of *N. perurans* virulence over time. Validation of virulence retention and loss in cultures was achieved using gill scoring, histopathology, and qPCR of gill samples from the *in vivo* AGD virulence challenge trial.

Gill scoring of six fish at each time point throughout the duration of the trial revealed no gill pathology for the attenuated cohort. In comparison, fish sampled in the virulent cohort were assigned cumulative gill scores at each time point until reaching the pre-determined humane endpoint. At 21 dpi three out of the six fish sampled were at a gill score of 2, resulting in the termination of the trial. No AGD-like pathology was detected in the control and attenuated cohort for the duration of the trial. Assessment of virulent cohort histology (Fig. 1) revealed the presence of AGD pathology with infected gills displaying lamellar epithelium hyperplasia and fusion (Adams and Nowak, 2001; Adams, Ellard and Nowak, 2004b). qPCR assessment revealed that the majority of fish sampled in the virulent cohort were positive for *N. perurans*. In the attenuated cohort, only one in six fish sampled at each of 7 and 14 dpi was positive for *N. perurans* and all fish tested in this cohort were negative for the parasite at 21 dpi. These results confirm that using a combination of histology and qPCR is an invaluable approach in diagnosing AGD (Downes *et al.*, 2015), particularly in the early stage of the disease.

Due to the abundance of literature suggesting *N. perurans* microbiome may influence AGD progression (Bowman and Nowak, 2004; Embar-Gopinath *et al.*, 2008; McCarthy *et al.*, 2015; Benedicenti *et al.*, 2019), 16S rRNA sequencing was employed to clarify the role of the microbiome in the virulent-1 and attenuated isolates. Fig. 2, clearly shows that time in prolonged culture results in microbial community shifts. A much broader and diverse group of bacteria were present within the virulent-1 isolate. No gram-positive bacteria were identified in either isolate, in contrast to the study by Benedicenti *et al.,* (2019) where both gram positive and gram negative bacteria were identified in their polygonal and two clonal cultures. Several gammaproteobacterial classes were found in the virulent-1 isolate but were absent in the attenuated isolate. The presence of the genera *Vibrio* and the *Simkaniaceae* family warrants further investigation as these two microbes are causative agents of disease in marine shellfish and finfish aquaculture (Kashulin *et al.,* 2017; Pawlikowska-Warych and Deptula, 2016).

Characterisation of the virulence molecules involved in AGD was achieved by comparing the proteomes of several *N. perurans* cultures. The soluble proteomic profile of a 3-year (attenuated), 1-year (virulent-1) and 70-day (virulent-2) old *N. perurans* culture was analysed using 2D PAGE coupled with LC-MS/MS. The genome of *N. perurans* has recently been sequenced (Botwright, personal correspondence), enabling the comprehensive proteomic studies of *N. perurans* presented here. A total of 11 spots were excised from the virulent-2 2D PAGE gel (Fig. 4) based on the statistical criteria of ANOVA ($p \leq 0.05$) and maximum fold change of ≥ 2 using the virulent-2 culture as a reference. These 11 spots resulted in 24 protein identifications from the collated databases of *N. perurans* (Table 1), Amoebozoa and a discrete number of gram-negative bacteria chosen from the microbiome sequencing (Appendices I, II, III). The virulent-2 culture showed the highest expression for the excised proteins, excluding spot 69 which was more elevated in the attenuated culture. This protein may be considered a

biomarker for reduced virulence in *N. perurans* as its expression shows that is markedly reduced in the virulent-1 and virulent-2 cultures.

Three proteins, glyceraldehyde-3 phosphate dehydrogenase (spot 314), chaperone protein (spot 283), putative TonB dependent receptor (spot 69) were associated only with *V. rumoiensis*, *Vibrio sp. C7* and *P. haloplanktis* respectively. The identity of the protein from spot 69 is aconitate hydratase AcnA, an iron sulphur regulatory protein, an essential enzyme in both the tricarboxylic acid cycle (TCA) and glyoxylate cycles and its presence is widely regarded as a biomarker for iron and redox stress (Lushchak *et al.*, 2014). Aconitate hydratase was identified from both *N. perurans* and the largely abundant *T. xiamenensis* (primarily of the attenuated microbiome) database therefore indicating this protein may be a bacterial protein derivative. The anaerobic bacterium, *Escherichia coli* maintains the use of isozyme AcnB in the TCA cycle, however upon growth in iron deficient and reactive oxygen species (ROS) stress, AcnB is deactivated and AcnA is expressed to relieve the metabolic block in the TCA cycle (Varghese *et al.,* 2003). Aconitase AcnA elevated expression in the attenuated culture suggests long term cultured *N. perurans* withstands oxidative and/or iron deficiency stressors when isolated from the host. This is presumably from microbial community changes over time and plausibly as a result of non-optimal nutritional media. A putative TonB-dependent receptor from *P. haloplanktis* was also identified in this spot with a plausible role in signalling and siderophore activity. TonB–dependent receptors have been implicated in carbohydrate scavenging (Blanvillain *et al.*, 2007), further suggesting *N. perurans* may face nutritional deficiencies during prolonged culture.

The proteins elevated in the virulent-2 culture compared to the attenuated and virulent-1 culture can be attributed to cytoskeletal, oxidative and immunomodulatory roles. Numerous cytoskeleton associated proteins; actin cytoplasmic A3a isoform (spot 599), actin depolymerization factor-like-domain-containing protein (spot 487), tubulin beta chain (spot 314) and fragmin A (spot 283) were elevated in the virulent-2 culture. Actin is a major cytoskeleton protein involved in motility, phagocytosis and tissue invasion in protozoan parasites (Burri *et al.*, 2012; Manich *et al.*, 2018; Weber *et al.*, 2016). Profilin (spot 582) and ADF-like-domain, an actin binding protein (ABP), were both found to be elevated in the virulent-2 culture. ABP's are recruited for the regulation of dynamic actin remodelling during phagocytosis in *Entamoeba histolytica* (Agarwal *et al.*, 2019), a key process relating to *E. histolytica's* pathogenesis. An allergic response to *N. perurans* has been postulated previously (Benedicenti *et al.*, 2015) and profilin is commonly regarded as an allergen protein in many pollen and foods capable of inducing the production of IgE and IgM (Sirvent *et al.*, 2011). It is therefore plausible to think that this protein may also induce a similar response in the gills of Atlantic salmon.

Song *et al.* (2018) inoculated recombinant *Acanthamoeba* profilin intranasally in mice to determine the proteins potential for triggering allergic airway inflammation. Mice affected with the recombinant protein displayed classical signs of an allergic response; increased mucin production and hyperplasia of epithelial cells, accompanied by an elevated Th2 cytokine response. Apicomplexans (a large phylum of parasitic alveolates) require profilin for actin polymerisation and furthermore, profilin has been shown to be crucial in host tissue invasion, egress from the cell and stimulates host toll like receptors of the innate system (Plattner *et al.*, 2008). Profilins role as an immunomodulator in AGD warrants extensive investigation based on its previous role as an immunogen in these protozoan and apicomplexan studies.

Elongation factor 1 alpha (EF1α) (spot 283) is vital for cytoskeleton integrity (Gross and Kinzy, 2005) however it can serve numerous canonical functions in the cellular milieu hence being coined a moonlighting protein (Sasikumar *et al.,* 2012). Elongation factors have been considered a virulence factor in both parasitic kinetoplastids and pathogenic bacteria as immunodominant antigens (Sabur *et al.*, 2018). Macrophage deactivation occurs when *Leishmania donovani* EF1α binds to the hosts Src homology 2 domain containing protein tyrosine phosphatase -1 (SHP -1) and additionally inhibits inducible nitric oxide expression (Nandan *et al.*, 2002). The protein's potential as a potent vaccine antigen in *L. donovani* has been evaluated by Sabur *et al.* (2018) who generated liposomal forms of leishmanial elongation factor 1 vaccine and both the recombinant and truncated vaccine provided long term immunity in BALB/c mice against visceral leishmaniasis.

Elongation factor thermo unstable (EF-Tu) (spot 283) was also elevated in the virulent-2 culture compared to the attenuated and virulent-1, and its identity was found in both *N. perurans* and *Pseudoaltermonas spp.* databases. Ribosomal subunits as well as EF-Tu typically have roles in translation and this is conceivably the case here for *N. perurans*, however EF-Tu has also been reported as a cell surface protein that facilitates host cell attachment by binding of fibronectin (Yu *et al.*, 2018) and plasminogen (Koenigs *et al.,* 2015). Bacteria only express EF-Tu therefore, it appears *N. perurans* has either acquired this genetic material through lateral gene transfer from its commensal microbiome , a described occurrence associated with amoebae and bacteria (Moliner *et al.*, 2009), or this protein is of bacterial origin entirely; a very plausible consideration due to the homologous EF-Tu in *Pseudoaltermonas spp.*

Proteins that support a function in oxidative stress were found to be elevated in the virulent-2 culture compared to the attenuated and virulent-1, specifically superoxide dismutase (SOD) (spot 487). A correlation between oxidative stress in hyperplastic lesions and late stage AGD has been shown for naïve smolts (Marcos-Lopez *et al.*, 2018a). It has been previously reported that *E. histolytica* can directly interfere with host derived neutrophil ROS production and can also rely on a secondary ROS scavenging mechanism using Fe-superoxide dismutase's (Begum *et al.,* 2015). The significantly reduced catalase enzyme in smolts displaying gill score of 2 from Marcos- Lopez *et al.* (2018b) study highlights the localised oxidative stress in the gill and suggests *N. perurans* is capable of tolerating the hostile environment by maintaining the expression of its own SOD.

Further protection from the host immune response is provided by heat shock proteins (HSP) that facilitate protein synthesis and simultaneously offer protective measures during episodes of oxidative, nutritional and heat stress in parasites (Perez-Morales and Espinoza, 2015). The presence of HSP8 (spot 326) from *N. perurans,* HSP85 (spot 326) from *Perkinsela* spp. and HSP70 (spot 334) from *P. pallidum* suggests the role of oxidative protection is crucial in the virulent-2 culture before adaptation slows its production in the virulent-1 and attenuated cultures. HSP85 was the only protein identified from *Perkinsela* spp. however, the genome of *N. perurans* was constructed to include the endosymbiont, and therefore many *N. perurans* proteins may be of *Perkinsela* spp. origin. A chaperone protein (spot 283) from *Vibrio sp. C7* was also found to be elevated in the virulent-2 isolate, potentially playing a similar role of oxidative protection. Malate dehydrogenase (spot 348) of *N. perurans* and glyceraldehyde-3 phosphate (spot 314), exclusively identified as *Vibrio rumoiensis*, are key enzymes involved in the TCA cycle that were elevated in the virulent-2 culture, highlighting the increased contribution of metabolism for *N. perurans* and its microbiome in the maintenance of virulence mechanisms.

Other metabolic proteins found to be elevated in the virulent-2 culture included citrate synthase (spot 283), ATP synthase (spot 511), ADP-ribosylation factor 4 and 1 (spot 511), and lipoxygenase (lox) homology domain-containing protein 1-like isoform X2 (spot 487). Citrate synthase is the first enzyme in the TCA cycle and ATP synthase is an essential nanomotor in the electron transport chain of the TCA cycle and thus, is responsible for the production of cellular ATP. Increased ATP production may be a virulence requisite for *N. perurans* as evidenced by the elevation of these enzymes in the virulent-2 culture with increased energy expenditure in the parasite due to replication and locomotion. Exploitation of ATP synthase is being considered as a drug target due to the novel subunits governing this protein in *T. gondii* (Salunke *et al.*, 2018). This finding may offer a therapeutic avenue if *N. perurans* nanomotor shares homology with this ATP synthase structure. ADP-ribosylation factor 4 and 1 displayed a higher expression in the virulent-2 culture with these GTP-binding proteins playing a role in protein tracking and cellular signalling (Field, Ali and Field, 1999). Lipoxygenase constitutes a family of iron containing enzymes that serve as dioxygenases catalysts. In the nosocomial pathogen, *Pseudomonas aeruginosa*, lipoxygenase has been linked to increasing the bacterium's persistence in the host, destroying the lipid membrane of epithelial cells (Aldrovandi *et al.*, 2018) and inhibiting the expression of chemokines (Morello *et al.*, 2019).

The ATP binding cassette (ABC) membrane transporter proteins (spot 198) in protozoan parasites were best described as exporters of parasite waste, until *T. gondii* was shown to import host derived cholesterol (Ehrenman *et al.*, 2010), which therefore suggests that transport may be bidirectional. They also represent an aspect of multi drug resistance in parasites (Anwar and Samudrala, 2018), therefore their characterisation is imperative in how future therapeutics may be effectively designed.

A characteristic of protozoan parasitic pathogenesis is the variation in antigenic representation to fulfil their parasitic lifecycle and gene management strategies remain at the forefront of this process. Chromatin is central to this role and histones are the building blocks of the nucleosomes that create chromatin structure. The elevation of histone H2B (spot 511) seen here in the virulent-2 culture may highlight the underestimated role histones undertake in regulating transcription dependent on the parasite's environment (Sullivan Jr, Naguleswaran and Angel, 2006). The most peculiar protein upregulated in the virulent-2 culture was peptidase C53 family protein (spot 198), a protease homologue from pestiviruses, that would be typically located in the extracellular matrix as an excreted protease. The presence of a protease family in *N. perurans* however is not surprising as proteases assist in promoting pathogenesis through tissue destruction, invasion and facilitate immune evasion (Sibley, 2013). The identification of this protein in the cytoplasmic fraction suggests this protein remained in its pro-peptide form when it was isolated, a form seen when the protein is translocating towards the plasma membrane for extracellular secretion and release.

In conclusion, these findings suggest the attenuated *N. perurans* culture has a markedly reduced ability to establish disease in the host model as revealed by qPCR, histopathology and gill score. Microbial communities characterised by 16S rRNA gene sequencing successfully identified differences in diversity and relative abundances between the attenuated and virulent-1 isolate. 2DE and LC-MS/MS successfully revealed the identity of 11 spots, of which 24 proteins were characterised and discussed here. Proteins in ten of the 11 spots were found to be significantly upregulated in the newly acquired culture and proteins in one spot, spot 69 containing bacterial protein derivatives; aconitate hydratase and TonB-dependent receptor proteins, was found to be significantly elevated in the attenuated isolate. Many of the differential proteins followed a linear trend of expression, starting with the highest expression in the newly acquired, followed by intermediate expression in the virulent-1 culture and the

lowest expression in the attenuated culture. The current study focused on the soluble, cytoplasmic proteins of the *N. perurans* parasite and their association with pathogen virulence. This is supported by the elevated proteins identified from the virulent-2 culture which include the biological processes of cell cytoskeletal re-organisation, protein synthesis, oxidative stress and immunomodulation, all functions which ultimately suggest their involvement in acute AGD virulence. To further the identification of all proteins associated with AGD pathogenesis, future studies will investigate the proteomic profile of membrane-bound and extracellular proteins from the *N. perurans* cultures presented in this report.

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CHAPTER 3

Differential exoproteome and biochemical characterisation of

Neoparamoeba perurans

3.1. Abstract

Infection with the protozoan ectoparasite *Neoparamoeba perurans,* the causative agent of AGD, remains a global threat to salmonid farming. This study aimed to analyse the exoproteome of both an attenuated and virulent-3 *N. perurans* isolate using proteomics and cytotoxicity testing. An amalgamation of bacterial, *N. perurans* and Amoebozoa proteins constituted the database that was searched against and revealed a disproportionate presence of proteins from the co-cultured microbiota of *N. perurans*. LC-MS/MS identified 33 differentially expressed proteins, the majority of which were significantly elevated in the attenuated exoproteome. Proteins of interest found in both exoproteomes were maltoporin, PPM type phosphatase, ferrichrome-iron receptor, flagelllin, putative ferric enterobactin receptor and a putative iron transport regulation protein (FecR). Protease activity remained significantly elevated in the attenuated exoproteome compared with the virulent-3 exoproteome. Similarly, the attenuated exoproteome had a significantly higher cytotoxic effect on RTgill W1 cells compared with the virulent-3 exoproteome. The presence of a phosphatase and a serine protease in the virulent-3 exoproteome may facilitate AGD infection but does not appear to be a key player in causing cytotoxicity. Altogether this study reveals prolonged culture of *N. perurans* affects the exoproteome composition in favour of nutritional acquisition, and that the current culturing protocol for virulent *N. perurans* does not facilitate the secretion of virulence factors.

3.2. Introduction

Neoparamoeba perurans (synonym *Paramoeba perurans*) is the marine ectoparasitic agent of amoebic gill disease (AGD) in finfish aquaculture (Young *et al.*, 2007). Previously confined to Tasmania (Munday, 1986), AGD's prevalence and geographic range has expanded to all marine *Salmo salar L.* farming countries including Norway (Steinum *et al.*, 2008), Chile (Bustos *et al.*, 2011), Scotland (Rodger, 2014) and Ireland (Rodger and McArdle, 1996). High morbidity and mortality of infected finfish and economic losses from expensive, curative treatments has led to AGD becoming a significant health problem for aquaculture (Rodger, 2014; Oldham, Rodger and Nowak, 2016). Infection presents as raised white mucoid lesions on the gill surface (Peyghan and Powell, 2006). Histopathological assessment shows epithelial hyperplasia on the host's primary and secondary lamellae (Adams and Nowak, 2004; Adams and Nowak, 2001).

The secretome or exoproteome of pathogenic organisms has gained increased attention in recent years, as many secreted proteins are known to be involved in host-pathogen associations, such as: adhesion, morphogenesis and toxicity to the host's immune system (Ranganathan and Garg, 2009; Hiery *et al.*, 2015). Secreted proteins can be regarded as virulence factors of infectious diseases as they play a role in disease progression (Serrano-Luna *et al.*, 2013). It is well documented that protozoan parasites are known to secrete glycoproteins, proteinases such as elastases, metalloproteases, as well as serine and cysteine proteases that facilitate host tissue invasion (Gonçalves *et al.*, 2018) and augment tissue destruction (Ralston and Petri, 2011). Furthermore, pathogenic amoebae have the ability to produce hydrolytic enzymes that cause detrimental damage to a host's membrane (Siddiqui and Khan, 2014). Protease activity and inhibition assays were also employed to confirm the presence and class of proteases in the extracellular proteome of both *N. perurans* cultures. The cytotoxic effect of each exoproteome on the rainbow trout gill cell line, RTgill W1, was evaluated using the Alamar Blue assay as per Dayeh *et al.* (2005b). The microbiome influence on the exoproteome

was also assessed by including antibiotics in subcultures of the *N. perurans* to reduce the bacterial load, prior to protein identification and protease assays. Proteomic approaches such as liquid-chromatography tandem mass spectrometry (LC-MS/MS) represent a high throughput technique that can map the extracellular proteome of significantly important parasites (Bayer-Santos *et al.*, 2013; Gonçalves *et al.*, 2018; Rubin *et al.*, 2015), thus elucidating their pathogenicity.

It has been hypothesised that *N. perurans* secretes an extracellular product during culture that has virulence implications for AGD development and host-parasite interactions (Butler and Nowak, 2004; Bridle *et al.*, 2015). The authors hypothesising such an extracellular product, suggest a cytolytic enzyme was responsible for the observed cytopathic effects in the fish cell line, CHSE- 214. Furthermore, Bridle *et al.* (2015) demonstrated the inability of an avirulent isolate of *N. perurans* (>3 years in culture), to establish the cytopathic effect that was observed in the virulent isolate. Despite Bridle *et al*. (2015) demonstrating a cytopathic effect from a virulent culture, the causative proteins responsible for the observed host cytopathic response were not identified. Therefore, this study aims to describe the complete extracellular products of an attenuated (4-year-old culture) and virulent (>70 days old) *N. perurans* culture cultivated on marine yeast agar, using a gel-free LC-MS/MS approach.

3.3. Materials and Methods

3.3.1. *Neoparamoeba perurans* culture

Neoparamoeba perurans trophozoites were isolated from AGD infected Atlantic salmon located on a commercial farm in the west of Ireland. An attenuated culture of *N. perurans*, previously shown to have lost virulence (Ní Dhufaigh *et al.,* 2020; in review) was used as the attenuated isolate. The virulent culture (virulent-1) of *N. perurans* (Ní Dhufaigh *et al.,* 2020; in review) became too old to continue in the analyses, therefore the virulent-2 culture from Ní Dhufaigh *et al.* (2020; in review) was propagated beyond 70 days in culture, and therefore its isolate name became 'virulent-3'.

Briefly, the methods to obtain these cultures were: fresh trophozoites were collected by swabbing AGD infected gills and placing swabs in 0.2 µm filtered sterile seawater for 4 h. This combined mixture was subsequently plated and maintained xenically at 16°C on marine yeast agar plates (MYA; 0.01% malt, 0.01% yeast, 2% Bacto Agar, sterile seawater at 30% ppt) overlaid with 7 mL of 0.2 µm filtered sea water (Crosbie *et al.*, 2012). Inoculated plates were washed weekly with 7 mL of sterile seawater to control bacterial growth. Amoebae were sub-cultured weekly by transferring free-floating cells to fresh MYA plates. Confirmation of *N. perurans* identity was performed using real time PCR as previously described by Downes *et al.* (2015).

3.3.2. Antibiotic treatment of *Neoparamoeba perurans* cultures

MYA was prepared in 24 well plates (Thermo Scientific ™Nunc ™) with the addition of 1:100 penicillin–streptomycin (pen–strep MYA; 10,000 U of penicillin and 10 mg of streptomycin per mL in sterile 0.9% [w/v] sodium chloride; Sigma-Aldrich). Trophozoites from the

attenuated and virulent-3 cultures were subcultured onto the pen-strep MYA wells in a final volume of 1 mL. After seven days of antibiotic exposure the extracellular proteome was extracted as described in section 3.3.3.

3.3.3. Extraction of extracellular proteome

Both the attenuated and virulent-3 cultures were harvested upon reaching 70% confluency (approximately two weeks of culturing). Trophozoites were mechanically scraped from several MYA plates and pooled into 50 mL falcon tubes. The trophozoites were centrifuged at 1,000 x g for 10 min at 4°C. The supernatant of the cultures was aspirated into fresh 50 mL falcon tubes followed by centrifugation at 10,000 x g for 10 min at 4°C to further remove bacterial and amoebic debris. After centrifugation, the supernatant was passed through a .45 µm filter (Pall Corporation) and subsequently a .25 µm filter (Pall Corporation) to obtain a cell free filtrate containing the putative extracellular proteome or exoproteome. The filtrate was concentrated 10-fold using Amicon centrifugal filters with a 3 kDa molecular-weight cut off (Millipore Corp). Protein quantification was measured with the BCA assay (Pierce™Thermofisher Scientific).

3.3.4. In-solution trypsin digestion

Concentrated exoproteomes of the attenuated and virulent cultures, and pen-strep treated cultures were adjusted to 100 µg/mL of protein. Buffer was exchanged using centrifugal concentrators in 20 mM Tris-HCl pH 7.5 for desalting purposes. Sample preparation occurred using the PreOmics kit (Munich) according to the Pellet and Precipitated Protein iST protocol with modifications (Kulak *et al.*, 2014). Briefly, desalted exoproteomes were concentrated to 100 µL and 300 µL of the initial kit buffer (described as “lysis buffer”) was added. The sample

was concentrated to a final volume of 50 µL and heated at 95°C for 5 min on a heating block. Samples were then placed in iST cartridges for trypsin digestion (Trypsin/LysC) at 37 °C for 2.5 h with mixing. Peptides were acidified and subsequently washed using wash 1 and wash 2 buffers. Peptides were eluted in 120 µL and dried using a vacuum centrifuge (Savant Speedvac, Thermo Fisher Scientific). After drying, peptides were resuspended in LC load buffer.

3.3.5. Liquid chromatography tandem mass spectrometry

Peptide fractions were analysed on a quadrupole Orbitrap (Q-Exactive, Thermo Scientific) mass spectrometer equipped with a reversed-phase NanoLC UltiMate 3000 HPLC system (Dionex LC Packings, now Thermo Scientific). Peptide samples were loaded onto C18 reversed phase columns (10 cm length, 75 µm inner diameter) and eluted with a linear gradient from 1 to 27% buffer B containing 0.5% AA and 97.5% ACN in 58 min at a flow rate of 250 nL/min. The injection volume was 5 µL.

3.3.6. Raw data processing and label free quantification (LFQ)

Raw data from the Orbitrap Q-Exactive was processed using MaxQuant version 1.6.14.0 for identification of proteins (Cox and Mann, 2008), incorporating the Andromeda search engine and MaxQuant's contaminants fasta file (Cox *et al.*, 2011). To identify peptides and proteins, MS/MS spectra were matched to a custom database of proteins from *N. perurans* (20,887 proteins [v2, 07/08/2019, CSIRO]), combined with a UniProt reference proteome database of Amoebozoa proteins (109,415 proteins), as well as proteins from a *Paramoeba* UniProtKB taxonomy search (5,001 proteins). The inclusion of proteins from a broad range of amoebae in the Amoebozoa dataset was to both facilitate and validate the identification of amoeba-specific proteins. Co-culturing bacteria of *N. perurans* contributed to the protein

identifications in the cytoplasmic protein investigation of *N. perurans* (Ní Dhufaigh *et al.,* 2020; in review), therefore it is imperative to distinguish between *N. perurans* specific and bacterial specific proteins. The microbial communities of the attenuated and a virulent-1 cultures were previously evaluated using 16S rRNA sequencing (Ní Dhufaigh *et al.,* 2020; in review). The key genera identified as part of this analysis were used to inform the creation of a bacterial protein database consisting of 148,582 proteins. This bacterial database was used in conjunction with the aforementioned amoebic databases. All proteins, except for those from the *N. perurans* database, were downloaded on May 8th 2020 from UniProt. Details of the total protein count from each species included in the database can be found in Appendices I, II, and III.

All searches were performed with tryptic specificity allowing two missed cleavages. The database searches were performed with carbamidomethyl (C) as fixed modification and acetylation (protein N terminus) and oxidation (M) as variable modifications. Match between runs was allowed and proteins were quantified using the LFQ algorithm in MaxQuant (Cox *et al.*, 2014). Mass spectra were searched using the default setting of MaxQuant namely, a false discovery rate of 1% on the peptide and protein level. For bioinformatic analysis, LFQ values obtained in MaxQuant were imported into Perseus (v. 1.6.10.50) software. Reverse hits, contaminants and peptides identified by site were filtered out. LFQ values were log2 transformed and differentially expressed proteins between samples were identified using a two-way Students t-test followed by Benjamin-Hochberg false discovery rate (FDR) correction. Hierarchical clustering was generated on the statistically significant proteins using Z-score protein intensities for the proteins with $p < 0.05$. For the antibiotic treated parasite exoproteome, comparative parameters were based on the total number of razor and unique peptides in each sample.

3.3.7. Enzymatic characterisation

Protease activity of the attenuated and virulent-3 concentrated exoproteomes was evaluated as previously described (Marcos-López *et al.*, 2017; Ross *et al.*, 2000) with modifications. Briefly, 250 µL of 2% azocasein dye (Sigma-Aldrich) was added to 100 µL of sodium phosphate buffer pH 8, serving as the reaction buffer. A 100 µL of either concentrated exoproteome or exoproteome of pen-strep treated cultures (at a concentration of 40 µg/mL), was added to the reaction buffer for both the attenuated and virulent-3 exoproteomes. 100 µL of 2 mg/mL Trypsin (Sigma-Aldrich) served as the positive control and 100 µL sterile seawater (.45 µm filtered and autoclaved) served as the negative control. Samples were incubated overnight at room temperature (20 – 22 °C) on a rocker. Reactions were stopped by the addition of 250 µL of 10% TCA and centrifuged at 6,000 x g for 5 min at room temperature. 100 µL of the sample supernatants were added to 100 µL of 1 N NaOH in a 96 well plate. Optical density was recorded at 450 nm on a Multiskan Sky microplate reader (ThermoFisher) using SkanIt software (ThermoFisher). The absorbance of the sterile seawater sample was subtracted from all other assay absorbance values. Protease activity was presented as a % of the positive control.

3.3.8. Enzyme inhibition assay

The protease assay as described in section 3.3.7. was modified to include the presence of various protease inhibitor classes to determine protease families found in the *N. perurans* exoproteome. Two protease inhibitors were selected, Phenylmethylsulfonyl fluoride (PMSF) targeting the serine protease family and Ethylenediaminetetraacetic acid (EDTA) targeting metalloproteinases. Exoproteome samples, trypsin and seawater controls were preincubated with 1 mM PMSF or 1 mM EDTA at room temperature for 2 min prior to the addition of azocasein dye and phosphate buffer. The experiment followed as described in section 3.3.7.

3.3.9. RTgill W1 culture and assay preparation

The *Oncorhynchus mykiss*, (rainbow trout) gill cell line RTgill W1 was purchased from the American Tissue Culture Type Collection (CRL 2523) and cultivated according to Bols *et al.* (1994) and Dayeh *et al.* (2005a). RTgill W1 cells were cultivated at 19°C on tissue culture treated T-75 cm^2 flasks (VWR Collection) in Leibovitz's L-15 media (Hyclone Laboratories) supplemented with 10% foetal bovine serum (Gibco, Thermofisher Scientific) and an antibiotic/antimycotic solution (10,000 U/ml Penicillin G, 10,000 µg/mL Steptomycin, 25 µg/mL Amphotericin B [Hyclone Laboratories]- complete media). RTgill W1 cells were cultivated in 100% atmospheric O_2 in a water jacketed CO_2 incubator, Forma Series II (Thermo Scientific).

Once 70% confluency was achieved, RTgill W1 cells were prepared for the cytopathic assays. Briefly, cells were detached by trypsin/EDTA solution (Biowest, Nuaillé), resuspended in 5 mL of L-15 containing FBS and cell number enumerated by haemocytometer (Neubauer, Hawksley). Cells were seeded overnight at 19°C at a cell density of 25,000 cells/well in a fluorescent 96-well plate (Corning). After 24 h, media was removed from the wells and test media was added. Exoproteomes from *N. perurans* attenuated, virulent-3 and both pen-strep treated cultures were buffer exchanged into phenol red-free L-15 media using centrifugal concentrators. A total of 40 µg of protein from each exoproteome sample was added to test wells in a final volume of 200 µL. Positive controls were treated with phenol red-free L-15 media only, while 0.5% Triton X-100 (Sigma-Aldrich) served as the negative control. Media only was added to no-cell wells to monitor background fluorescence. The final volume in each well was 200 µL. After 24 h of test exposure, RTgill W1 viability was assessed.

3.3.10. Cytotoxicity alamar blue preparation

Alamar blue was prepared as per Dayeh *et al.* (2005a). A 5% (v/v) alamar blue solution (AB) was prepared in sterile filtered PBS pH 7.4, prior to starting the assay. This assay was performed three times independently with the exoproteomes of the attenuated and virulent-3 cultures. Evaluation of the exoproteome from pen-strep treated cultures was only performed once. Media was removed from the 96-well plate and 200 µL of AB was pipetted into each well. The plate was incubated in the dark for 30 min at 19°C. Fluorescence measurements were carried out using a Fluoroskan™Microplate Fluorometer (Thermofisher) at 530 nm (excitation) and 590 nm (emission).

3.4. Results

3.4.1. Exoproteome identification using differential proteomics

Results of the LC-MS/MS showed that 38 proteins were identified from a combination of bacterial and amoebic (Amoebozoa group and *N. perurans*) protein databases in total. A final 33 significant proteins were found to be differentially expressed between the exoproteomes of the attenuated and virulent-3 parasites. These 33 significantly, differentially expressed proteins are plotted in a clustered heatmap (Figure 2). A total of 11 *N. perurans* (Table 1) proteins contributed to the differential proteomic expression observed between parasite exoproteomes and the remaining 22 protein identifications were assigned to bacteria, from genera *Pseudoaltermonas* spp and *Vibrio* spp. All bacteria-associated protein identifications, significant and non-significant, are listed in Appendix V. Table 2 depicts the top seven bacterial protein identifications, based on relevance to extracellular function and cytotoxicity potential to RTgill W1.

Only one protein, flagellin of *Vibrio tasmaniensis* (strain LGP32), displayed significant elevation in the virulent-3 exoproteome, meaning 32 proteins remained significantly reduced in the virulent-3 exoproteome. Therefore indicating a diminished role for secreted proteins in *N. perurans* virulence repertoire. Contrarily, the attenuated exoproteome maintained significant elevation in these 32 proteins, including all 11 *N. perurans* protein identifications. Of the 33 significant proteins, three *N. perurans* proteins were identified as hypothetical proteins and two *N. perurans* were assigned 'NA' or unidentified. Five non-significant proteins that were shared between parasite exoproteomes were maltoporin, PPM-type phosphatase domain-containing protein, ferrichrome-iron receptor, flagellin and putative ferric enterobactin receptor. A PPM-type phosphatase domain-containing protein (*P. fungivorum*) was the only non-*N. perurans* amoebic protein identified. This identification is likely to represent a protein

that has high homology to a native *N. perurans* phosphatase and was therefore grouped with the *N. perurans* proteins in Table 1. Proteins that were significantly elevated in the attenuated exoproteome relate to serine protease and endopeptidase activity, cytoskeletal and membrane associated proteins and suggest functional roles in nutritional acquisition.

3.4.2. Antibiotic treated exoproteome identification

The analysis of one sample from the exoproteome of the pen-strep treated *N. perurans* culture resulted in the identification of 30 proteins by LC-MS/MS, from a combination of bacterial and amoebic (Amoebozoa group and *N. perurans*) protein databases. No differential statistical analysis is reported here for these proteins as no technical replicates were included in this experiment. A total of six *N. perurans* proteins and one actin-3 protein from the soil-dwelling amoeba *Dictyostelium discoideum* contributed to the 30 identifications (Table 3). The latter identification is again likely to represent an *N. perurans* actin homologue. The remaining 23 proteins were of bacterial origin (Appendix VI), from *Pseudoaltermonas* spp, *Thalassospira xiamenensis* and *Vibrio* spp. Two hypothetical proteins and two unidentified *N. perurans* proteins were present in both pen-strep exoproteomes and one uncharacterised bacterial protein of *Pseudoalteromonas haloplanktis*, was present in the attenuated exoproteome only (based on number of razor and unique peptides). Despite the introduction of antibiotics, the attenuated exoproteome maintained a higher razor and unique peptide count.

Table 1: All 11 *N. perurans* protein identifications and one putative homologous protein associated with *Planoprotostelium fungivorum* from MaxQuant analysis of the attenuated and virulent-3 exoproteomes, characterised by a two-way Students *t*- test in Perseus.

Gene Bank IDs	Protein IDs	Fasta headers	Mol. weight [kDa]	Fold change[a]	Attenuated peptides[b]	Virulent peptides[b]	T-test[c]
	A0A2P6N765	PPM-type phosphatase domain-containing protein (*Planoprotostelium fungivorum*)	190.44	-0.144883	14	19	NS
	PPER_00018933-RA	---NA---	281.31	-4.72831	40	17	+
	PPER_00016120-RA	TolAlike protein	36.42	-3.89208	11	0	+
	PPER_00020708-RA	Hypothetical protein	64.3	-5.01281	10	0	+
	PPER_00011993-RA	---NA---	35.25	-1.15085	8	6	+
	PPER_00013024-RA;PPER_00016390	Hypothetical protein; calmodulin	13.01	-4.76979	8	3	+
	PPER_00019895-RA	Multifunctional chaperone	28.08	-3.49453	9	2	+
	PPER_00008268-RA	Urea ABC transporter substrate-binding protein Branched-chain amino acid	47.26	-3.18528	6	0	+
	PPER_00011992-RA	Hypothetical protein	39.76	-2.86775	6	6	+
	PPER_00012939-RA	Cu-Zn superoxide dismutase	15.50	-3.72917	6	2	+
	PPER_00014132-RA	Physarolisin	49.49	-4.06437	5	1	+
	PPER_00011189-RA	Peptidase S8 and S53 domain-containing protein	58.69	-2.78376	3	0	+

The + sign = statistically significant differential proteins (based on q-value); NS = non-significant; Mol= molecular; kDa = kilodalton; [a] Fold change of virulent proteins related to attenuated proteins; [b]Razor and unique peptide counts; [c]Student's t test significance.

Table 2: Top six non-*Neoparamoeba perurans* proteins of bacterial origin, identified by Max Quant analysis of the attenuated and virulent-3 *N. perurans* exoproteomes. Proteins are ranked based on relevance to extracellular function. The complete set of identifications are provided in Appendix V.

Gene	Protein IDs	Fasta headers	Organism	Mol. weight [kDa]	Fold change[a]	Att. Peptides[b]	Virulent Peptides[b]	T-test[c]
ST37_14230	A0A1X1MR06	ABC transporter substrate-binding protein	Vibrio sp. qd031	47.26	-3.18528	6	0	+
VS_II0220	B7VQI9	Maltodextrin-binding protein	Vibrio tasmaniensis (strain LGP32	42.39	-4.90439	13	4	+
VS_0355	B7VIC7	Immunogenic protein	Vibrio tasmaniensis (strain LGP32)	34.44	-5.20998	11	1	+
ahpCB	Q3IE21	Peroxiredoxin 2 (TSA) (PRP)	Pseudoalteromonas haloplanktis (strain TAC 125)	22.12	-4.75659	9	0	+
PSHAa2977; B1199_19990	Q3IK06; A0A244CKW5	Secreted alkaline phosphatase; Alkaline phosphatase	Pseudoalteromonas haloplanktis (strain TAC 125); Pseudoalteromonas ulvae	56.1	-4.1791	10	2	+
PCIT_22080	U1KK96	Peptidase S8/S53 subtilisin kexin sedolisin	Pseudoalteromonas citrea DSM 8771	127.37	-3.70559	6	3	+

The + sign = statistically significant differential proteins (based on q-value); Mol= molecular; kDa = kilodalton; [a] Fold change of virulent proteins relative to attenuated proteins; [b]Razor and unique peptide counts; [c]Student's t test significance

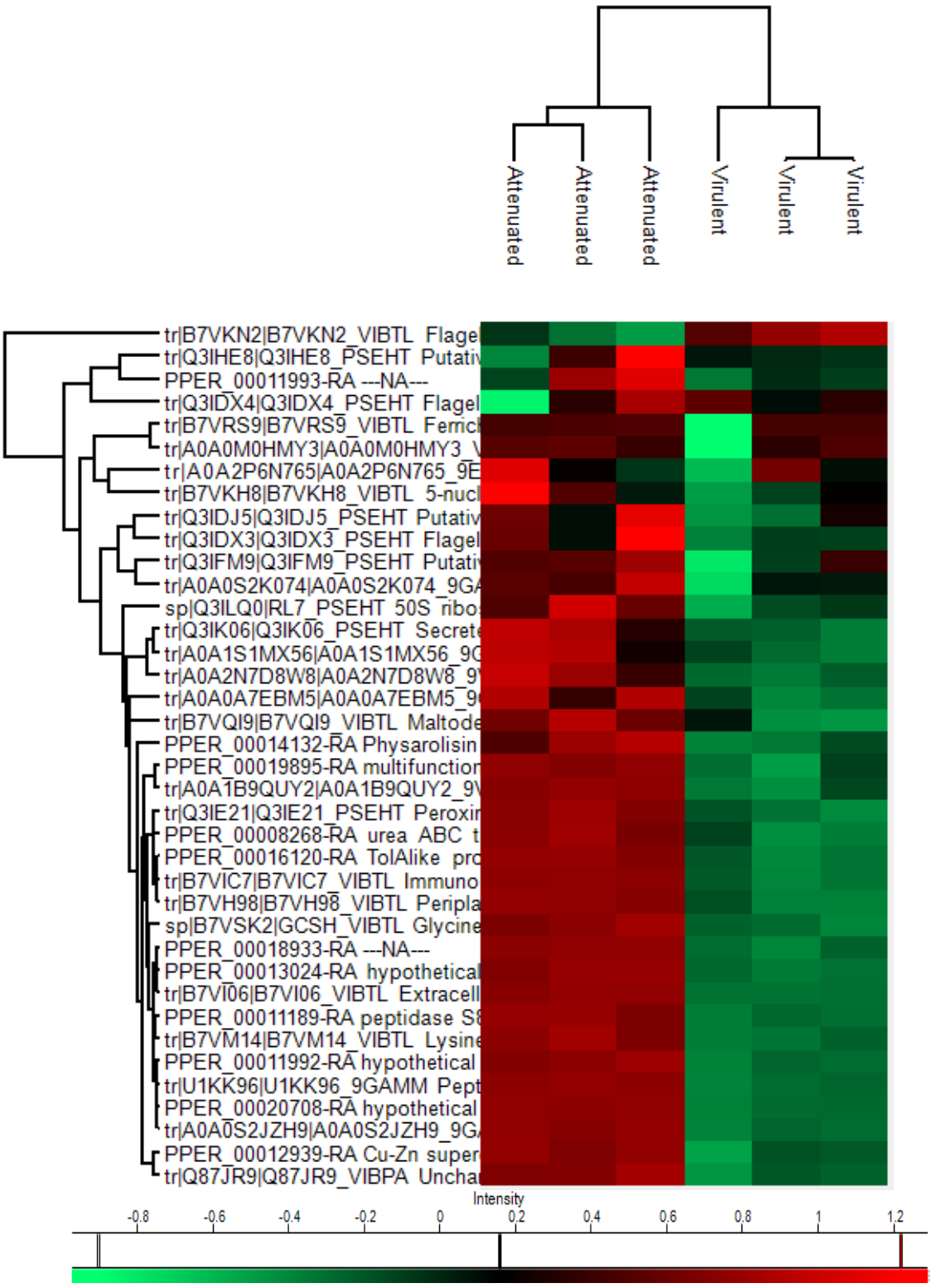

Figure 1: Hierarchical clustering of statistically significant proteins between the attenuated and virulent-3 exoproteome replicate samples. LFQ intensity values were normalised by z- scoring with red colours showing increased abundance and green displaying a decreased expression of proteins.

Table 3: All six *Neoparamoeba perurans* proteins and one putative homologous protein associated with *Dictyostelium discoideum* identified using Max Quant analysis of the exoproteomes of pen-strep treated attenuated and virulent-3 cultures.

Protein IDs	**Fasta headers**	**Mol. weight [kDa]**	**Attenuated peptides[a]**	**Virulent peptides[a]**
P07829	Actin- 3 (*Dictyostelium discoideum*)	41.84	2	2
PPER_00011992-RA	Hypothetical protein	39.76	4	4
PPER_00011993-RA	--NA---	35.25	4	4
PPER_00013030-RA	Cell envelope biogenesis	74.1	4	0
PPER_00011994-RA	Leucine-rich repeat-containing protein C10orf11-like	40.9	3	2
PPER_00017145-RA	Hypothetical protein	32.89	2	2
PPER_00018933-RA	---NA---	281.31	6	4

[a]Razor and unique peptide counts; NA refers to unidentified protein

3.4.3. Enzymatic characterisation and inhibition assay

All exoproteome isolates hydrolysed the nonspecific protease substrate, azocasein, relative to the positive control (Figure 2), excluding the sample from virulent-3 pen-strep treated culture and PMSF treated sample. The attenuated and virulent-3 exoproteome exhibited different protease activity, with the attenuated having significantly more protease activity than that of the virulent-3 ($p < 0.0001$) and virulent-3 pen-strep treated ($p < 0.0001$) exoproteome.

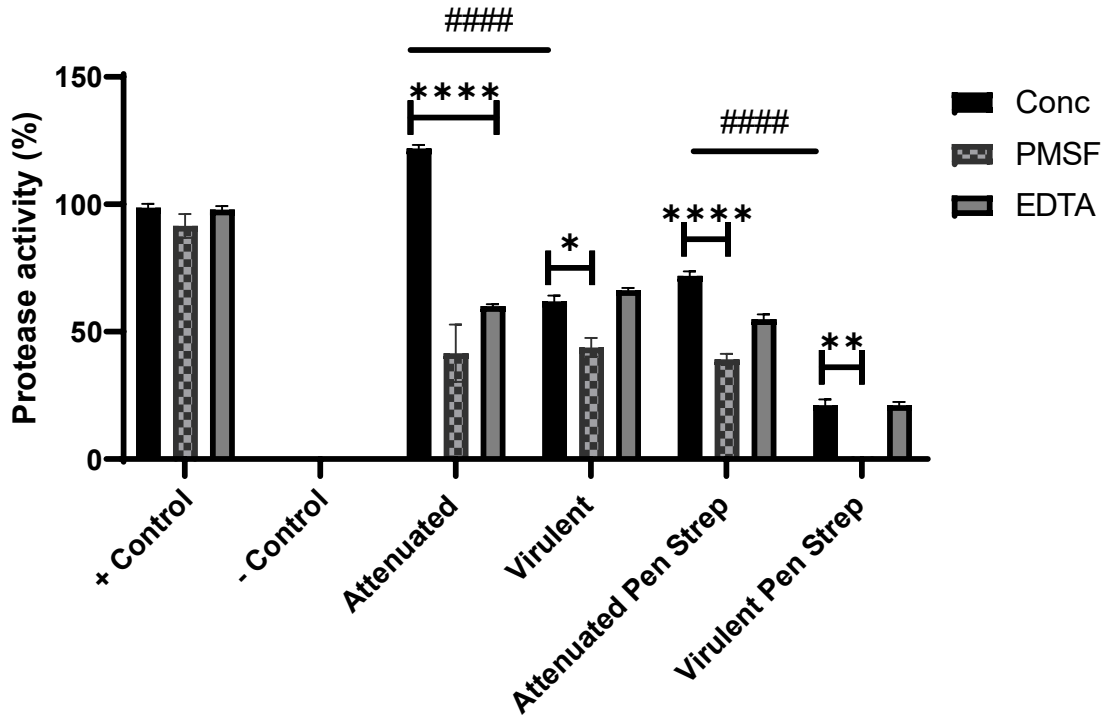

Figure 2: Protease activity and inhibition assay of exoproteome samples from the attenuated, virulent-3 and antibiotic (pen-strep) treated attenuated and virulent-3 cultures using azocasein as a substrate. Trypsin (2mg/mL) was used as positive control and sterile seawater as a negative control. Results are expressed as a percent of the positive control. All data sets are represented as ±SEM from biological replicates. Two-way ANOVA followed by a Tukey post hoc test for multiple comparisons: Attenuated vs PMSF and EDTA, ****(p <0.0001); Virulent vs PMSF,*(p < 0.0284), EDTA non-significant (ns); Attenuated vs Virulent, #### (p <0.0001); Attenuated Pen Strep vs PMSF ****(p <0.0001); EDTA ns; Virulent Pen Strep vs PMSF, ** (p <0.0044), EDTA ns; Attenuated Pen Strep vs Virulent Pen Strep, #### (p <0.0001).

Presence of inhibitors in both the attenuated (p <0.0001) and virulent-3 (p <0.0284) exoproteome significantly reduced protease activity, except for EDTA in the virulent-3 exoproteome, which was found to be non-significant. The introduction of PMSF to both the exoproteome samples of pen-strep treated cultures was statistically significant (pen-strep attenuated p <0.0001; pen-strep virulent-3 p <0.0044), suggesting the presence of serine and threonine proteases in all *N. perurans* exoproteomes. EDTA had a non-significant impact on the exoproteome samples from both pen-strep treated cultures, suggesting *N. perurans* does not rely on proteases of this family.

3.4.4. Cytotoxicity assay

The attenuated exoproteome caused significant cell cytotoxicity compared with the virulent-3 exoproteome, revealed by the metabolic impairment dye, Alamar blue (Figure 3). The RTgill W1 cells treated with the virulent-3 exoproteome load remained largely unaffected by the overnight incubation. This result indicates that the more virulent-3 isolate of *N. perurans* does not release cytotoxic molecules when cultured in the laboratory with MYA agar.

3.4.5. Cytotoxicity of exoproteome from antibiotic-treated *Neoparamoeba perurans* cultures

As shown in Figure 3b, once the cultures were treated with antibiotics, the cytotoxicity of the attenuated exoproteome was reduced and the virulent-3 exoproteome had an increased cytotoxic effect on the RTgill W1 cells. This shift in the secretion of cytotoxic proteins upon exposure to antibiotics, could be a survival response in the amoeba as it loses its symbiotic diversity.

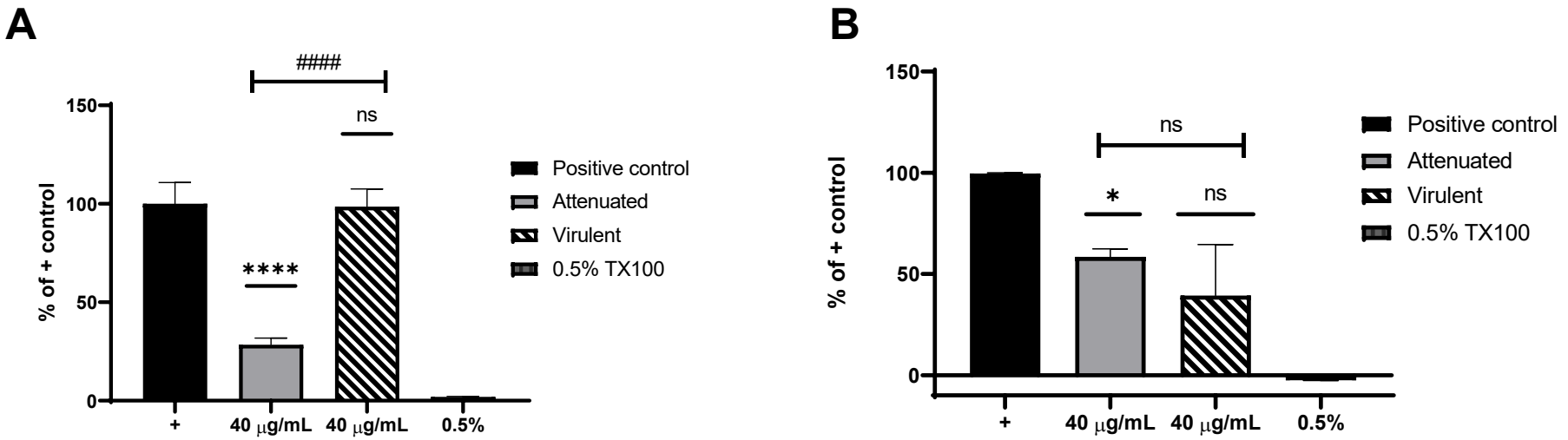

Figure 3: Cytotoxicity assay using RTgill W1. A) 40 µg/mL of attenuated and virulent-3 exoproteome. B) 40 µg/mL of pen-strep treated attenuated and virulent-3 exoproteome. RTgill W1 cells in sterile PBS were used as positive (+) control and sterile seawater as a negative control. Results are expressed as a percent of the positive control and are representative of one independent experiment. All data sets are represented as ±SEM from biological replicates. One-way ANOVA followed by Tukey post hoc test for multiple comparisons: A) Attenuated vs + control, ****(p <0.0001); Virulent vs + control, ns; Virulent vs +control, ns; Attenuated vs virulent, #### (p <0.0001). B) Attenuated Pen Strep vs + control, * (p <0.0278); Virulent Pen Strep vs + control; ns; Attenuated Pen Strep vs Virulent Pen Strep, ns.

3.5. Discussion

The aim of this study was to characterise the differences in protein expression in the exoproteome of an attenuated versus a virulent-3 culture of *N. perurans* and antibiotic-treated attenuated and virulent-3 *N. perurans*. This antibiotic combination, penicillin–streptomycin, was introduced to subcultures of the attenuated and virulent-3 isolates to determine if *N. perurans* associated microbiota was responsible for the secretion of proteins identified in the exoproteome. Biochemical tests using a protease assay and protease inhibitors were performed to detect and identify specific protease families in all exoproteomes. Previous studies of pathogenic amoebae such as *E. histolytica* (Serrano-Luna *et al.*, 2013) and *N. fowleri* (Kim, Kim and Shin, 2008) report the cytotoxic role of the secretions from the respective parasites during infection, therefore this study also sought to investigate the cytotoxicity of *N. perurans* exoproteomes using the gill epithelial cell line, RTgill W1.

Encompassing the complexity of *N. perurans* and its microbiota, protein databases of several key genera were included from a recent microbiome study utilising these two amoeba isolates (Ní Dhufaigh *et al*, 2020; in review). Furthermore, to facilitate the identification of amoebic proteins the Amoebozoa reference proteome from UniProt was included in the analysis, in conjunction with the new *N. perurans* database (CSIRO, 2019). Overall, the LC-MS/MS results revealed a stark contrast between exoproteomes of the two parasites and most remarkably, that the attenuated parasite significantly secretes more proteins than the virulent-3 culture. A disproportionate quantity of proteins identified originated from the microbiome of the parasites, suggesting the commensal bacteria cohabiting in *N. perurans* are actively releasing proteins into their extracellular milieu. The significant presence of bacterial protein identifications (11 *N. perurans* proteins to 22 bacterial proteins) highlights the intrinsic yet predominant role of the parasite's microbiome in attenuated culture and has clear implications

when performing comparative exoproteomic analysis. Only one protein, flagellin of *Vibrio tasmaniensis* (strain LGP32), was found to be present at a significantly higher level in the virulent-3 exoproteome. This protein is non-amoebic in nature. Flagellin is the main subunit of the bacterial flagellae and is likely present due to the significant presence of *Vibrio* bacteria in the culture. Although present at a non-significantly elevated level, five additional proteins were found in both exoproteomes, four of which are membrane-associated. These proteins; maltoporin, ferrichrome-iron receptor, flagelllin and putative ferric enterobactin receptor were associated with the *Vibrio* and *Pseudoaltermonas* bacterial genera. Interestingly, both maltoporin and ferrichrome-iron receptor were found exclusively in the virulent-3 exoproteome. Given their function, these proteins could potentially be bacterial-associated virulence factors of *N. perurans*. Maltoporin is a conserved antigen in *Vibrio* species (Lun *et al.*, 2014) and ferrichrome-iron receptor is a known bacterial virulence factor associated with iron-scavenging in environments with low iron-bioavailability (Payne *et al.,* 2016). The presence of iron acquisition proteins reflects the iron-limited growth conditions in which *N. perurans* is cultured in *in vitro* which suggests that the parasite may rely on its microbiota to scavenge iron.

The majority of protein identifications which were present at a significantly higher level, came from the attenuated exoproteome compared to the virulent-3, and mostly were of bacterial association. No proteins of *N. perurans* endosymbiont, *Perkinsela* spp. were found despite the inclusion of *Perkinsela* spp. Database and , the genome of *N. perurans* was constructed to include the endosymbiont.. *N. perurans* specific proteins were identified, many of which had endopeptidase activity which correlates with cytotoxicity, such as Cn-Zn superoxide dismutase (SOD) and physarolisin, (Table 1). SOD expression has previously been found to be significantly higher in the cytoplasmic fraction of a virulent-2 isolate of *N. perurans* (Ní Dhufaigh *et al,* 2020; in review). Although SOD is reported to defend parasites against the host

response, we suggest its presence here is part of the attenuated isolate's defence mechanism in response to excess production of endogenous oxidative stressors from its own commensal microbiota.

TolA-like and urea ABC transporter proteins were present at significantly higher levels in the attenuated exoproteome, highlighting that membrane-associated proteins can be released into the extracellular milieu. The presence of ABC transporters has been associated with the exoproteomes of marine bacteria in nutrient poor environments, where increased transcription of transporter proteins increases scavenging potential for nutrients (Christie-Oleza *et al.*, 2015; Christie-Oleza and Armengaud, 2010). A tolA-like protein in *Acanthamoeba* has been described as an environmental adaptation to bacterial toxins (Shabardina *et al.*, 2018), a role that *N. perurans* may require during prolonged culture and thus explains the increased presence in the attenuated exoproteome.

Physarolisin, a carboxyl enzyme isolated here belongs to the serine peptidase family S53 (Takahashi, 2013) and was elevated in the attenuated exoproteome. A cysteine peptidase family (C53) was notably elevated in a virulent-2 isolate from its cytoplasmic proteome as a zymogen, discussed in Ní Dhufaigh *et al.* (2020; in review). Although this cysteine protease was not identified in this current study, the potential for identification of differing virulence phenotypes based on differential expression of protease families could be a useful biomarker of virulence. A peptidase, S8 and S53 domain containing protein was absent entirely from the virulent-3 exoproteome but remained elevated in the attenuated exoproteome. However, a peptidase S8/S53 subtilisin kexin sedolisin from *Pseudoaltermonas citrea* was present in both exoproteomes and this protein may explain the serine protease activity seen in both exoproteomes. Binding proteins such as hypothetical protein (PPER_00020708) and calmodulin associated with calcium binding were also identified in the attenuated

exoproteome. Hypothetical protein (PPER_00020708) was identified as calponin after performing a BLAST search (Altschul *et al.*, 1990). Multifunctional chaperone expression relating to proteomic homeostasis (Buchner, 2019) was increased in the attenuated exoproteome, which may be a result of maintaining protein stability from the commensal microbiotas nutritional stressors. However, most of the differentially expressed proteins of the virulent-3 and attenuated exoproteome were of bacterial origin. The significantly higher level observed in the attenuated *N. perurans'* exoproteome can be considered an artefact of prolonged culture and a result of the parasite's associated microbiota secreting soluble components into the culture supernatant. Both attenuated and virulent-3 isolates were cultured xenically and the presence of commensal bacteria is very evident in both cultures however, increased biofilm presence was observed in the attenuated isolate.

Based on these results, it would appear that the virulent-3 *N. perurans* isolate analysed here, does not secrete an abundance of proteins into its environment. This contrasts with observations made by Butler and Nowak (2004) and Bridle *et al.* (2015) that indicated the presence of an extracellular product was inducing a cytopathic effect on the CHSE-214 and RGE-2 cell lines, respectively. The virulent *N. perurans* isolate analysed in this study was in culture for over 70 days before harvesting and isolation of its exoproteome and cultured using MYA as per Crosbie *et al.,* (2012). It is imperative to note however, that cultures of *N. perurans* maintained for over 70 days on MYA can cause AGD, as outlined in Ní Dhufaigh *et al*. (2020; in review). These contrasting results indicate that either, secreted proteins do not play a role in inducing AGD *in vivo* or, more likely, that the culture conditions do not favour the secretion of virulence factors. The parasite may require the presence of certain environmental stimuli to activate such a mechanism. This has been shown in other studies e.g. Gonçalves *et al.* (2018) and Rubin *et al.* (2015) who reported that cultivation of parasites *in vitro* can facilitate or lessen protease secretion either by providing optimal nutritional growth or sustained growth. It has

also been shown that parasitic amoebae can be prompted to release cytotoxic proteases in response to sugars or host derived components. Mannose added to cultures of *Acanthamoeba spp.* triggers the release of MIP133, a 133 kDa serine protease (Hurt *et al.*, 2003) and in response to co-incubation with extracellular matrix components of host fibronectin, *E. histolytica* adheres to these fibronectin fragments and simultaneously causes proteolysis (Franco, Vazquez-Prado and Meza, 1997). Future endeavours relating to the study of the *N. perurans* ECP should explore culture media supplementation.

Due to the bacterial presence in the attenuated isolate, we sought to reduce the bacterial proteome background by incorporating antibiotics into the MYA of both *N. perurans* cultures and sub-culturing for seven days prior to exoproteome analysis. Proteomic changes were observed as a result of antibiotic addition, resulting in only six *N. perurans* proteins (Table 3), one homologous actin-3 of *Dictyostelium discoideum* (Table 3) and 23 proteins (Appendix V) of bacterial origin. Peculiarly, the introduction of penicillin-streptomycin only slightly reduced the presence of bacterial proteins but did change the protein's expression profile towards a more nutritional function (i.e 11 proteins can be molecularly categorised in the uptake of iron). Several proteins of unknown function, two of which were classed as unidentified or 'NA' (PPER_00011993 and PPER_00018933), and two proteins classed as 'hypothetical proteins' (PPER_00011992 and PPER_00017145) were identified in both exoproteomes. The two unidentified proteins were previously found in the non-antibiotic treated cultures, therefore a BLAST (Altschul *et al.,* 1980) search was performed to try and elucidate their function. Tentatively, the search revealed that the unidentified protein, PPER_0018933, has a low similarity (0.008 [E value], 23.67% identity) to a hypothetical protein from *Sorangium cellulosum* (BE08_15360). An additional BLAST search of this protein sequence resulted in numerous hypothetical proteins except for one protein, FecR family protein, an iron transport regulation protein. Once more, the presence of an iron scavenging protein in *N. perurans*

exoproteome has been putatively revealed therefore, future research efforts should address the link between iron availability and the synthesis of virulence factors. A new protein, hypothetical protein (PPER_000171145) identified by a BLAST search as HEPN-domain-like protein, was identified in the exoproteomes of both virulent-3 and attenuated pen-strep treated cultures. This protein can be associated with a toxin-antitoxin system and can regulate antibiotic resistance in prokaryotes (Yao *et al.*, 2015; Jia *et al.*, 2018). Whether this protein discovered here has a role as a toxin, or interestingly in antibiotic resistance, remains unknown until further functional studies are completed. One further protein (PPER_0013030_RA), whose function is associated with cell envelope biogenesis, was found in the pen-strep treated attenuated exoproteome suggesting the microbiome's attempt in repairing cellular damage from the antibiotic treatment.

A membrane-associated *N. perurans* protein, leucine rich repeat (LRR) protein, was found in the exoproteomes of both pen-strep treated cultures. LRR motifs are involved in pathogen recognition and host cell signalling and their location on the surface and as an excretion product of the membrane, allows for host interaction (Kedzierski *et al.*, 2004). Even though LRR motifs have been classified as virulence factors in many parasites, including *E. histolytica*, they are diverse and only some share homology to BspA protein, a LLR-containing protein responsible for bacterial-host colonisation (Davis *et al.*, 2006). Results of this study confirm the loss of virulence in the attenuated isolate as demonstrated in Ní Dhufaigh *et al.* (2020; in review). It is hypothesised that this isolate's inability to infect is directly correlated to the absence of necessary host cell attachment ligands, therefore it is unlikely that this LLR-containing protein is acting as a virulence factor under these conditions.

Protease activity remained significantly higher (p <0.0001) in the attenuated exoproteome compared with the virulent-3 exoproteome and the exoproteome of the pen-strep

treated virulent-3 culture. Based on the protein identifications from LC-MS/MS, we propose the involvement of physarolisin, peptidase S8 and S53 domain-containing protein (of *N. perurans*) and peptidase S8/S53 subtilisin kexin sedolisin (of *P. citrea*) from the attenuatedculture to the observed protease activity. Protease activities of all exoproteomes were significantly inhibited in the presence of PMSF indicating serine proteases are dominant in *N. perurans* exoproteome. The unknown protein, PPER_00011993, was identified in all exoproteomes and could plausibly represent an uncharacterised serine type protease, due to the significant inhibition of protease activity in the virulent-3 exoproteome by PMSF. Peptidase S8/S53 subtilisin kexin sedolisin of *P. citrea* was also present in the virulent-3 culture and may have also contributed to the production of protease activity. Protease inhibition using EDTA was also significantly reduced in both the attenuated exoproteome and that of the pen-strep treated attenuated culture, however no significant difference in metalloproteinase inhibition was noted between the virulent-3 exoproteome and that of the pen-strep treated virulent-3 culture. The reduced proteomic identifications from the exoproteomes of pen-strep treated cultures correlates with the reduced protease activity seen in the antibiotic treated cultures, thus directly suggesting the role of *N. perurans* microbiota.

RTgill W1 (trout gill) cells served as a model to test the cytotoxic effect of the exoproteomes of both the virulent-3 and attenuated *N. perurans.* Results of the cytotoxicity assays showed that the attenuated exoproteome caused significantly more cytotoxicity than the virulent-3 exoproteome (Figure 3, A). This is likely to derive from the presence of a combination of *N. perurans* physarolisin, Cu-Zn superoxide dismutase and bacterial proteins: Peroxiredoxin 2 (of *P. haloplanktis*) secreted alkaline phosphatase (of *P. haloplanktis*) and plausibly an immunogenic protein (of *V. tasmaniensis*) in the exoproteome of the attenuated parasite. Cells treated with the virulent-3 exoproteome remained unaffected, and levels of cytotoxicity were comparable to untreated controls. In contrast to this finding, an attenuated

culture of *N. perurans* was previously reported to cause no cytopathic effect to an embryonic cell line, CHSE-214 (Bridle *et al*, 2015). However, discrepancies in findings could be explained by the selection of the cell line model and furthermore, the method of culturing *N. perurans* prior to the collection of the parasite's supernatant. Previous studies with bacteria describe a higher susceptibility of CHSE-214 to infection during co-incubation with *Yersinia ruckeri* compared with salmon head-kidney and Atlantic salmon kidney cells (Menanteau-Ledouble, Lawrence and El-Matbouli, 2018) as well as fathead minnow and rainbow trout liver cell lines (Tobback *et al.*, 2010). Menanteau-Ledouble, Lawrence and El-Matbouli (2018) also identified a significant difference between high passage and low passage of CHSE-214, where younger cells adhered more tightly than older cells. Thus, the influence of passage number may have a direct effect on CHSE-214 cells during cytopathic effect studies. RTgill W1 are known precursor stem cells of the gill (Bols *et al.*, 1994) and under appropriate culturing conditions, secrete mucus from goblet-like cells (Lee *et al.*, 2009), therefore representing a more appropriate *in vitro* model for AGD. Upon commencing this experiment no salmon cell line was commercially available, thus RTgill W1 was selected. However the recent development of two salmon gill epithelial cell lines from Gjessing *et al.* (2018) should facilitate the use of a more AGD intrinsic model in the future.

The method of culturing *N. perurans* prior to the collection of the parasite's supernatant may influence the parasite's release of cytotoxic molecules. A described in section 3.3.1, both isolates of *N. perurans* were cultivated on MYA per Crosbie *et al*. (2012), however Bridle *et al*. (2015) maintained *N. perurans* in L-15 media for 24 h prior to the collection of the parasite's supernatant. Such an altered culturing state could have induced a secretion triggering mechanism from a more nutrient rich source than the standard culturing protocol according to Crosbie *et al.* (2012). Ideally, proteomic work should be undertaken on an *N. perurans*

exoproteome in L-15 medium culture to fully validate the influence of culture conditions on virulence factor production.

The introduction of antibiotics to the attenuated and virulent-3 cultures resulted in changes to their exoproteomes in terms of their cytotoxicity. As with the LC-MS/MS results, this analysis was also exploratory, and no statistical analysis was performed. However, the results relate to the reduced protease activity and reduced protein identifications observed previously from the exoproteome of pen-strep treated attenuated culture. Interestingly, the virulent-3 exoproteome has a cytotoxic effect on RTgill W1 when antibiotics are introduced to its culture, reiterating the parasite's response to foreign stimuli. Based on the collective results surrounding the attenuated exoproteome, we suggest the microbiome of the attenuated isolate sustains immunogenicity in culture and is responsible for the observed cytotoxicity here, but is incapable of establishing AGD *in vivo*. These findings have direct implications for future *in vitro* work using *N. perurans* isolates of differing age and presumably sampling location.

In summary, the *N. perurans* exoproteome differs in protein quantity, protease activity and cytotoxicity between phenotypes of an attenuated and virulent-3 isolate. Presently, the collective results suggest the exoproteome of *N. perurans* is not discriminative of virulence due to the complexity surrounding the comparative attenuated model and its associated microbiota. In essence, this study represents an evaluation of a long-term cultured isolate of *N. perurans*. As previously stated, we cannot discard the possibility that a virulent *N. perurans* isolate in enriched cultivation, as was the experimental design in Bridle *et al.* (2015), would reveal the extracellular product previously hypothesised. Furthermore, *N. perurans* may require a contact dependent mechanism by adhering to the host in order to trigger the extracellular product. Going forward with this work, we suggest exploring additional means of

N. perurans culture supplementation and mechanisms to reduce the bacterial secretion effect observed in the attenuated culture.

3.6. References

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CHAPTER 4

The cell surface and membrane proteome of *Neoparamoeba perurans*, the causative agent of amoebic gill disease

4.1. Abstract

Membrane and cell surface proteins are instrumental in host-parasite interactions and are central to diagnostic and therapeutic interventions in both medical and veterinary diseases. The membrane of *Neoparamoeba perurans* is suspected to be rich in antigens that are potential vaccine and diagnostic targets for the treatment of amoebic gill disease in farmed salmonids. Presently, little is known regarding the membrane of *N. perurans*, with only one putative mannose-like binding protein suggested as a membrane bound protein of the organism. Consequently, this study sought to identify all membrane-bound and surface exposed proteins of an attenuated and virulent-3 culture of *N. perurans*. Using label-free mass spectrometry and membrane surface protein biotinylation, 327 putative membrane proteins and 139 putative cell surface proteins were identified. Using a combined database of *N. perurans* specific proteins, bacterial proteins from the parasite's microbiome and Amoebozoa proteins, peptide identifications were mapped to both the parasite and its microbiota. Several proteins of potential interest to the aquaculture pharmaceutical industry were found, some of which play a role in cell-mediated adhesion, including: GAPDH, G-family proteins and some potentially novel, uncharacterised proteins. The therapeutic target potential of these proteins in relation to AGD is discussed.

4.2. Introduction

Membrane proteins and other membrane bound organelles are actively involved in pathophysiological processes within the cell (Blonder *et al.*, 2006). These proteins are of importance, constituting 30% of proteins that are encased in cellular membranes and representing 60% of all drug targets (Braun *et al.*, 2007; Tan *et al.,* 2008). Classification of membrane proteins is based on the protein-membrane interaction which can be integral (transmembrane) or peripheral membrane proteins. Integral membrane proteins transverse the phospholipid bilayer, in contrast to the hydrophilic nature of peripheral membrane proteins which are localised on the membrane surface (Tan *et al.,* 2008). *Neoparamoeba perurans* is the marine facultative parasite responsible for the gill disease of farmed Atlantic salmon (*Salmo salar L.*), referred to as amoebic gill disease (AGD) (Young *et al.*, 2008). Gill disease is a significant cause of morbidity and mortality in the aquaculture industry (Mitchell and Rodger, 2011). Presently, the only mechanism of AGD treatment is the reliance on freshwater or hydrogen peroxide baths, which cause significant economic burdens to farms (Rodger, 2014; Marcos-Lopez and Rodger, 2020). Therefore, research efforts in designing therapeutics for AGD is imperative to the sustainability of aquaculture production.

Previous proteomic efforts to establish the pathophysiological nature of the disease have involved profiling the soluble cytoplasmic fraction of *N. perurans* (Ní Dhufaigh *et al.,* 2020) and characterising the extracellular proteins (ECP) of the parasite in culture (Chapter 3). Such efforts have underpinned the role of metabolism for the parasite and the elevated expression of an allergen protein that may play a role in acute AGD virulence. Surprisingly, the exoproteome revealed a diminished role of protein secretion in a virulent culture versus an attenuated culture, indicating that the parasite's surface is likely to be orchestrating the initial steps in AGD. Indeed, this follows the main theory of how AGD is initiated (Adams and Nowak,

2004; Butler and Nowak, 2004; Lovy *et al.*, 2007; Nowak and Archibald, 2018 ; Villavedra *et al.*, 2010), in that enigmatic attachment receptors on the surface of the parasite enable membrane fusion and host-parasite interaction with the epithelium of *Salmo salar L*. gills.

There are scant publications to date investigating the role of membrane proteins in *N. perurans* virulence. Until now, two studies that have focused on this topic (Valdenegro-Vega *et al.*, 2014; Villavedra *et al.*, 2010). Genes encoding a homolog of mannose-binding protein from *Acanthamoeba* spp were identified by Valdenegro-Vega *et al.,* (2014). Salmon immunised with this recombinant protein developed functional antibodies but did not offer protection from subsequent AGD infection. The presence of a high molecular weight carbohydrate epitope on the parasite's surface was reported by Villavedra *et al.* (2010) however, immunisation did not lead to protection against AGD. It is possible several epitopes may be required to develop and maintain a systemic response to AGD, therefore the development of a divalent vaccine may be required (Xing *et al.*, 2018). To further our understanding of the parasite's membrane and surface proteins role, this work used label-free mass spectrometry and membrane surface protein biotinylation to identify proteins present in two *N. perurans* cultures of differing virulence. Using comparative proteomics, proteins of pivotal importance to a virulent-3, recently isolated culture of *N. perurans* were identified as potential anti-AGD vaccine targets.

4.3. Materials and Methods

4.3.1. *Neoparamoeba perurans* culture

Neoparamoeba perurans trophozoites were harvested from naturally infected Atlantic salmon located on a commercial farm in the west of Ireland. An attenuated culture of *N. perurans*, previously shown to have lost virulence (Ní Dhufaigh *et al.,* 2020; in review) was used as the attenuated isolate. The virulent-1 culture of Ní Dhufaigh *et al.* (2020; in review) became too old to continue in the analyses, therefore the virulent-2 culture from Ní Dhufaigh *et al.* (2020; in review) was propagated beyond 70 days in culture, and therefore its isolate name became 'virulent-3'.

Briefly, the methods to obtain these cultures were: fresh trophozoites were collected by swabbing AGD infected gills and placing swabs in 0.2 µm filtered sterile seawater for 4 h. This combined mixture was subsequently plated and maintained xenically at 16°C on marine malt yeast agar plates (MYA; 0.01% malt, 0.01% yeast, 2% Bacteriology agar, sterile seawater at 30 practical salinity units [PSU]). Species identification was confirmed by real time polymerase chain reaction (PCR) as described per Downes *et al.* (2015). The attenuated culture of *N. perurans* was isolated as above but was maintained in culture for over 4 years prior to harvesting cells.

4.3.2. Isolation of membrane bound proteins

Routine harvesting of both parasite cultures was undertaken to achieve the 2g wet cell pellet required for membrane protein isolation. Once confluency (~every 2 weeks) was achieved, *N. perurans* trophozoites were gently scraped from MYA and transferred to a 50 mL falcon tube. Trophozoites were collected by centrifugation at 1,000 x g for 10 min at 4°C. Seawater

supernatants were aspirated from the cell pellet and assessed for free floating trophozoites. Cell pellets were weighed dry and weight was recorded before storing the pellets at -80°C until required.

On the day of membrane protein extraction, multiple stocks of frozen trophozoite pellets were removed from the -80°C freezer and thawed on ice. ReadyPrep™ protein extraction kit (Membrane II) from Bio-Rad laboratories was used to isolate the membrane proteins. As frozen trophozoite pellets thawed, the membrane concentrating reagent and lysis buffer were prepared from the kit. All the attenuated pellets were amalgamated into one sample as were the virulent-3 pellets, resulting in two 15 mL falcon tubes containing trophozoites suspended in 10 mL of lysis buffer with 1 mM phenylmethanesulfonyl fluoride (PMSF). Cells were lysed by sonication on ice at 35 Hz for four cycles of 30 seconds "pulse on" and 15 seconds "pulse off".

Sonicated samples were centrifuged at 3,000 x g for 10 min at 4°C to pellet unbroken cells. The supernatant from both samples were added into separate 60 mL aliquots of chilled membrane concentrating reagent and stirred on ice for 60 min. After this incubation, samples were transferred to 30 mL ultracentrifuge tubes (Beckman Coulter) and ultracentrifuged at 65,000 rpm for 60 min at 4°C (Optima™ L-100 XP Ultracentrifuge [Beckman Coulter]). Supernatants were removed from each tube and membrane pellets washed in 3 mL of cold lysis buffer and incubated on ice for 2 min. This procedure was repeated once more. Membrane pellets were gently scraped from the ultracentrifuge tubes and transferred to sterile 2 mL microcentrifuge tubes for storage at -80°C. Membrane solubilisation buffer (MSB) was prepared fresh as: 4% sodium dodecyl sulfate (SDS), 20% glycerol, 10% 2-mercaptoethanol and 0.125 M Tris HCl, pH approximately 6.8. Membrane pellets from the 2 g cell pellet were thawed on ice and 2 mL of MSB was added to resuspend the pellets. The membrane pellets in MSB were incubated for 60 min at room temperature to ensure complete solubilisation with

intermittent vortexing. Solubilised pellets were centrifuged (Sorvall™ Legend™ Micro 21R Microcentrifuge, Thermo Scientific™) at room temperature at 21,100 x g for 1 min to remove non-solubilised material.

4.3.3. Isolation of cell surface proteins

Cell surface proteins of the attenuated and virulent-3 parasites were biotinylated using Pierce Cell surface Protein Isolation Kit (Thermo Fisher) following manufacturer's protocols with modifications. *N. perurans* trophozoites from 20 petri dishes containing MYA and a sterile seawater overlay were grown as described in section 4.3.1 to 90-95% confluence (approximately 2 weeks duration). The seawater overlay was aspirated and the trophozoites were gently washed with 8 ml phosphate-buffered saline (PBS) at room temperature. The PBS was subsequently aspirated. One vial of EZ-Link Sulfo-NHS-SS-Biotin (12 mg) was dissolved in 48 mL of ice-cold PBS and 10 mL of biotin solution was added to each petri dish. Petri dishes were added to a rocker and incubated for 30 min at 4 °C. To quench the biotin, 500 µL of quenching solution was added to each dish. Trophozoites were gently scraped using a mechanical scraper into a 50 mL falcon tube and collected by centrifugation at 1, 000 x g for 10 min at 4 °C. The supernatant was aspirated and the pellets were resuspended in 5 mL Tris buffered saline by gentle pipetting and centrifuging once more.

Trophozoite pellets were lysed by pipetting in 500 µL lysis buffer containing 1 mM phenylmethanesulfonyl fluoride (PMSF) inhibitor and sonicated at 35 Hz on ice for four cycles of 30 seconds "pulse on" and 15 seconds "pulse off". Samples were incubated on ice for 30 min with vortexing every 5 min for complete membrane solubilisation. The cell lysates were centrifuged at 10,000 x g for 2 min at 4 °C and the supernatants containing the solubilised, biotin-labelled, membrane proteins were transferred to a new microcentrifuge tube. To isolate

the biotin-labelled proteins, the supernatants were added to a column containing NeutrAvidin Agarose for 60 min at room temperature (20- 22 °C) on a rocker. The column was washed four times using 500 µL of Wash Buffer to remove contaminants with centrifugation at room temperature for 1 min at 1,000 x g. To elute the biotin-labelled proteins, the column was incubated with SDS-PAGE sample buffer (4% SDS, 20% glycerol, and 0.125 M Tris HCl, pH approx. 6.8) containing 50 mM dithiothreitol, at room temperature for 60 min with continuous rocking, followed by centrifugation at 1,000 x g for 2 min at room temperature.

4.3.4. Protein quantification and one-dimensional (1D) gel

Protein quantification was performed using the bicinchoninic acid assay (BCA assay [Thermo Scientific, Pierce]). A total volume of 50 µL of solubilised membrane protein (concentration 1 µg/mL) and 50 µL of cell-surface, biotin-labelled protein (concentration 1 µg/mL) was transferred to new microcentrifuge tubes. Bromophonol blue (Bio-Rad) was added at a final concentration of 0.004%. Samples were incubated at 60°C for 10 min on a heat block after which, they were allowed to cool on ice.

To remove excess detergent prior to mass spectrometry analysis, samples were briefly subjected to gel electrophoresis which separated excess detergent from the proteins. Samples were loaded onto a 4% stacking and 12% separating Acrylamide/Bis gel (Acrylamide/Bis 30% 37.5:1; 0.5 M Tris- HCL, pH 6.8; 1.5 M Tris- HCL, pH 8.8; diH_2O; 10% SDS, TEMED, 10% ammonium persulfate) using a Mini PROTEAN Tetra cell (Bio-Rad, CA) with SDS PAGE running buffer (25 mM Tris, 192 mM glycine and 0.1% SDS). 25 µL of each sample was loaded in duplicate into the wells of the gel. Electrophoresis proceeded for a limited time of 8 minutes at constant voltage (100 V) to allow proteins to migrate into the separating gel but prevent band separation. Gels were stained using QC Colloidal Coomassie (Bio-Rad, CA) overnight and de-

stained by washing in deionised water in triplicate, followed by 10% ethanol and 7.5% acetic acid.

4.3.4. In gel digestion

Enzymatic in-gel digestion was performed as described by Shevchenko *et al.* (2006). Gels were rinsed with deionised water and the two bands were excised and cut into pieces using a sterile scalpel. Gel pieces were centrifuged in a Sorvall™ Legend™ Micro 21R Microcentrifuge (Thermo Scientific™) at 21,000 x g for 1 min to collect excess water, destained three times with 100 µL of 100 mM ammonium bicarbonate/acetonitrile (1:1, v/v) and incubated for 30 min with vortexing every 10 min. Following the addition of ammonium bicarbonate, 500 µL of neat acetonitrile was added and incubated at room temperature until gel pieces were destained and decreased in size. Acetonitrile was removed and the gel pieces were dried in vacuum centrifuge (Savant,Speedvac, Thermo Fisher Scientific). Gel pieces were rehydrated with trypsin buffer for 18 hrs at 37°C (0.5 µg trypsin in 50 mM ammonium bicarbonate with 1 µL trypsin solubilisation reagent [Trypsin singles, Sigma-Aldrich) with intermittent shaking.

A total volume of 100 µL of extraction buffer (1:2 [vol/vol] 5% formic acid/ acetonitrile) was added to each tube for 15 min at 37°C with intermittent shaking. Supernatants containing the peptides were aspirated and desalted using C18 zip tips with Equilibration Buffer (0.1% trifluoroacetic acid [TFA] in MS grade water) and Elution Buffer (50% acetonitrile 0.1% TFA in MS grade water). After drying in vacuum centrifuge (Savant,Speedvac, Thermo Fisher Scientific), peptides were stored at -20°C until LC-MS/MS.

4.3.5. Liquid chromatography tandem mass spectrometry

Peptide fractions were analysed on a quadrupole Orbitrap (Q-Exactive, Thermo Scientific) mass spectrometer equipped with a reversed-phase NanoLC UltiMate 3000 HPLC system (Dionex LC Packings, now Thermo Scientific). Peptide samples were loaded onto C18 reversed phase columns (10 cm length, 75 µm inner diameter) and eluted with a linear gradient from 1 to 27% buffer B containing 0.5% AA and 97.5% ACN in 58 min at a flow rate of 250 nL/min. The injection volume was 5 µL.

4.3.6. Raw data processing and label free quantification (LFQ)

Raw data from the Orbitrap Q-Exactive was processed using MaxQuant version 1.6.6.0 for identification of proteins (Cox and Mann, 2008), incorporating the Andromeda search engine (Cox *et al.*, 2011). To identify peptides and proteins, MS/MS spectra were matched to a custom *N. perurans* database containing 20,887 proteins (v2, 07/08/2019, CSIRO). Reference proteomes of Amoebozoa containing 109,415 proteins, *Paramoeba* containing 5,001 proteins and bacteria proteins containing 148,582 proteins were downloaded on May 8th from UniProt and were included in the search to distinguish between amoeba specific and co-culturing bacteria proteins (Appendices I, II, III). All searches were performed with tryptic specificity allowing two missed cleavages. LFQ was computed using the ion intensities for protein profiles, intensity of corresponding peptides in different LC MS/MS runs were matched by MaxQuant (Cox *et al.*, 2014). The database searches were performed with carbamidomethyl (C) as fixed modification and acetylation (protein N terminus) and oxidation (M) as variable modifications. Mass spectra were searched using the default setting of MaxQuant namely a false discovery rate of 1% on the peptide and protein level. For bioinformatic analysis, LFQ values obtained in MaxQuant were imported into Perseus (v. 1.6.10.50) software. LFQ values were log2

transformed and missing values were imputed with the assumption of a normal distribution (width 0.3, down shift 1.8). Differentially expressed proteins between samples were identified using a two-way Students t-test followed by Benjamin-Hochberg FDR correction. Hierarchical clustering was performed on Z-score protein intensities for the proteins with $p < 0.05$.

4.3.7. In silico analysis

Gene ontology (GO) was performed to describe the location, molecular function and biological process of each significant *N. perurans* protein. At present, no UniParc identifers are available for *N. perurans*. therefore, protein orthologes from characterised species were used in the analysis. To obtain UniParc identifiers (known characterised proteins) for GO enrichment in iProClass (Wu *et al.*, 2004), *N. perurans* upregulated protein sequences were searched against the non-redundant database in Blast-p for orthologous proteins. Any potential eukaryotic results from the amoebozoa database were also added to the *N. perurans* enrichment. A separate GO enrichment was performed for any results originating from the microbiome.

4.4. Results

4.4.1. One dimensional gel

In order to prepare the membrane samples for LC-MS/MS, SDS PAGE (as outlined in Section 4.3.4) was employed to remove detergent (Figure 1). Bands were excised, further destained with deionised water and subjected to enzymatic in-gel digestion as outlined in Section 2.4.

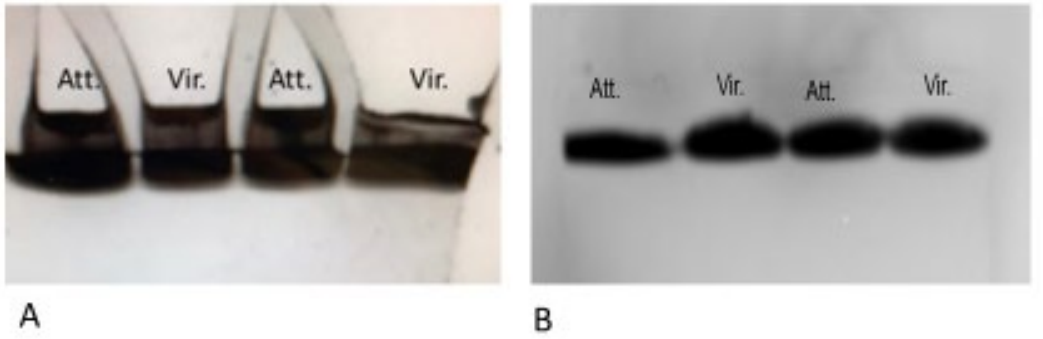

Figure 1: SDS-PAGE of A) membrane proteins and B) biotin-labelled, cell surface proteins from attenuated and virulent-3 *Neoparamoeba perurans* cultures. Att. = protein sample from attenuated *N. perurans*. Vir. = protein sample from virulent-3 *N. perurans*. Note: 25 µg of protein loaded in each lane. Bands were excised and digested overnight with 0.5 µg trypsin.

4.4.2. Membrane protein identification

A total of 327 proteins from the technical replicates of both the attenuated (three sample injections) and virulent-3 (three sample injections) membrane proteomes were identified using label-free LC-MS/MS. The application of a two-way sample t-test yielded 302 significantly, differentially expressed proteins, 93 of which were associated with *N. perurans* and Amoebozoa and 209 of which were associated with the *N. perurans* microbiome. The 302 proteins that encompassed all significant identifications are clustered in a heat map (Figure 2). Of the 93 proteins associated with *N. perurans* and Amoebozoa, 56 proteins were elevated in

the virulent-3 parasite, and 37 proteins had reduced expression. It is worth noting that only two proteins, cytochrome oxidase subunit 2 from *Paramoeba aparassomata*, and histone 4 of *Acanthamoeba castellanii str. Neff* respectively were identified from the Amoebozoa database. These proteins are likely to be homologues of *N. perurans* proteins. All other amoebic proteins were *N. perurans* in origin. Bacterial identifications dominated the analyses overall, with 133 bacterial proteins elevated in the virulent-3 parasite and 76 bacterial proteins had reduced expression. . The bacterial proteins, both elevated and reduced originated from genera *Pseudoaltermonas spp*, *Vibrio spp* and *Thalassospira spp*.

The protein with the largest overall positive fold change, (9.1) in the virulent-3 proteome was identified as maltoporin of *Vibrio tasmaniensis* (strain LGP32) (Table 1). The *N. perurans* protein with the largest positive fold change (8.23) in the virulent-3 proteome was identified as quinolinate synthase, 8.23 (Table 2). The protein with the largest overall negative fold change, (-6.68) in the virulent-3 proteome was identified as phasin protein from *Thalassospira xiamenensis* (Table 3). The *N. perurans* protein with the largest negative fold change, (-3.3) in the virulent-3 proteome was identified as a hypothetical protein (Table 4).

GO enrichment using IProClass revealed the varying roles of proteins in a virulent-3 versus attenuated *N. perurans* and its associated microbiome. Apart from cellular structural function, transporter activity and protein and nucleic acid binding were broadly the functions assigned to those elevated *N. perurans* proteins (Table 2). Two of the 56 *N. perurans* significantly elevated proteins could not be assigned a component location. Overall, 24 of these proteins (44%) were classed as membrane proteins. A relatively large proportion of *N. perurans* proteins (14 proteins; 26%) were classified as ribosomal and involved in translation (Figure 3). A further two membrane proteins (3%) were associated both with the membrane and the mitochondrion and 1 other protein (2%) was identified as a proton transporting two-sector

ATPase complex. Five of the non-membrane proteins (9%) were found to be cytoplasmic in origin with 2 cytoskeletal proteins (4%) also present. Of the remaining eight proteins, two were assigned to the nucleus, three were of the myosin complex, and finally three proteins were described as uncharacterised.

Processes varied, but several interesting, upregulated protein processes in membrane of the virulent-3 *N. perurans* were found, including: four proteins involved in signal transduction (8%); two proteins of cell communication (intraspecies interaction with organisms – 4%) and one protein of cell adhesion mediated by integrin, and signal transduction (2%). Ten percent of the process identifications were found to be associated with membrane transport.

A greater diversity of significantly elevated proteins was found in the microbiome membrane analysis (Figure 4), the majority of which were identified as membrane components. Using IProClass to reveal the cellular components, 10 of the 133 bacterial proteins were uncharacterised and could not be assigned a component location. Of the 123 remaining proteins, 57 (46%) were classified as membrane proteins. Another 25 proteins (20%) were found to be membrane and external encapsulating structure and cell envelope proteins. 20 proteins were associated with the ribosome with a further eight proteins associated with the small ribosomal subunit. Four cytoplasmic proteins were present and the remaining nine proteins of the component analysis were classified as: membrane, respiratory chain complex II (1 protein); membrane, proton- transporting two-sector ATPase complex (2 proteins); membrane, mitochondrial (1 protein); pyruvate dehydrogenase complex (1 protein); bacterial type flagellum (1 protein); ATP-binding (ABC) transporter (1 protein); oxoglutarate dehydrogenase complex (1 protein) and protein containing complex (1 protein). Overall, enzymes predominated the proteins found in the functional groups of the microbiome membrane analysis including: 32 transferases proteins (33% coupled with transporter activity), four

peptidases (4%), seven oxioreductases (7%), one isomerase protein (1%), and three hydrolase proteins (3%). Similar to the *N. perurans* specific membrane analysis, 28 proteins were involved in translation (41%) along with eight proteins relating to carbohydrate metabolism (19%).

Both the virulent-3 and attenuated *N. perurans* membrane proteomes shared 25 proteins with no significant differential expression. These comprised 13 ribosomal proteins (52%) related to translation, followed by two carbohydrate metabolic proteins (8%), two cytoskeletal proteins (8%), one cytoplasmic protein with GTPase activity (4%), one endoplasmic reticulum protein related to ATP binding and another involved in the cellular response to glucose starvation (4%) and finally six proteins of membrane origin (24%). Unusually, none of these six proteins had transporter activity and all had various functions: iron ion binding, GTPase actvity, hydrolase activity (part of LysM domain), transcription, catalytic activity and one of unknown function.

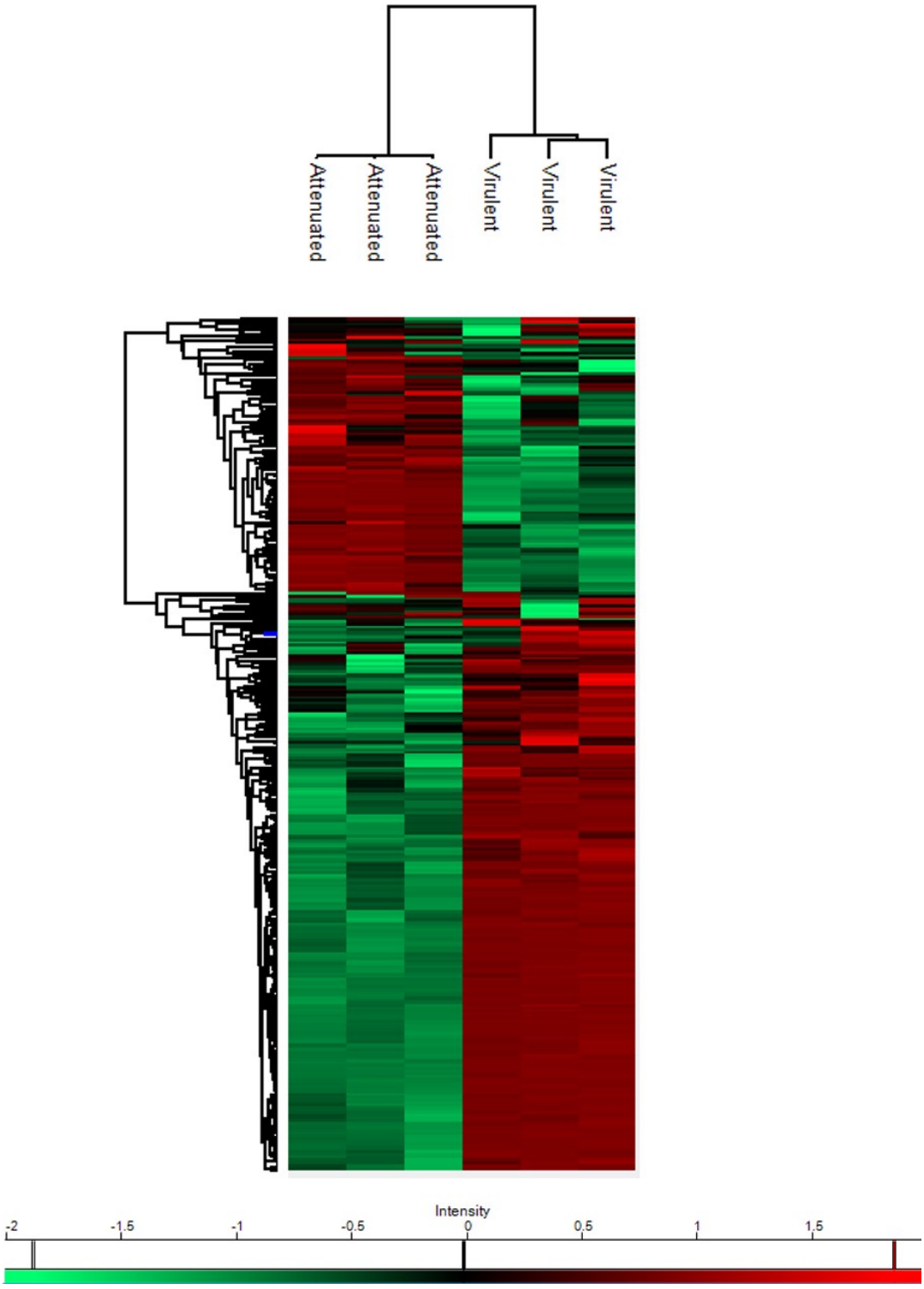

Figure 2: Normalised cell membrane LFQ values achieved using Z- scoring of the 302 significantly expressed proteins. Hierarchical clustering was performed using Euclidean distance. Protein intensity is represented as a colour chart with red colours showing increased abundance and green displaying a decreased expression of proteins.

Table 1: Top 10 significantly elevated membrane proteins based on fold enrichment in the virulent-3 *Neoparamoeba perurans* culture (including the associated microbiome)

Gene	Protein IDs	Fasta headers	Organism	Mol. weight [kDa]	Fold change[a]	Atten. Peptides[b]	Vir. Peptides[b]
VS_II0158	B7VQC8	tr|B7VQC8|B7VQC8_VIBTL Maltoporin	*Vibrio tasmaniensis* (strain LGP32)	50.47	9.09	0	30
PSHAa1989	Q3IIU7	tr|Q3IIU7|Q3IIU7_PSEHT Uncharacterized protein	*Pseudoalteromonas haloplanktis* (strain TAC 125)	94.4	8.71	4	53
rplB	B7VLF4	sp|B7VLF4|RL2_VIBTL 50S ribosomal protein L2	*Vibrio tasmaniensis* (strain LGP32)	29.91	8.58	6	23
	PPER_00007094-RA	PPER_00007094-RA quinolinate synthase	*Neoparamoeba perurans*	122.46	8.23	0	15
	PPER_00007011-RA	PPER_00007011-RA tRNA uridine-5-carboxymethylaminomethyl(34) synthesis enzyme	*Neoparamoeba perurans*	136.85	7.67	1	12
PP2015_1053	A0A0S2K0N7	tr|A0A0S2K0N7|A0A0S2K0N7_9GAMM TonB-dependent receptor	*Pseudoalteromonas phenolica*	95.59	7.44	0	18
BCU70_04105	A0A2N7D2I8	tr|A0A2N7D2I8|A0A2N7D2I8_9VIBR Porin_4 domain-containing protein	Vibrio sp. 10N.286.49.	36.85	7.27	1	9
atpF	B7VN00	tr|B7VN00|B7VN00_VIBTL ATP synthase subunit b	*Vibrio tasmaniensis* (strain LGP32)	17.55	7.25	0	27
PSHAb0340	Q3IC00	tr|Q3IC00|Q3IC00_PSEHT Putative Outer membrane protein with a TonB box	*Pseudoalteromonas haloplanktis (*strain TAC 125)	113.29	7.15	3	11
NqrF	A0A0C3E7I8	tr|A0A0C3E7I8|A0A0C3E7I8_9VIBR Na(+)-translocating NADH-quinone reductase subunit F	*Vibrio mytili*	45.11	7.07	2	16

Mol = molecular; kDa= kilodalton; Atten= attenuated; Vir = virulent; [a] Fold change of virulent proteins related to attenuated proteins; [b]Razor and unique peptide counts.

Table 2: *Neoparamoeba perurans* top 10 significantly elevated membrane proteins based on fold enrichment in the virulent-3 parasite.

Protein IDs	**Fasta headers**	**Mol. weight [kDa]**	**Fold change[a]**	**Atten. Peptides[b]**	**Vir. Peptides[b]**
PPER_00007094-RA	PPER_00007094-RA quinolinate synthase	122.46	8.23	0	15
PPER_00007011-RA	PPER_00007011-RA tRNA uridine-5-carboxymethylaminomethyl(34) synthesis enzyme	136.85	7.67	1	12
PPER_00011993-RA	PPER_00011993-RA ---NA---	35.25	6.23	4	32
PPER_00011992-RA	PPER_00011992-RA hypothetical protein	39.76	5.73	0	9
PPER_00011394-RA	PPER_00011394-RA ---NA---	42.16	5.65	2	14
PPER_00014494-RA	PPER_00014494-RA FAM49 family protein	28.22	5.2	6	9
PPER_00011994-RA	PPER_00011994-RA leucine-rich repeat-containing protein C10orf11-like	40.9	4.95	1	11
PPER_00010491-RA	PPER_00010491-RA 50S ribosomal protein L16	14.14	4.9	0	7
PPER_00017905-RA	PPER_00017905-RA V-type H+-transporting ATPase subunit c	15.72	4.63	3	3
PPER_00020055-RA	PPER_00020055-RA Nif3-like dinuclear metal center hexameric protein	17.23	4.61	3	3

Mol= molecular; kDa= kilodalton; Atten= attenuated; Vir = virulent; [a] Fold change of virulent proteins related to attenuated proteins; [b]Razor and unique peptide counts; NA = unknown protein

Table 3: Top 10 significantly reduced membrane proteins based on fold enrichment in the virulent-3 *N. perurans* culture (including the associated microbiome). These proteins reflect significantly elevated proteins in an attenuated culture.

Gene	Protein IDs	Fasta headers	Organism	Mol. weight [kDa]	Fold change [a]	Atten. Peptides [b]	Vir. Peptides [b]
rpsC	A0A367W4Z9	tr|A0A367W4Z9|A0A367W4Z9_9PROT 30S ribosomal protein S3	*Thalassospira xiamenensis*	25.46	-3.28	9	3
	PPER_00001494-RA	PPER_00001494-RA hypothetical protein	*Neoparamoeba perurans*	15.42	-3.3	15	0
atpF	A0A154KXX9	tr|A0A154KXX9|A0A154KXX9_9PROT ATP synthase subunit b	*Thalassospira xiamenensis*	18.53	-3.34	6	1
pal	A0A367WZK2	tr|A0A367WZK2|A0A367WZK2_9PROT Peptidoglycan-associated protein	*Thalassospira xiamenensis*	18.09	-3.34	18	13
rpsM	A0A154KXE6	tr|A0A154KXE6|A0A154KXE6_9PROT 30S ribosomal protein S13	*Thalassospira xiamenensis*	13.47	-3.61	9	3
psd	A0A0B4XUR1	tr|A0A0B4XUR1|A0A0B4XUR1_9PROT_Phosphatidylserine decarboxylase proenzyme	*Thalassospira xiamenensis* M-5 = DSM 17429	25.11	-3.78	9	0
TH24_04130	A0A367WK15	tr|A0A367WK15|A0A367WK15_9PROT Uncharacterized protein	*Thalassospira xiamenensis*	39.7	-3.91	23	5
PCIT_05990	U1KRH1	tr|U1KRH1|U1KRH1_9GAMM Biopolymer transport protein	*Pseudoalteromonas citrea* DSM 8771	14.97	-4.17	6	0
SAMN05428964_101719	A0A154KZR4	tr|A0A154KZR4|A0A154KZR4_9PROT Cell envelope biogenesis protein OmpA	*Thalassospira xiamenensis*	32.43	-6.01	21	5
SAMN05428964_103144	A0A154KQK5	tr|A0A154KQK5|A0A154KQK5_9PROT Phasin	*Thalassospira xiamenensis*	16.35	-6.68	21	9

Mol= molecular; kDa= kilodalton; Atten= attenuated; Vir = virulent; [a] Fold change of virulent proteins related to attenuated proteins; [b]Razor and unique peptide counts.

Table 4: *Neoparamoeba perurans* top 10 significantly reduced membrane proteins based on fold enrichment in the virulent-3 parasite. These proteins reflect significantly elevated proteins in an attenuated culture.

Protein IDs	Fasta headers	Mol. weight [kDa]	Fold change[a]	Atten. Peptides[b]	Vir. Peptides[b]
PPER_00002901-RA	PPER_00002901-RA efflux RND transporter permease subunit	327.02	-2.03	6	3
PPER_00012760-RA	PPER_00012760-RA Rho GTPase	28.58	-2.06	9	3
PPER_00019684-RA	PPER_00019684-RA 50S ribosomal L16	13.54	-2.14	6	2
PPER_00013586-RA	PPER_00013586-RA 60S ribosomal L35	14.21	-2.14	6	6
PPER_00001535-RA	PPER_00001535-RA FKBP-type peptidyl-prolyl cis-trans isomerase	29.25	-2.22	9	0
PPER_00014001-RA	PPER_00014001-RA hypothetical protein	15.91	-2.66	12	3
PPER_00017781-RA	PPER_00017781-RA nascent polypeptide-associated complex subunit alpha 1	24.49	-2.98	9	3
PPER_00002289-RA	PPER_00002289-RA hypothetical protein	97.82	-3.07	6	0
PPER_00000694-RA	PPER_00000694-RA DUF4398 domain-containing protein	13.38	-3.11	12	0
PPER_00001494-RA	PPER_00001494-RA hypothetical protein	15.42	-3.3	15	0

Mol= molecular; kDa= kilodalton; Atten= attenuated; Vir = virulent; [a] Fold change of virulent proteins related to attenuated proteins; [b]Razor and unique peptide counts.

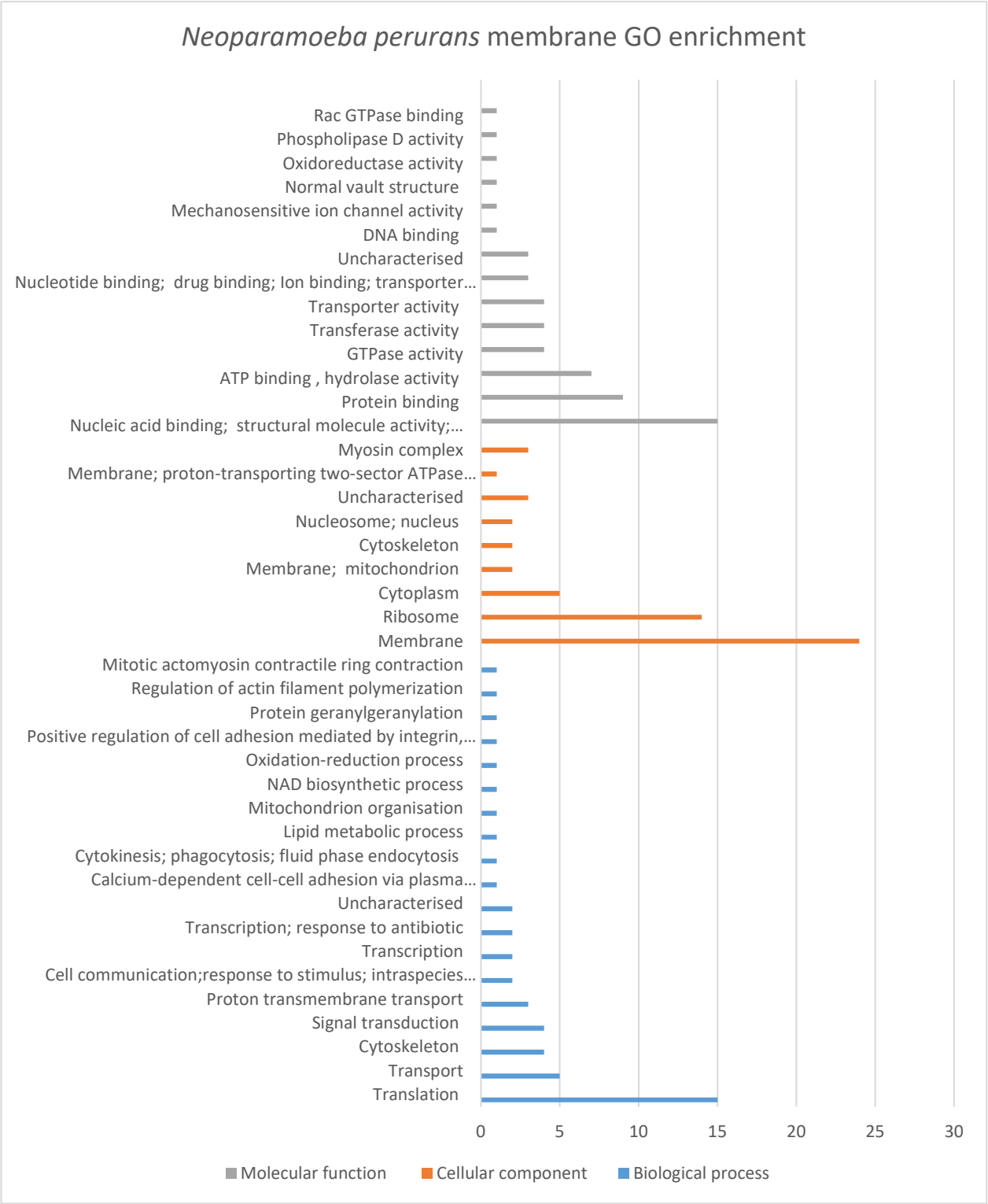

Figure 3: Gene ontology (GO) enrichment analysis of the 56 significantly elevated *N. perurans* membrane proteins from the virulent-3 culture plus two of the Amoebozoa identifications; histone 4 and cytochrome c oxidase subunit 2 of *A. castellanii str, Neff* and *P. aparasomata*, respectively.

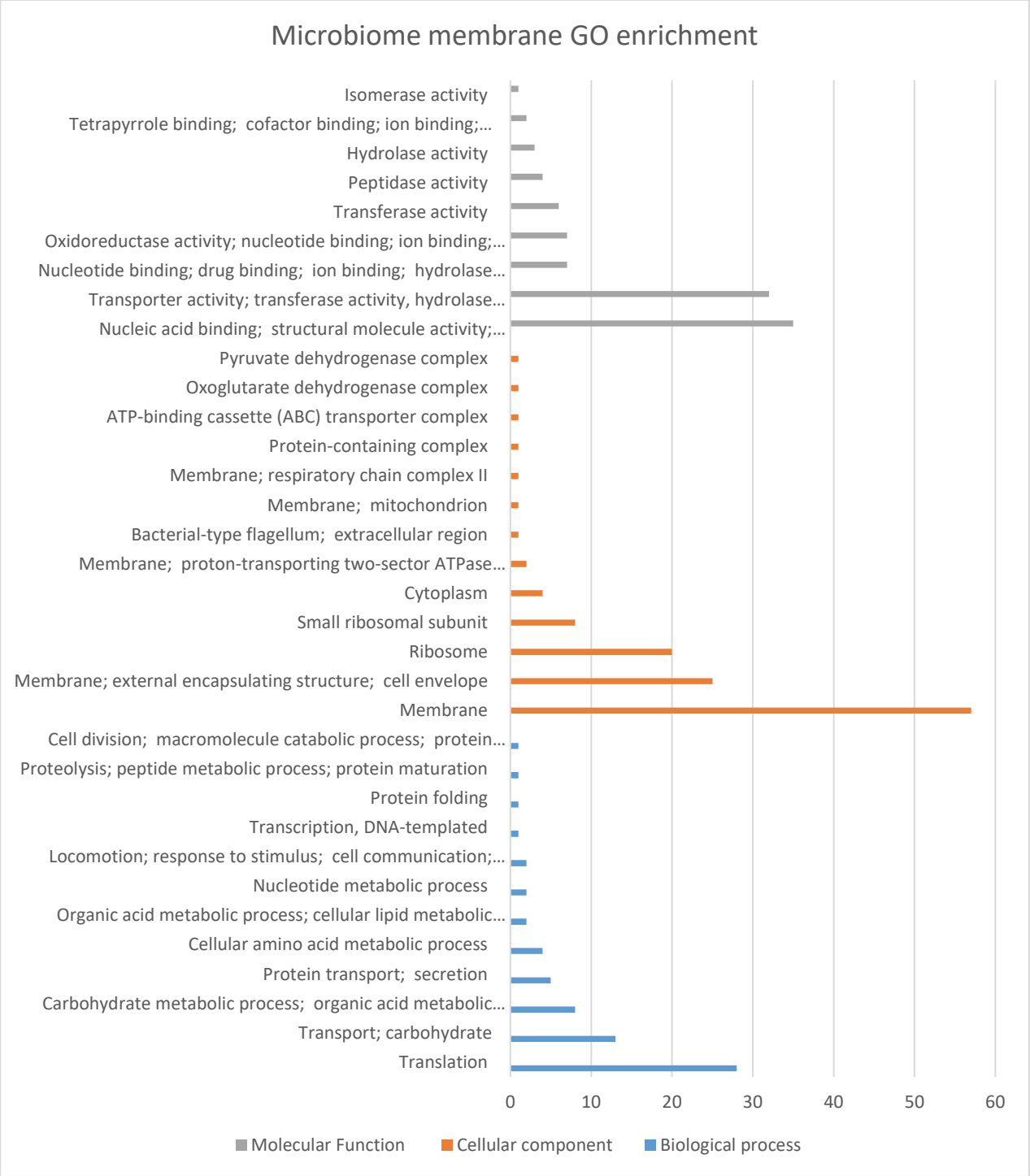

Figure 4: Gene ontology (GO) enrichment analysis of the 133 significantly elevated proteins from the microbiome of the *Neoparamoeba perurans* virulent-3 culture.

4.4.3. Cell-surface protein identification

A total of 139 putative cell- surface proteins were identified using label free LC-MS/MS in the attenuated and virulent-3 cultures. Applying the same criteria for the membrane protein analysis, a two-way sample t-test identified 118 significantly, differentially expressed proteins in the virulent-3 culture: 23 significantly elevated proteins associated with *N. perurans* and Amoebozoa and 17 significantly elevated proteins associated with the parasite's microbiome. Similar to the membrane analysis, the virulent-3 culture also had significantly reduced expression of some cell- surface proteins, including 24 *N. perurans* associated proteins and 54 proteins associated with the microbiome. The 118 proteins that encompassed all significantly, differentially expressed identifications are clustered in a heat map (Figure 5). Only one other eukaryotic identification excluding *N. perurans* was found, namely an uncharacterised protein associated with *Cavenderia fasciculata (strain SH3)*. This protein is likely to be a homologue of an *N. perurans* protein.

The protein with the largest overall positive fold change (7.3) in the virulent-3 cell surface proteome was identified as a putative TRAP transporter solute receptor (TAXI family) of *Vibrio nigripulchritudo* (Table 5). The *N. perurans* protein with the largest positive fold change (5.74) in the virulent-3 cell-surface proteome was identified as a branched-chain amino acid ABC transporter substrate-binding protein (Table 6). The protein with the largest overall negative fold change (-6) in the virulent-3 cell-surface proteome was a TonB dependent receptor of *N. perurans* (Table 7, 8).

A component analysis of the elevated proteins, using the GO enrichment tool, IProClass identified a relatively high number cytoplasmic proteins in the *N. perurans* and Amoebozoa identifications (Figure 6) at 11 of 23 proteins (48%). This suggests that some cross-contamination of cytoplasmic proteins occurred during the biotinylation process.

However, 7 of the 23 proteins (31%) were membrane proteins with a further 1 protein aligning to the membrane and proton-transporting two-sector ATPase complex (4%). Unusually, three proteins were mitochondrial in origin (13%) and one protein was assigned to a chaperone complex (4%). These 23 proteins were involved in various molecular binding activities, namely protein binding (four proteins, [20%]), two proteins of nucleotide binding, drug binding (10%), one chaperone binding protein (5%), two cofactor binding and metal cluster binding proteins (10%) and one nucleic acid binding (5%) were prevalent. Oxioreductase activity, four proteins at 20% and transferase activity, two proteins at 10% were also top functions.

With respect to the cell-surface microbiome identifications, 17 significantly elevated proteins were found in the virulent-3 culture (Figure 7). The component analysis identified eight membrane proteins (44%), followed by three cytoplasmic proteins (17%). The remaining six proteins were assigned to periplasmic space, ribosomal subunit, sulfopyruvate decarboxylase complex and mitochondria. Carbohydrate metabolic processes represented the highest proportion of identifications of the biological processes assigned to the microbiome identifications (3 out of 17 proteins [14%]). A further three surface proteins (14%) were involved in amino acid transport. Three of the significantly elevated cell-surface proteins (17%) of the microbiome had a carbohydrate metabolic function. However, a large proportion (9 of the 17 proteins [50%]) were found to be involved in transport of carbohydrate molecules. Similar to the identifications found in *N. perurans* and Amoebozoa, binding proteins were also noted in the microbial analysis. This included: four proteins of transmembrane transporter activity and ATP binding were abundant (22%); three proteins of transferase activity and nucleic acid binding (17%); and three proteins of nucleotide binding, drug binding, ion and ligase binding (17%).

Both the virulent-3 and attenuated *N. perurans* cell-surface proteomes shared 21 proteins that were not significantly, differentially expressed, comprised of 13 membrane proteins (65%), followed by six cytoplasmic proteins (30%) and one ribosomal protein (5%). Roles in transport were evident among these shared proteins and profilin, a previously documented cytoplasmic protein associated with *N. perurans* virulence was also found (Ní Dhufaigh *et al.,* 2020; in review). Enzymes were also found namely, one protein of isomerase activity and one protein of kinase activity. An additional enzyme known in signalling pathways, glutathione S-transferase P, was identified, in conjunction with a number of proteins of unknown function.

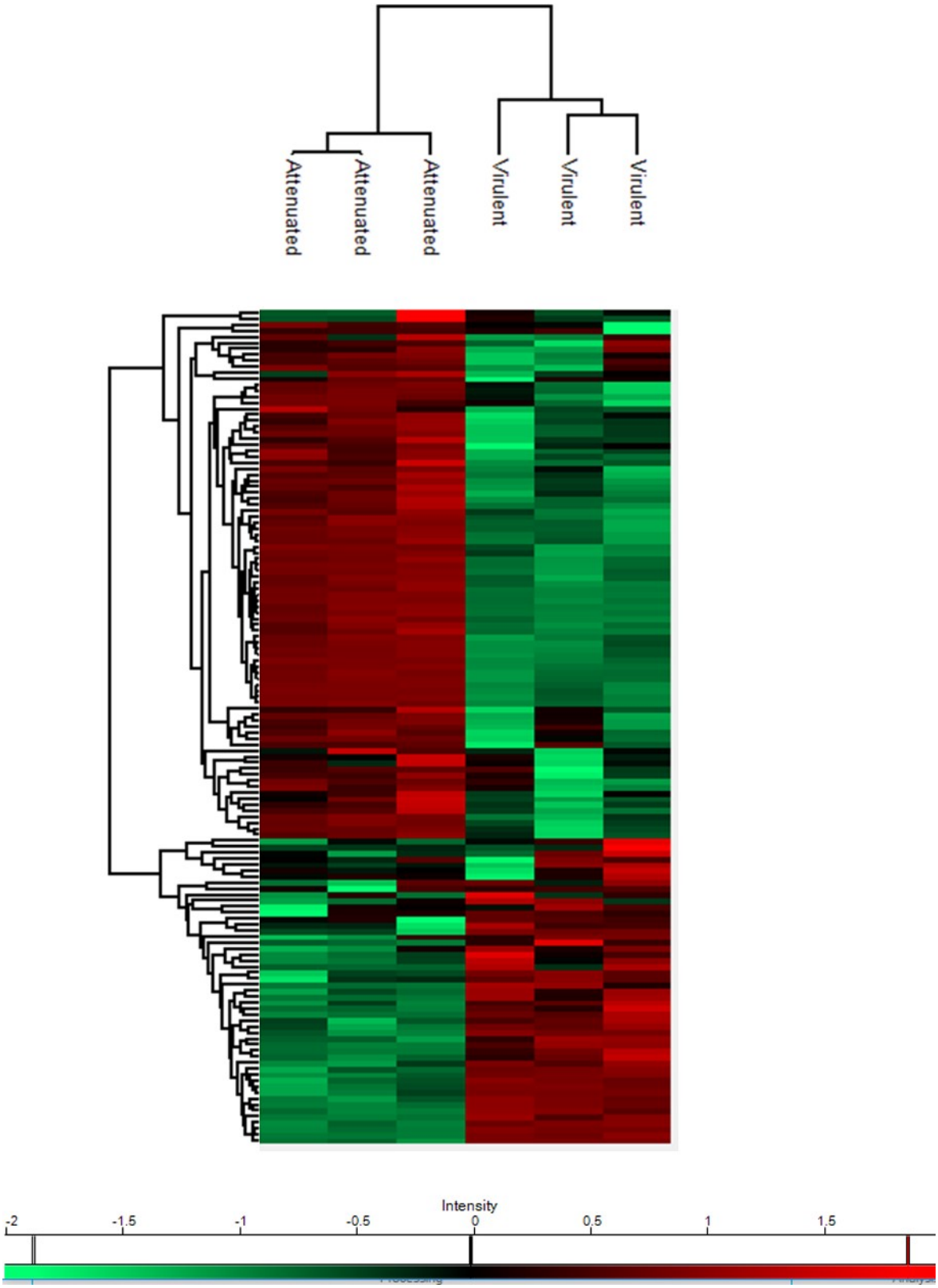

Figure 5: Normalised cell- surface proteins LFQ values achieved using Z score of the 118 significantly expressed proteins of the attenuated and virulent-3 parasites. Hierarchical clustering was performed using Euclidean distance.

Table 5: Top 10 significantly elevated cell-surface membrane proteins based on fold enrichment in the virulent-3 *Neoparamoeba perurans* culture (including the associated microbiome).

Gene	Protein IDs	Fasta headers	Organism	Mol. weight [kDa]	Fold change[a]	Atten. Peptides[b]	Vir. Peptides[b]
VIBNI_A1034	U4K3N7	tr|U4K3N7|U4K3N7_9VIBR Putative TRAP transporter solute receptor, TAXI family	*Vibrio nigripulchritudo*	34.77	7.28	3	6
	PPER_0000 6972-RA	PPER_00006972-RA branched-chain amino acid ABC transporter substrate-binding protein	*Neoparamoeba perurans*	73.65	5.75	3	3
	PPER_0000 5536-RA	PPER_00005536-RA 2,3-bisphosphoglycerate-independent phosphoglycerate mutase	*Neoparamoeba perurans*	390.86	5.62	2	9
TH24_03600	A0A367WL P1	tr|A0A367WLP1|A0A367WLP1_9PROT Sugar ABC transporter	*Thalassospira xiamenensis*	35.52	5.61	7	9
VS_0355	B7VIC7	tr|B7VIC7|B7VIC7_VIBTL Immunogenic protein	*Vibrio tasmaniensis (strain LGP32)*	34.44	5.58	3	16
VS_II0220	B7VQI9	tr|B7VQI9|B7VQI9_VIBTL Maltodextrin-binding protein	*Vibrio tasmaniensis (strain LGP32)*	42.39	5.34	2	19
VS_1403	B7VNJ0	tr|B7VNJ0|B7VNJ0_VIBTL Amino acid ABC transporter	*Vibrio tasmaniensis (strain LGP32)*	36.76	5.23	7	14
	PPER_0000 4423-RA	PPER_00004423-RA 2-methylcitrate dehydratase	*Neoparamoeba perurans*	86.05	5.07	8	12
	PPER_0000 7094-RA	PPER_00007094-RA quinolinate synthase	*Neoparamoeba perurans*	122.46	4.90	4	6
EES38_20050	A0A3N9TB 87	tr|A0A3N9TB87|A0A3N9TB87_9VIBR Glutamine synthetase	*Vibrio sp. LJC006*	51.24	4.62	3	3

Mol = molecular; kDa = kilodalton; Atten =attenuated; Vir = virulent; [a] Fold change of virulent proteins related to attenuated proteins; [b]Razor and unique peptide counts.

Table 6: *Neoparamoeba perurans* top 10 cell-surface membrane proteins based on fold enrichment significantly elevated in the virulent-3 parasite relative to the attenuated parasite**.**

Protein IDs	**Fasta headers**	**Mol. weight [kDa]**	**Fold change[a]**	**Atten. Peptides[b]**	**Vir. Peptides[b]**
PPER_00006972-RA	PPER_00006972-RA branched-chain amino acid ABC transporter substrate-binding protein	73.65	5.75	3	3
PPER_00005536-RA	PPER_00005536-RA 2,3-bisphosphoglycerate-independent phosphoglycerate mutase	390.86	5.62	2	9
PPER_00004423-RA	PPER_00004423-RA 2-methylcitrate dehydratase	86.05	5.07	8	12
PPER_00007094-RA	PPER_00007094-RA quinolinate synthase	122.46	4.9	4	6
PPER_00005534-RA	PPER_00005534-RA glutamate-ammonia ligase	51.76	4.44	9	12
PPER_00005561-RA	PPER_00005561-RA ABC transporter ATP-binding protein	115.61	4.14	0	9
PPER_00020574-RA	PPER_00020574-RA ADF-like domain-containing protein	18.58	2.89	6	6
PPER_00002073-RA	PPER_00002073-RA DNA-directed RNA polymerase subunit beta	454.87	2.62	4	9
PPER_00014426-RA	PPER_00014426-RA hypothetical protein AK812_SmicGene31809	70.78	1.92	5	6
PPER_00006080-RA	PPER_00006080-RA 2-oxoglutarate dehydrogenase E1 component	356.65	1.76	3	8

Mol = molecular; kDa = kilodalton; Atten =attenuated; Vir = virulent; [a] Fold change of virulent proteins related to attenuated proteins; [b]Razor and unique peptide counts.

Table 7: Top 10 significantly reduced cell- surface membrane proteins based on fold enrichment in the virulent-3 *Neoparamoeba perurans* culture (including the associated microbiome) relative the attenuated culture.

Gene	Protein ID	Fasta header	Organism	Mol. weight [kDa]	Fold change[a]	Atten. Peptides[b]	Vir. Peptides[b]
TH44_06080	A0A367XEF4	tr|A0A367XEF4|A0A367XEF4_9PROT ABC transporter substrate-binding protein	*Thalassospira xiamenensis*	35.77	-4.11	7	1
tuf	A0A0F4NMA3	tr|A0A0F4NMA3|A0A0F4NMA3_PSEO7 Elongation factor Tu (Fragment)	*Pseudoalteromonas piscicida*	40.45	-4.39	6	2
JCM19236_343	A0A0B8PZ63	tr|A0A0B8PZ63|A0A0B8PZ63_9VIBR TRAP transporter solute receptor	*Vibrio sp. JCM 19236*	33.52	-4.58	4	3
TH44_00945	A0A367XGM1	tr|A0A367XGM1|A0A367XGM1_9PROT C4-dicarboxylate ABC transporter	*Thalassospira xiamenensis*	38.62	-4.8	10	0
metL	A0A1X1MWY	tr|A0A1X1MWY2|A0A1X1MWY2_9VIBR Bifunctional aspartokinase/homoserine dehydrogenase	*Vibrio sp. qd031*	88.38	-4.96	9	0
dapB	A0A1A6KDK8	tr|A0A1A6KDK8|A0A1A6KDK8_9VIBR 4-hydroxy-tetrahydrodipicolinate reductase	*Vibrio sp. UCD-FRSSP16_10*	28.87	-5.15	6	1
TH24_03085	A0A367WNY5	tr|A0A367WNY5|A0A367WNY5_9PROT Branched-chain amino acid ABC transporter substrate-binding protein	*Thalassospira xiamenensis*	44.71	-5.38	12	3
TH24_06215	A0A367WIS2	tr|A0A367WIS2|A0A367WIS2_9PROT Nitrate ABC transporter substrate-binding protein	*Thalassospira xiamenensis*	35.69	-5.67	17	5
TH24_16540	A0A367W8E6	tr|A0A367W8E6|A0A367W8E6_9PROT Branched-chain amino acid ABC transporter substrate-binding protein	*Thalassospira xiamenensis*	46.32	-5.83	22	4
	PPER_00016575-RA	PPER_00016575-RA TonB-dependent receptor	*Neoparamoeba perurans*	104.5	-6.00	3	2

Mol = molecular; kDa = kilodalton; Atten =attenuated; Vir = virulent; [a] Fold change of virulent proteins related to attenuated proteins; [b]Razor and unique peptide counts

Table 8: *Neoparamoeba perurans* top 10 significantly reduced cell- surface membrane proteins based on fold enrichment in the virulent-3 parasite.

Protein IDs	**Fasta headers**	**Mol. weight [kDa]**	**Fold change[a]**	**Atten. Peptides[b]**	**Vir. Peptides[b]**
PPER_00016125-RA	PPER_00016125-RA alpha-actinin A	68.19	-1.96	24	5
PPER_00019812-RA	PPER_00019812-RA hypothetical protein	126.63	-2.19	23	3
PPER_00020149-RA	PPER_00020149-RA Isocitrate lyase	30.67	-2.2	3	3
PPER_00006085-RA	PPER_00006085-RA nitrate ABC transporter substrate-binding protein	187.58	-2.26	9	5
PPER_00020852-RA	PPER_00020852-RA elongation factor 1	49.26	-2.31	7	1
PPER_00017666-RA	PPER_00017666-RA actin related 2 3 subunit 2	23.38	-2.35	12	3
PPER_00020708-RA	PPER_00020708-RA hypothetical protein	64.3	-2.69	22	9
PPER_00017815-RA	PPER_00017815-RA actin-like protein	47.12	-2.73	24	9
PPER_00014582-RA	PPER_00014582-RA unbiquitin-conjugating enzyme E2	17.11	-3.04	6	1
PPER_00016575-RA	PPER_00016575-RA TonB-dependent receptor	104.5	-6.0	3	2

Mol = molecular; kDa = kilodalton; Atten =attenuated; Vir = virulent; [a] Fold change of virulent proteins related to attenuated proteins; [b]Razor and unique peptide counts.

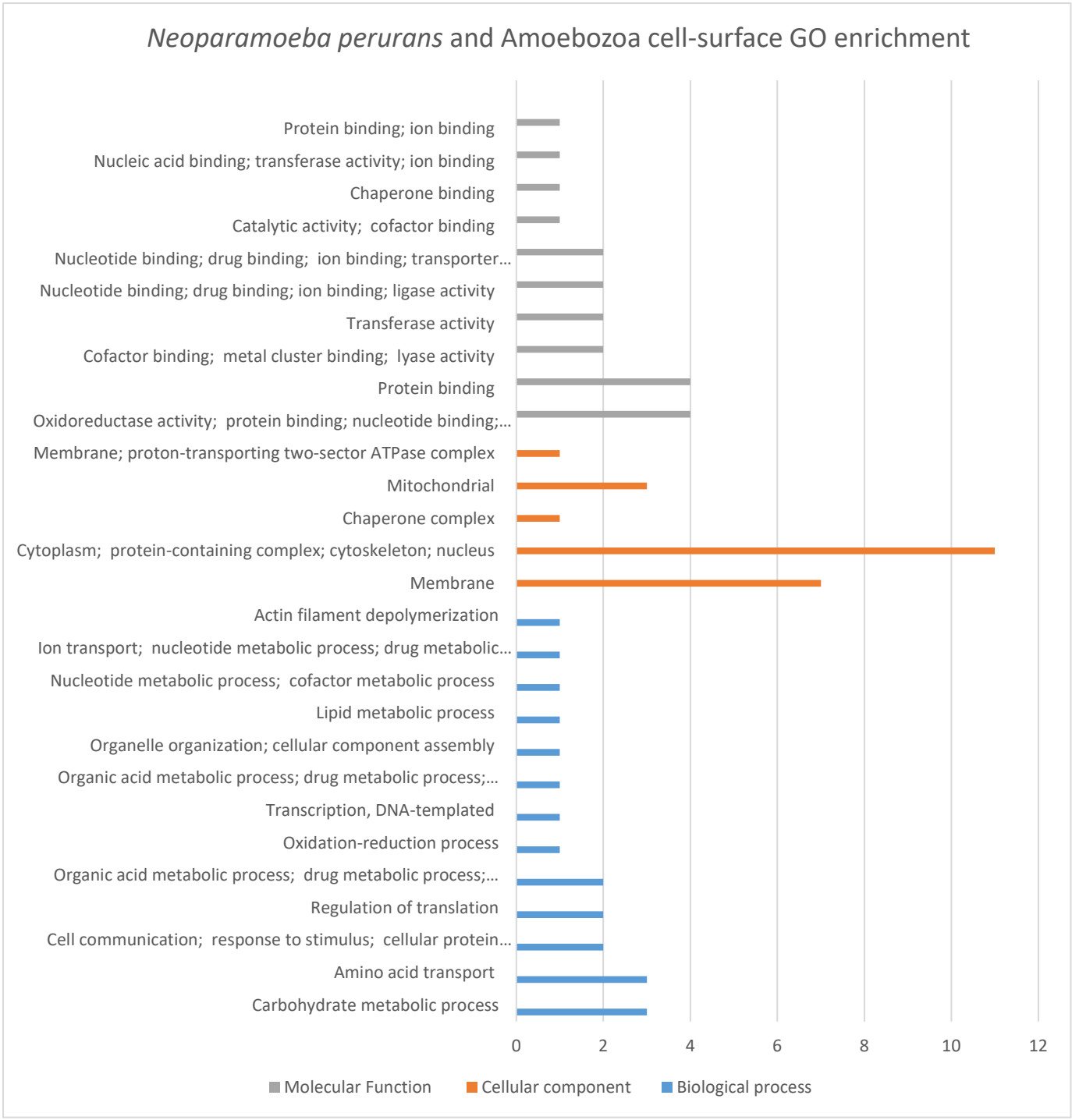

Figure 6: Gene ontology (GO) enrichment analysis of *N. perurans* significantly expressed cell- surface proteins. Proteins described are significantly elevated in the virulent-3 parasite.

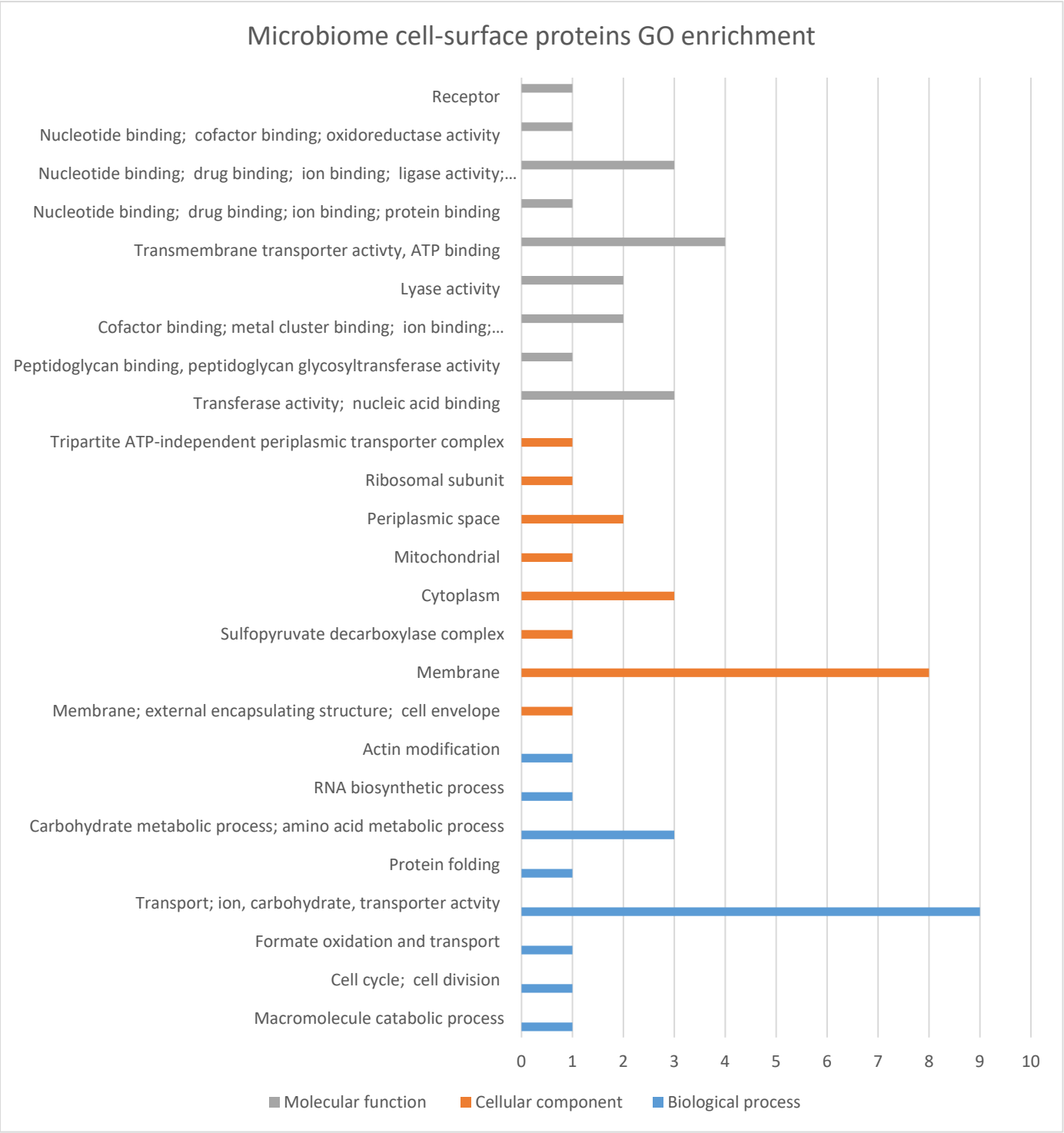

Figure 7: Gene ontology (GO) enrichment analysis of *N. perurans* microbiome significantly expressed cell-surface proteins. Proteins described are elevated in the virulent-3 culture.

4.5. Discussion

This study availed of LC-MS/MS analysis of unlabelled and biotinylated membrane proteins to identify proteins that were associated with *N. perurans* virulence, through comparative proteomics. The proteomes of a virulent-3 and an attenuated *N. perurans* culture were compared. Given the unknown role of the parasite's membrane microbiome, several genera of marine bacteria were also included in the database search for differentiation between parasitic and bacterial proteins. No proteins of *N. perurans* endosymbiont, *Perkinsela* spp. were found despite the inclusion of the *Perkinsela* spp. database and that the *N. perurans* proteome database was created with *Perkinsela* spp. proteins.

From the membrane analysis, 189 proteins were significantly elevated in the virulent-3 parasite and can therefore be considered as potential effector proteins. Separating the parasite and its microbiome, 56 proteins of *N. perurans* and Amoebozoa (two proteins) origin were found, and 133 were of bacterial origin, comprising genera *Pseudoaltermonas spp.* and *Vibrio spp*. IProClass, an integrated bioinformatic resource for genomic and proteomic research, was used to classify significantly elevated identifications by their component or location, molecular function and biological process. Of those proteins that were significantly elevated in the virulent-3 culture, membrane proteins represented approximately 44% of *N. perurans* and Amoebozoa identifications, and 46% of microbiome identifications. The remaining proteins were identified as either cytoplasmic or ribosomal.

A lesser abundance of proteins was found on the cell-surface of the parasite, in contrast to the total count of membrane bound proteins. This is expected given the diverse functions of phospholipid embedded integral proteins that provide structure to a membrane in comparison to

surface tethered peripheral proteins that typically transduce signals (Lodish H, 2000). For example, the human bacterium *Staphylococcus aureus* has approximately 24 surface proteins (Jan-Roblero *et al.*, 2017), while its cell membrane contains on average 143 proteins (Gurung *et al.*, 2011). In total, 118 significantly, differentially expressed cell-surface proteins were identified, with 23 proteins assigned as significantly elevated in *N. perurans* and Amoebozoa and, only 17 significantly elevated microbiome proteins identified. Of these 23 *N. perurans* and Amoebozoa surface proteins, 31% were membrane proteins. IProClass classified 44% of the microbiome proteins as membrane in origin.

Based on the relatively high abundance of membrane protein identifications, it can be concluded that the present method of isolating the membrane and surface protein fractions has been successful. Biotinylation has many advantages but one significant limitation of this method has been previously shown in Yu *et al.* (2006) , where renal inner medullary collecting duct cells were biotinylated for the purpose of apical and basolateral plasma membrane identification. Previously known membrane proteins were not detected by LC-MS/MS after biotin labelling and it was found that only membrane proteins that have amino acids with primary amines exposed on the surface will be labelled, therefore underrepresenting membrane proteins that lack these residues. For this reason, a combination of biotinylation and density gradient ultracentrifugation was used to extract the peripheral and integral membrane proteins of *N. perurans* in this study. Yu *et al.* (2006) also noted that cytoplasmic contamination occurred during the labelling process. *N. perurans* surface proteins had an unusual cell component count of 13% for mitochondrial proteins. The presence of mitochondrial proteins may be accounted for by cell lysis that occurred during the biotinylation process. Alternatively, metabolic activities and endocytosis were not reduced enough

in *N. perurans* during the process and the biotin may have transversed the membrane and labelled proteins present on the mitochondrial surface.

To date, no conclusive proteomic analysis has been conducted on *N. perurans* membrane and surface-bound proteins however, two studies confirmed the presence of a mannose binding protein (Valdenegro-Vega *et al.*, 2014) and high molecular weight antigens on the parasite's surface (Villavedra *et al.*, 2010). Alluding to these previously identified putative proteins, this work found no direct association to a putative mannose binding protein or a carbohydrate associated glycoprotein. The reasons for this are numerous. Due to the presumed rapid turnover of eukaryotic membranes, the proteins in question may not have been expressed at the time of the experiment. The time in culture prior to biotinylation may have exceeded the point of production of these proteins, perhaps this is a protein that is observed rapidly after *in vivo* infection and becomes reductant upon culturing. Finally, the high incidence of microbial identifications in the membrane analysis may have masked potentially interesting and relevant proteins of *N. perurans* in AGD and thus, future work should reduce the occurrence of the microbiota. Note, this work did not aim to modify the current culturing xenic methodology for *N. perurans* as the AGD community has not established if they contribute to AGD or, are simply an artefact of culture.

A carbohydrate-binding maltoporin of the microbiome membrane fraction (*Vibrio tasmaniensis* [strain LGP32]) was found to have a 9.1 fold increase in the overall virulent-3 membrane proteome (Figure 4). Maltoporin (maltose-inducible porin) is also referred to as LamB and is widely recognised as an immunogen in enteropathogenic bacteria (Upadhyaya, Singh and Dixit, 2007). Particularly, the epitopes of maltoporin in *Vibrio* species are conserved and immunisation with LamB protein had a protective effect against *Vibrio* infection in zebrafish (Lun *et al.*, 2014). It is tempting to think that this identification may be a derivative of lateral gene

transfer from the bacteria to *N. perurans*, rather than a true *Vibrio*-derived protein. Lateral gene transfer or horizontal gene transfer is a described phenomenon in amoeba where intra-bacterial species can exchange information within amoebae but also genetic exchange can occur from bacteria to amoebae (Moliner, Raoult and Fournier, 2009).

Expanding behind the current knowledge available for the *N. perurans* membrane proteome, proteinases (Kim *et al.*, 2006; Irmer *et al.*, 2009), adhesion proteins such as mannose binding protein (Garate *et al.*, 2005), Gal/GalNAc lectin (Faust and Guillen, 2012), integrin like protein (Han *et al.*, 2004), pore forming proteins (Herbst *et al.*, 2002) (Young *et al.*, 1982), and more recently GTPase signalling proteins (Bosch and Siderovski, 2013; Aqeel *et al.*, 2015) are well reported virulence factors of clinically important free-living amoebae. Proteins involved in signal transduction or cell-cell signalling were found in *N. perurans* membrane fraction. The genome of *Entamoeba histolytica* contains a remarkably large amount of Ras genes that are involved in motility, invasion, phagocytosis and evasion of the host immune response (Bosch and Siderovski, 2013). Several Ras family proteins were identified in this study as significantly elevated proteins in the virulent-3 parasite: Rho family (small GTP binding-protein Rac3); Ras related RIC1-like; Ras-related protein O-RAL; and Ras-related Rac1. These subunit proteins are effectors in diseases and play roles in disease migration and invasion (Bosch and Siderovski, 2013). It is not described how *N. perurans* develops tropism towards gill tissue and initiates pathology, however given the functions of Ras proteins in other infectious diseases in directing cell motility and communication, a plausible role for Ras is host-parasite mediation between *N. perurans* and host cell epithelial cells. Interestingly, an additional Rac GTPase binding protein was identified as FAM49 family protein, recently coined CYFIP-related Rac interactor (CYRI), which binds to Rac1 and mediates host cell invasion (Whitelaw *et al.*, 2019). CYRI is required for cellular polarity

and plasticity during cell locomotion and is used as an environmental cue for the size of cell protrusion made during movement (Fort *et al.*, 2018). This is clearly an important mechanism in how *N. perurans* interacts with its environment and how it may interact during the onset of host cell attachment.

The largest positive *N. perurans* protein fold change found in the membrane analysis was quinolinate synthase (Table 2). Quinolinate synthase has an enzymatic role in NAD and cofactor biosynthesis and NAD homeostasis has been shown to be indispensable in *Leishmania* virulence (Gazanion *et al.*, 2011). A reduction in quinolinate synthase expression was observed in the virulent-3 membrane proteome, therefore reflecting significantly elevated proteins of the attenuated culture. With respect to the largest negative protein fold change in the microbiome, a phasin protein of *Thalassospira xiamenensis* was identified. Phasin proteins are responsible for carbon storage upon growth under unfavourable conditions (de Almeida *et al.*, 2007), which can be presumed given the length of culturing time. The largest negative fold change observed in *N. perurans* (Table 4) specifically was a hypothetical protein and therefore remains uncategorised. Proteins of undescribed function warrant further investigation, even proteins present at reduced expression, as their role may be prominent in cellular homeostasis.

Assessing the cell-surface proteins, the largest overall fold change in the analysis was a putative TRAP transporter solute receptor (TAXI family) of *Vibrio nigripulchritudo*. Furthermore, many additional members of this TRAP transporter family were found in the microbiome cell-surface analysis. The best studied of this protein family is, SiaPQM from *Haemophilus influenzae* that is involved in scavenging sialic acid from its hosts cell- surface glycoproteins (Severi *et al.*, 2005). Tentatively, this may be the case for *Vibrio nigripulchritudo* in scavenging glycoproteins from Atlantic salmon gill epithelial cells or even *V. nigripulchritudo* may scavenge from *N.*

perurans. It is interesting to note that the proteins with the largest positive fold change in both the membrane and cell-surface proteomes were of *Vibrio spp* origin, namely maltoporin and TRAP transporter respectively. Therefore, it is likely that these proteins, are of great significance to the parasite and possibly play a role in AGD. The role of bacteria in AGD has been postulated previously (Bowman and Nowak, 2004), with reports of certain bacterial taxa intensifying the development of AGD lesions in gill tissue, such as *Psychroserpens spp* (Bowman and Nowak, 2004) and *Winogradskyella* sp. (Embar-Gopinath, Butler and Nowak, 2005). It is worth noting that a bacterial endosymbiont of filarial nematodes, *Wolbachia*, enhances infectivity during onchocerciasis disease (Tamarozzi *et al.*, 2011). Whether these bacterial proteins have implications in establishing AGD remains unknown until further investigation is performed.

An unusual finding of this work was the reduced expression of certain protein types in the cell-surface proteome of the virulent-3 *N. perurans* (Table 7). A TonB-dependent receptor, that mediates scarce but vital substrate-specific transport into the cell, had the largest negative fold change in the cell surface analysis. TonB-dependent receptors transport numerous substrates into a cell such as siderophores, vitamin B12, and maltodextrin (Hickman *et al.*, 2017). It is therefore plausible to believe that attenuated strains of *N. perurans*, during culture, require more of a particular substrate that TonB- dependent receptor transports from the environment. The largest *N. perurans* specific positive fold change of the cell-surface analysis was a branched-chain amino acid ABC transporter substrate binding protein. Transport proteins are well described with regards to bacterial pathogenesis but the literature is lacking in pathogenic amoebae studies. ABC transport proteins however are described as essential in apicomplexan (parasitic alveolate) virulence (Rajendran *et al.*, 2017) which suggests a synonymous role for these proteins in *N. perurans*. It is interesting to note the opposing roles of the two transporter proteins here and that one is more

associated with an attenuated culture of *N. perurans* (TonB-dependent receptor) and the other is more associated to the virulent-3 culture (ABC transporter).

One of the top molecular functions identified amongst the proteins of the virulent-3 *N. perurans* cell surface, was protein binding at 20% and oxidoreductase activity, also at 20% (Figure 6). Datasets resulting from the cell-surface analysis were explored further to identify proteins that were common in both *N. perurans* and its microbiome. One protein with oxidoreductase activity appeared in both *N. perurans* and the microbiome identifications (from *Vibrio spp*), namely glyceraldehyde-3-phosphate dehydrogenase; (GAPDH). GAPDH has been described as a moonlighting protein that is typically identified as a glycolytic enzyme in both bacterial and protozoan cells (Tristan *et al.*, 2011). GAPDH was found on *Entamoeba histolytica* cell surface proteome by surface biotinylation (Biller *et al.*, 2014) and has been outlined as a plasminogen receptor (Figuera, Gomez-Arreaza and Avilan, 2013). It has been previously shown that GAPDH facilitates pathogen adhesion to host extracellular components such as fibronectin, mucin and laminin and behaves as a direct virulence factor in protozoan and bacterial disease by enhancing pathogenesis (Kinoshita *et al.*, 2008; Alvarez *et al.*, 2007; Zhu *et al.*, 2017; Lama *et al.*, 2009; Seidler and Seidler, 2013). Therefore, it could be postulated that its position on the surface of the parasite is a direct link to host ligand attachment. Drawing correlation from the host immune response in AGD, mucin 5 has been identified as a putative biomarker of the host response (Marcos-Lopez *et al.*, 2018). An intriguing find, due to GAPDH mediating adhesion and colonisation to host epithelial mucins. Furthermore, GAPDH has been suggested as a broad spectrum vaccine candidate for microbial disease in aquaculture (Li *et al.*, 2012).

Several unidentified proteins were found to be elevated in the membrane of the virulent-3 *N. perurans* (Table 2). Interestingly PPER_00011993 RA was previously found as an elevated

secreted protein from the attenuated parasite (Chapter 3), in contrast to its reduced expression seen here in the membrane. Ectodomain shedding or regulated membrane protein release is a common feature seen in protozoan cells that are typically involve in antigenic variation of the membrane surface (Muller *et al.*, 2012; Gruszynski *et al.*, 2003). Although antigenic variation is utilised to remain undetected by the host system, our hypothesis here is during long term culture *N. perurans* is differentiating its lifestyle to suit its *in vitro* environment and this has stimulated the release of membrane proteins. Another method of analysing label free data is to observe the total count for razor and unique peptides, in conjunction with fold change. Protein PPER_00011993 RA has a fold change of 6.23 and an incredibly high razor and unique peptide count of 32 in the virulent-3 parasite compared with just 4 in the attenuated peptide count. This protein undoubtedly has relevance to the physiological and plausibly pathophysiological state of a virulent-3 isolate of *N. perurans.* Protein PPER_00011992 RA has a fold change of 5.7 and 9 razor and unique peptide counts in the virulent-3 parasite in comparison to 0 in the attenuated peptide count. Again, the relevance of this protein to virulent-3 *N. perurans* isolates must not be underestimated and further investigation is required to identify the proteins and what precise role they play in virulent *N. perurans.*

In conclusion, several proteins of potential interest to the aquaculture pharmaceutical industry have been revealed. This work showed that *N. perurans* membrane proteome is rich in potential diagnostic proteins given the described functional roles of cell mediated adhesion in maltoporin, GAPDH and GTPase signalling proteins and undescribed yet highly relevant proteins to virulent-3 *N. perurans* cultures. Underpinning the precise mechanism of host cell attachment in AGD is pivotal in the design of vaccine or chemotherapy treatments and thus, the described proteins are a promising start to this process.

Future work should consider gene knockout experiments to fully elucidate the functional role of these select proteins in *N. perurans* survivability and pathogenicity. Furthermore, systemic characterisation of *N. perurans* glycobiology through functional and structural means will further our understanding of the exact mechanisms of host cell-parasite interaction in AGD.

4.6. References

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CHAPTER 5

General discussion

5. General discussion

Despite over 30 years of AGD research, the virulence factors of *Neoparamoeba perurans* remain unknown and this lack of knowledge has hindered the development of therapeutic agents. Current treatments of freshwater and hydrogen peroxide bathing cause significant economic burdens to the aquaculture industry (Oldham, Rodger and Nowak, 2016) and thus, the need to develop therapeutic agents that merit organic status, such as the production of a vaccine, are required. By defining the putative virulence proteins in AGD, select proteins can be targeted for therapeutic intervention.

This study primarily focussed on comparatively analysing isolates of *N. perurans* of differing virulence, for changes in proteomic expression. Bridle *et al.* (2015) established the loss of virulence in a long-term cultivated clonal isolate, offering an invaluable method to analyse *N. perurans* physiology in controlled laboratory conditions. The virulence factors contributing to AGD pathogenesis are suspected to be of proteomic origin (Butler and Nowak, 2004; Bridle *et al.*, 2015) and with this information in mind, experimental tasks were designed to identify these putative virulence proteins. The research presented in chapters 2 to 4 have met the aims and objectives listed for this thesis and have identified numerous protein candidates of significance to virulent (virulent-2, virulent-1 and virulent-3) *N. perurans* cultures and furthermore, have identified the complex interaction *N. perurans* shares with its microbiota. To address the findings of each chapter, this general discussion will cover the following headings: virulence factors, uncharacterised proteins, microbiome characterisation, limitations of the study and future research.

5.1 Virulence factors

Protozoan parasites are a leading cause of morbidity and mortality in both medical and animal health (Kirk *et al.*, 2015; Muller and Hemphill, 2016). Determining the virulence factors of protozoan parasites enables the identification of disease causative molecules and thus, facilitates methods to disrupt the function and/or activity of these molecules. All experimental chapters listed in this thesis explored the potential for virulence factors across four fractions of a virulent and attenuated *N. perurans* isolate, namely the: hydrophilic, extracellular, membrane bound and cell-surface proteins as to fully elucidate the role of each fraction in AGD. LC-MS/MS was the chosen analytical tool for this work, as proteomic identification captures information such as protein phosphorylation as well as protein trafficking (Chandramouli and Qian, 2009; Rang *et al.*, 2015), and can therefore provide a better representation of cell homeostasis than genomic technologies. An *in vivo* challenge trial, using naïve Atlantic salmon smolts, confirmed the virulence and attenuation of the *N. perurans* isolates (attenuated and virulent-1) studied.

A specific set of protein databases were used throughout this work to match peptides sequences from LC-MS/MS analyses to specific proteins. These databases included a defined set of *N. perurans* protein sequences provided by collaborators at CSIRO, Australia (which included a sub-set of *Perkinsela* sp. protein identifications), all known protein sequences from the microbiome of *N. perurans* (based on the bacterial identifications from 16S rRNA gene Illumina MiSeq sequencing) and all known Amoebozoa protein sequences. Only one significantly elevated protein of *N. perurans* endosymbiont, *Perkinsela* spp. was found as a heat shock protein from the 2D gel analysis. Several other *Perkinsela* spp. proteins were identified in the proteomic analysis of all chapters, however after performing a quality control filter of the proteins, the *Perkinsela* spp. proteins did not pass the razor and unique peptide cutoff. Chapter one aimed to identify the

hydrophilic virulence factors by comparatively profiling a virulent-2, virulent-1 and attenuated isolate of *N. perurans* using 2D gel electrophoresis and LC-MS/MS.

Several proteins were found to be significantly upregulated in the virulent-2 that comprised proteins involved in cytoskeletal, oxidative and immunomodulatory roles. Actin and actin-associated proteins were also significantly elevated in the virulent-2 parasite, an expected find, as actin is a major component of the cytoskeleton of amoebae and plays a role in tissue invasion, motility and phagocytosis (Burri *et al.*, 2012; Manich *et al.*, 2018). Actin processes are instrumental in the pathogenicity of *Entamoeba histolytica* and it was shown by Bolanos *et al.* (2014) that a downregulation of actin, myosin II heavy chain and alpha-actinin occurred in response to the administration of (−)-epicatechin, resulting in amoebic adhesion reduction on Caco-2 cells. Future investigation should explore known anti-amoebic flavonoids on *N. perurans* cells for therapeutic screening.

Profilin, also an actin associated protein of amoebae, was elevated in the virulent-2 isolate and this protein has been shown to trigger allergic airway inflammation in mice infected with *Acanthamoeba* sp. (Song *et al.*, 2018). In late stage AGD, the Th2 pathway of *Salmo salar L*. has been shown to be upregulated, which suggests either *N. perurans* elicits an immune evasion strategy or the infection is a result of an allergic response (Benedicenti *et al.*, 2015). Given the previous role of profilin in *Acanthamoeba sp*., future work should aim to analyse the effect of inhibiting profilin in *N. perurans* prior to a challenge trial and to observe gill scoring and histology for the development of mucoid patches. Interestingly, profilin was found as a cell-surface protein of shared, non-significance of the virulent-3 and attenuated parasite in Chapter 4. This provides evidence that this protein can be localised at the surface, however upon culturing *in vitro,* the

protein may become readily redundant. Furthermore, this protein could be a suitable candidate for vaccine design, given its extracellular position on the surface of the parasite.

Lipoxygenase, an iron containing enzyme and ATP binding cassette (ABC) membrane transporter protein were also found as significantly elevated in the virulent-2 parasite. Lipoxygenase has been shown to increase *Pseudomonas aeruginosa* persistence in the host and destroy the lipid bilayer of the host membrane (Aldrovandi *et al.*, 2018). Lipoxygenase is typically released into the cellular milieu however chapter three did not find this protein in the virulent exoproteome. Reasons for not identifying any putative virulence factors of the virulent-3 exoproteome are further discussed in sections 5.3 and 5.4. Nonetheless, lipoxygenase may have a function in inducing necrosis of the gill epithelium membrane. The ABC membrane transporter has been suggested as bidirectional in the uptake of host cholesterol (Ehrenman *et al.*, 2010) and can be implicated in drug resistance. Interestingly, transporter proteins were also identified in the cell-surface fraction as a branched chain amino acid ABC transporter substrate binding (of *N. perurans*) and TRAP transporter solute receptor (TAXI family) (of *Vibrio nigripulchritudo*), however an ATP binding cassette ABC transporter was not found. Transport proteins are not well described in parasitic amoebae virulence, however transport proteins bind and carry nutritional molecules into the cell, and they therefore represent a desirable drug target for AGD due to the direct access to the cytoplasm of the cell. Given the predescribed role of the ABC membrane transporter in uptaking host cholesterol, *N. perurans* may require this function in scavenging host-gill derived cholesterol.

It has been postulated that the mechanism which *N. perurans* uses to adhere to the gill epithelium to initiate AGD infection may be an adhesion protein (Villavedra *et al.*, 2010; Valdenegro-Vega *et al.*, 2015), therefore as part of this work, the membrane and cell-surface of the

parasite was targeted for investigation. Despite the presumed identification of mannose binding proteins, this investigation did not find such proteins. Overall, a maltoporin protein of *Vibrio* sp. origin was identified as the protein with the largest positive fold change in the virulent-3 membrane proteome compared with the attenuated strain. Indeed, a protein from *Vibrio* sp. also had the largest positive fold changes in the cell-surface proteome of the virulent-3 culture, which may be suggestive of virulence factor contribution by the microbiota. AGD lesions have been reported as intensified in the presence of *Psychroserpens* sp. (Bowman and Nowak, 2004) and *Winogradskyella* sp. (Embar-Gopinath *et al.,* 2005) compared with lesions on gills infected with *N. perurans* alone. The contribution of bacteria in increasing parasitic virulence and infectivity has previously been demonstrated. For example, enhanced infectivity has been previously reported in the bacterial endosymbiont of filarial nematodes, *Wolbachia* during onchocerciasis disease (Tamarozzi *et al.*, 2011). Therefore, it is plausible to believe that the microbiome of *N. perurans* is playing a significant role in AGD infectivity by producing proteinaceous virulence factors. In order to address this, future challenge trials should include bath inoculation of naïve smolts with species from the *Vibrio* genus to discriminate this putative role of virulence in AGD. Only recently, a correlation between AGD outbreaks and *Tenacibaculum dicentrarchi* has been observed (Slinger *et al.,* 2020), thus highlighting the need to closely monitor and investigate the relationship of *N. perurans* and bacterial associated gill pathogens.

A potential virulence factor that was found on the cell-surface of the virulent-3 membrane is glyceraldehyde-3-phosphate dehydrogenase (GAPDH). This protein was also found in the hydrophilic fraction but as a *Vibrio sp* protein. GAPDH was identified as both a *N. perurans* and a *Vibrio* sp. protein and has been previously shown to aid in attachment and colonisation of pathogens to host mucins (Zhu *et al.*, 2017). Therefore, it may be hypothesised that its position on

the surface of the parasite is a direct link to host ligand attachment. Mucin 5 has been identified as a putative biomarker of the host response in AGD (Marcos-Lopez *et al.*, 2018) , and it is tempting to think GAPDH may recognise this as its target host ligand. Several GTPase signalling proteins involved in signal transduction or cell-cell signalling were found in *N. perurans* membrane fraction. GTPase signalling proteins such as Ras proteins are involved in motility, invasion, phagocytosis and evasion of the host immune response in *E. histolytica* (Bosch and Siderovski, 2013). Several Ras family proteins were identified in this study as significantly elevated proteins in the virulent-3 parasite: Rho family (small GTP binding-protein Rac3); Ras related RIC1-like; Ras-related protein O-RAL; and Ras-related Rac1. It is plausible that *N. perurans* develops tropism towards gill tissue and initiates pathology through the use of Ras proteins. A Rac GTPase binding protein was also identified in the virulent-3 membrane as FAM49 family protein, recently coined CYFIP-related Rac interactor (CYRI), that binds to Rac1 and mediates host cell invasion (Whitelaw *et al.*, 2019). CYRI is imperative for cellular polarity and plasticity during cell locomotion (Fort *et al.*, 2018), which therefore has implications in how *N. perurans* interacts during the onset of host cell attachment. Future efforts should identify methods that impede and target this cell signalling pathway in *N. perurans* to identify any deleterious effects on locomotion and host cell attachment.

Overall, several proteins of potential virulence capabilities have been described in this thesis, and Table 1 describes the degree of overlap observed between the hydrophilic, exoproteome (ExP), membrane bound and cell-surface fractions. Proteins that were shared across fractions were actin, profilin allergen, maltoporin (*Vibrio* spp. in origin), ABC transporter, and glyceraldehyde 3 –phosphate dehydrogenase of both *N. perurans* and *Vibrio* spp. Finding several proteins across different spatial and temporal scales indicates that *N. perurans* maintains

expression of these proteins and as such, these proteins may have a significant role in *N. perurans* physiology and pathogenicity. Three of these putative virulence proteins are found within the membrane, namely, maltoporin, GAPDH and ABC transporter, which offers an easier therapeutic target route as these proteins are exposed to the external environment than cytosol bound proteins such as elongation factor 1 alpha.

Profilin is an actin associated protein and this was found as significantly elevated, along with actin in the virulent-2 parasite. Proteins that are italicised were found as non-significantly expressed but are included here as these were the only proteins that originated from the virulent-3 exoproteome, and experimental chapter 3 highlighted the required optimisation of culturing for the production of virulence factors.. These non-significant exoprotoeme proteins are involved in iron acquisition, and it is interesting to note that lipoxygenase from the hydrophilic fraction, is a non-heme iron family enzyme (Hansen *et al.,* 2013). Iron metabolism has been suggested as a role in the pathogenesis of amebiasis (Lee *et al.,* 2008) .Similar to the iron acquisition proteins that chelate iron from the host, the ABC transporter and the TRAP TAXI family transporter scavenge cholesterol from the host. This is indicative of a nutritional acquisition role for these proteins in *N. perurans*. Cell-cell communication and cross talk between *N. perurans* and the host gill epithelium may be facilitated by FAM49 protein, RAS family protein and even lipoxygenase (Kurakin *et al.,* 2020). Finally, GAPDH and maltoporin are here posited as host cell attachment mediators due to their previously described roles in host cell receptor binding (Zhu *et al.,* 2017; Yang et al., 2019).

Table 1: Summary table of all virulence associated *Neoparamoeba perurans* and microbiome associated proteins identified across the proteomic fractions and their potential functions

IN SUMMARY **: VIRULENCE FACTORS**						
Protein	**Species**	**Hydrophilic**	**ExP**	**Membrane**	**Cell-surface**	**Function**
Actin	*N. perurans*	☑	☑			Cytoskeleton, tissue invasion
Elongation factor 1 alpha	*N. perurans*	☑				Macrophage deactivation
Lipoxygenase	*N. perurans*	☑				Increase persistence in host, destroys lipid bilayer
Profilin allergen	*N. perurans*	☑			☑ NS	Activates Th2 response, mucin overproduction
Maltoporin	*Vibrio neresis; tasmaniensis*		☑ *NS*	☑		*LamB, immunogen, conserved antigen*
Ferrichrome-iron receptor	*Vibrio tasmaniensis*		☑ *NS*			*Iron acquisition*
Ferric enterobactin receptor	*P. haloplanktis*		☑ *NS*			*Iron acquisition*
ABC transporter	*N. perurans*	☑		☑		Host cholesterol scavenging
TRAP TAXI family transporter	*Vibrio nigripulchritudo*				☑	Host cholesterol scavenging
FAM49	*N. perurans*			☑		Binds to Rac1, host cell invasion
RAS family proteins	*N. perurans*			☑		Tropism, signalling
Glyceraldehyde 3 –phosphate dehydrogenase GAPDH	*Vibrio rumoiensis; N. perurans*	☑			☑	Attachment and colonisation to host mucins

5.3 Uncharacterised proteins

Many of the proteins listed in this thesis were successfully characterised however, a number of proteins could not be identified and thus their role in *N. perurans* and furthermore, as a virulence factor of AGD remain unknown. One protein, PPER_00011993, appeared as a significantly elevated protein in the attenuated exoproteome of chapter three and subsequently appeared as a significantly elevated protein of the virulent-3 membrane proteome in chapter four. The opposing role of this protein is highly suggestive of a virulence state and that the attenuated parasite may be releasing or shedding the protein into the environment. The controlled release of proteins or ectodomain shedding is a common feature seen in protozoan cells that are typically involved in antigenic variation of the membrane surface (Muller *et al.*, 2012; Gruszynski *et al.*, 2003). Given the confirmed loss of virulence of the attenuated parasite, a plausible reason for shedding is that long-term cultured *N. perurans* differentiates its lifestyle to suit its *in vitro* environment, thus stimulating the release of membrane proteins. Another unknown protein, PPER_00011992, was also found as a significantly elevated protein of the virulent-3 membrane. Table 2 describes the degree of overlap of the unknown and hypothetical proteins found across the membrane, exoproteome and pen-strep exoproteome. The fold changes relate to the statistical analysis from the membrane and exoproteome only, as no technical replicates were included for the pen-strep exoproteome. Although no description for these proteins is available at present, further analysis through cloning or structural characterisation of these proteins should be attempted in order to identify the proteins and ultimately their relevance to AGD.

Table 2: Summary table of the uncharacterised proteins, their fold change and total razor and unique peptide count found in *Neoparamoeba perurans* from all experimental chapters.

Fasta headers	Mol. Weight [kDa]	Fold change		Membrane Atten. Peptides	Membrane Vir. Peptides	Exo Atten. Peptides	Exo Vir. Peptides	Pen Strep Atten. Peptides	Pen Strep Vir. Peptides
PPER_00011993-RA—NA-'Unidentified'	35.25	6.23	-1.15	4	32	8	6	4	4
PPER_00011394-RA—NA-'Unidentified'	42.16	5.65		2	14				
PPER_00011992-RA—Hypothetical protein	39.76	5.73	-2.86	0	9	6	6	4	4
PPER_00018933-RA—NA-'Unidentified'	281.31	-4.73				40	17	6	4
PPER_00020708-RA Hypothetical protein	64.3	-5.01				10	0		
PPER_00013024-RA Hypothetical protein	13.01	-4.77				8	3		
PPER_00017145-RA Hypothetical protein	32.89	-						2	2

Mol= molecular weight; kDa = kilodalton; Atten = attenuated; Vir = virulent

5.4 Microbiome characterisation

Endosymbionts of protozoan parasites represent another facet of parasite complexity, in that genetic exchange can occur amongst intra-bacterial species but information can also be exchanged from bacteria to amoebae (Moliner *et al.,* 2009). There has been considerable speculation in the literature about the involvement of bacteria in AGD, both host-derived (Embar-Gopinath *et al.*, 2008; Birlanga *et al.*, 2020; Slinger *et al.*, 2020) and *N. perurans*-derived (McCarthy *et al.*, 2015; Benedicenti *et al.*, 2019). Therefore, chapter one included 16S rRNA Illumina MiSeq sequencing of the virulent-1 and attenuated cultures to aid identification of the bacterial species present within.

The presence of bacterial biofilms was evident in both the attenuated and virulent (virulent 1,2) cultures of *N. perurans* used in this work. The attenuated long-term culture, in particular, harboured a considerable quantity of bacteria that was easily observable under a light microscope. Proteins secreted by these bacteria were suspected to have caused the unexpected cytotoxic effect observed with the RTgill W1 cells, as discussed in chapter 3. Therefore, there was a clear need to investigate the bacteria present in each of the *N. perurans* cultures. Similar to McCarthy *et al.* (2015) and Benedicenti *et al.,* (2019), gram negative bacteria predominated the results. A distinct difference was noted between the richness and diversity of bacterial communities present in the virulent-1 and attenuated isolates. Diversity was depleted in the attenuated isolate, which was not unexpected, as long-term culture confinement would lead to natural selection of amoeba-resisting organisms that can proliferate in stressful environments. The presence of *Thalassospira xiamenensis* belonging to the class Proteobacteria was markedly high in the attenuated isolate. This result was corroborated in the LC-MS/MS analysis of all experimental chapters where proteins related to this bacterium appeared as significantly elevated proteins of the attenuated culture.

Pathogenic bacteria of finfish health importance such as *Vibrio spp* and *Simkaniaceae spp* were found in the virulent-1 microbiome (Pawlikowska-Warych and Deptula, 2016; Kashulin *et al.,* 2017). This interesting find presents the hypothesis that wild, virulent *N. perurans* can act as a Trojan horse and transport potentially dangerous bacteria to susceptible hosts. Such an idea is not unfamiliar and has been reported in *Acanthamoeba castellanii Neff strain* (Siddiqui and Khan, 2012), therefore the potential for this form of interaction in *N. perurans* is possible. The majority of hydrophilic proteins identified in chapter 2 of this work were derived from the *N. perurans* proteome (21 proteins), with only three proteins associated with bacterial symbionts.

In contrast, most of the protein identifications in the secretory proteome (outlined in chapter 3) were mapped to bacterial peptides of the attenuated culture. The results of the secretome analysis in chapter 3 provided insight into the exoproteomic changes that occur in the attenuated-3 culture. Many of the proteins identified from the LC-MS/MS were bacterial proteins, with protease activity and associated with cytotoxicity, such as secreted alkaline phosphatase and peptidase S8/S53 subtilisin kexin sedolisin. Chapter three highlighted the reduced role of the exoproteomic fraction of a virulent-3 isolate and revealed the difficulty in performing comparative protein expression with a microbial rich attenuated isolate. It is imperative to note that 11 *N. perurans* proteins were identified on both exoproteomes, however significant elevation was noted in the attenuated culture. As previously outlined, the exoproteome of the attenuated isolate showed unexpectedly high cytotoxicity with these responsible proteins presumed to be from the microbial community, such as an immunogenic protein of *V. tasmaniensis* and peroxiredoxin 2 of *P. haloplanktis*. Therefore, an antibiotic was introduced to dampen the microbial effect seen in the cytotoxicity assay. Although a reduction of protease activity, protein identifications and cytotoxicity were obtained in the attenuated isolate, the complete removal of the microbiome was

not entirely possible. Some biofilm formation was still observed in the attenuated culture, but not in the virulent-3 culture and bacterial proteins were still identified in the LC-MS/MS analysis. It is important to note that the attenuated culture did not induce AGD *in-vivo*. This was shown in the results of chapter one, therefore it was considered a valid comparator to aid identification of hydrophilic and membrane-associated virulence factors in the virulent-3 isolate. However, the increased protein identifications of the attenuated exoproteome created complexity in confidently identifying secretion virulence factors. *N. perurans* secreted proteins may have implications in AGD but the current experimental conditions could be masking their presence with high quantities of bacterial secretions. Some suggestions for tackling this issue are discussed in section 5.5.

5.5 Limitations of the study

The cultivation procedure for the various isolates of *N. perurans* was followed as previously described by Crosbie *et al.* (2012). To date, three separate *N. perurans* cultures have been successfully established in the Marine and Freshwater Research Centre of GMIT and the species identity confirmed using qPCR (Downes *et al.*, 2015). The culturing method of *N. perurans* is quite simplistic and has never been re-evaluated since its inception, in comparison to other pathogenic organisms, such as *Acanthamoeba sp.* where the cultivation procedure has been repeatedly explored and revised (Adam, 1959; Adam *et al.,* 1967; Byers *et al.,* 1980; Xuan *et al.,* 2009). As a result of this simplistic and untailored media for *N. perurans* cultivation, additional organisms such as prokaryotes and potentially other eukaryotes propagate in *N. perurans* culture.

In relation to the culturing of *N. perurans* in the laboratory, the question of whether to produce a clonal isolate or to use a xenic culture of the parasite is an important one. *N. perurans*

cultures are generally initiated by spreading a swab from an infected salmon on a simple malt-yeast agar (MYA) plate that is subsequently overlaid with sterile-filtered seawater. In xenic cultures, bacteria carried by the swab from the salmon, will co-cultivate in the absence of antibiotics. Indeed, when *N. perurans* cultures are left for extended periods of time, bacteria can become the dominant organisms. The role of the bacterial species that co-habit the gills of AGD infected salmon, alongside *N. perurans,* is unknown. The scientific community have speculated the potential involvement of bacterial species in proliferating AGD (Bowman and Nowak, 2004; Embar-Gopinath et al., 2005). Thus, this work sought to maintain *N. perurans* in a xenic culture due to the unknown role that co-cultured bacteria may play in *N. perurans* virulence. This approach does have its complications, particularly in proteomics studies, where the assignment of proteins to specific species is an important part of the identification process. However, this work was designed to capture as detailed an overview as possible in relation to the proteomic virulence factors associated with AGD, be they bacterial or amoebic in origin. Nonetheless, future research may consider the use of clonal cultures (Collins *et al.*, 2017) isolated by flow cytometry, prior to performing proteomic studies, to identify any disparities in proteomic expression between polyclonal and clonal *N. perurans* isolates.

Part of this work outlined in chapter two sought to investigate the virulence factors in the exoproteome of *N. perurans*. The cytotoxicity of the secretome of an attenuated and virulent-3 strain of *N. perurans* was compared using a rainbow trout cell line (RTgill W1). Unexpectedly, the secreted fraction of the attenuated *N. perurans* isolate exhibited a much stronger cytotoxic effect on the cell line compared with that of the virulent-3 isolate. The virulent-3 and attenuated nature of both the *N. perurans* cultures used in this experiment was validated in the *in vivo* trial outlined in chapter one, therefore the cytotoxicity observed in RTgill W1 with the attenuated culture was

unexpected. Compounding this, it was previously reported by Bridle *et al.* (2015) that the secreted fraction of a virulent *N. perurans* isolate elicited a cytopathic effect on another salmonid cell line, CHSE-214 from the Chinook embryo, pointing towards the existence of a cytotoxic extracellular product (ECP) in the secretome of *N. perurans*. Upon examining the differences in the proteomic profile of the secreted fractions, the total number of proteins identified was significantly greater in the attenuated culture-3 rather than the virulent-3 culture. Secretory proteins of the attenuated-2 microbiota were more pronounced in the protein identifications and this is to be expected due to the long term culturing process as previously discussed. It is possible that *N. perurans* may require the presence of host-derived material to trigger the release of secreted virulence factors. Indeed, Cano *et al.* (2019) has suggested that the use of *ex vivo* gills or Transwell inserts may be a more appropriate means to investigate this protein fraction.

Release of secreted virulence factors has been demonstrated in other amoebae, for example, *Acanthamoeba spp*. releases a 133 kDa serine protease in response to co-incubation with extracellular matrix components of host fibronectin (Hurt *et al.*, 2003). *E. histolytica* binds to host-derived fibronectin when co-incubated and simultaneously causes proteolysis (Franco, Vazquez-Prado and Meza, 1997). Culture enhancement for the purpose of maintaining virulent isolates should be a requisite going forward with secretion virulence factor investigation.

5.6 Future research

Since commencing this research, freshwater and hydrogen bathing remain the only effective treatment strategies for AGD. Currently no research efforts have devised a strategy to combat *N. perurans* itself, and this lack of research development is correlated to the knowledge gap relating to *N. perurans* virulence factors. One of the major goals of this thesis was to identify

N. perurans virulence factors for therapeutic intervention. As a result of this work, a select list of proteins from several fractions of the parasite have been described that are now proposed as playing a crucially important role in *N. perurans* virulence. The variation in the function of these proteins offers numerous options for intervention. For example, the targeting of virulence factors such as GAPDH or profiling, as part of an antivirulence strategy would be a potentially viable approach as many of the proteins identified from *N. perurans* and its associated microbiome are not shared with the host, therefore offer a safe and non- deleterious effect to the host during treatment. Potentially, profilin may be a suitable candidate for such an antivirulence strategy, as a recombinant form of the protein increases the Th2 response in a mouse model (Song *et al.,* 2018). This approach was taken by Ghosh *et al.* (2020) for the treatment of severe amebic colitis using an antibody macrophage migration inhibitory factor (MIF) of *E. histolytica* in combination with metronidazole, which successfully reduced chronic infection in the mouse model. The combination of antiparasite MIF blocking antibodies with metronidazole significantly improved tissue damage and inflammation in the mouse model, versus using metronidazole alone. An additional therapeutic approach could combine several *N. perurans* virulence factor targets, as shown by Katalani *et al.* (2020) where three virulence factors of *Clostridium perfringens* incorporated as trivalent vaccine, significantly increased host cell viability upon exposure to the virulence factors with the vaccine. A potential experimental plan could incorporate the creation of mutations in one or more of these virulence protein targets using RNA interference technology (Lima *et al.,* 2013). An assessment of the resulting *N. perurans* mutated strain's ability to establish AGD in naïve smolts could be carried out in a controlled challenge trial. If any of the mutants' virulence was attenuated, the target protein in question would be worthy of further analysis, either by expression and cloning for recombinant vaccine production or structural elucidation by X- ray crystallography for drug design.

AGD is a multifactorial disease that encompasses the host, parasite and environment. Virulence of *N. perurans* is only one facet of AGD, nonetheless it's one of the most underrepresented aspects of AGD research. Furthering the complexity of AGD, is the association of *N. perurans* and its microbiome. The recently described link of *Vibrio* species occupying the parasites cytoplasm by MacPhail *et al.* (2021) correlates with the findings derived in this thesis, where a proportion of bacterial identifications were directly derived from *Vibrio* species. Future research should aim to incorporate the identified *Vibrio* derived proteins in this thesis in future vaccine trials in conjunction with *N. perurans* cultures as to fully understand the direct or indirect virulence contribution by this bacteria species. The outcome of this work is expected to facilitate progress in the design of *N. perurans* targeted therapies for the treatment of AGD in farmed fish. Developing treatments that target and disrupt *N. perurans* will improve gill health in salmonids and will promote aquaculture sustainability and productivity.

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APPENDICES

Appendix I: Bacterial reference proteome downloaded on the 8th of May 2020 from UniProt .

Proteome ID	Organism	Organism ID	Protein count	BUSCO	CPD	Genome representation (RefSeq)
UP000198862	*Pseudoalteromonas denitrificans DSM 6059 (Strain: DSM 6059)*	1123010	5332	C:98.7%[S:98.2%,D:0.4%],F:0.4%,M:0.9%,n:452	Close to Standard	full
UP000033452	*Pseudoalteromonas rubra (Strain: S2471)*	43658	4390	C:98.7%[S:98.5%,D:0.2%],F:0%,M:1.3%,n:452	Standard	full
UP000033511	*Pseudoalteromonas piscicida (Strain: S2040)*	43662	4134	C:97.1%[S:96.7%,D:0.4%],F:0.2%,M:2.7%,n:452	Close to Standard	full
UP000179786	*Pseudoalteromonas amylolytica (Strain: JW1)*	1859457	4015	C:99.3%[S:98.9%,D:0.4%],F:0.4%,M:0.2%,n:452	Standard	full
UP000030341	*Pseudoalteromonas piratica (Strain: OCN003)*	1348114	3997	C:99.8%[S:99.3%,D:0.4%],F:0%,M:0.2%,n:452	Standard	full
UP000033664	*Pseudoalteromonas ruthenica (Strain: S3137)*	151081	3470	C:98.5%[S:98%,D:0.4%],F:0.2%,M:1.3%,n:452	Standard	full
UP000061457	*Pseudoalteromonas phenolica (Strain: KCTC 12086)*	161398	4164	C:98.9%[S:98.2%,D:0.7%],F:0.4%,M:0.7%,n:452	Standard	full
UP000006843	*Pseudoalteromonas haloplanktis (strain TAC 125) (Strain: TAC 125)*	326442	3484	C:99.3%[S:98.9%,D:0.4%],F:0.2%,M:0.4%,n:452	Standard	full
UP000016487	*Pseudoalteromonas citrea DSM 8771 (Strain: DSM 8771)*	1117314	4457	C:99.6%[S:99.3%,D:0.2%],F:0.2%,M:0.2%,n:452	Standard	full
UP000194841	*Pseudoalteromonas ulvae*	107327	3964	C:99.3%[S:98.9%,D:0.4%],F:0%,M:0.7%,n:452	Standard	full
UP000076643	*Pseudoalteromonas luteoviolacea DSM 6061 (Strain: DSM 6061)*	1365250	5008	C:99.6%[S:98.9%,D:0.7%],F:0%,M:0.4%,n:452	Standard	full
UP000007127	*Thalassospira xiamenensis M-5 = DSM 17429 (Strain: M-5)*	1123366	4340	C:98.2%[S:98.2%,D:0%],F:0.5%,M:1.4%,n:221	Standard	full
UP000252266	*Thalassospira xiamenensis (Strain: IB13)*	220697	4080	C:96.4%[S:96.4%,D:0%],F:0.9%,M:2.7%,n:221	Standard	full
UP000253064	*Thalassospira xiamenensis (Strain: S27-11)*	220697	4028	C:97.3%[S:97.3%,D:0%],F:0.5%,M:2.3%,n:221	Standard	full
UP000219068	*Thalassospira xiamenensis (Strain: USBA 78)*	220697	5070	C:98.2%[S:95.9%,D:2.3%],F:0.5%,M:1.4%,n:221	Outlier	full

Proteome ID	Organism	Organism ID	Protein count	BUSCO	CPD	Genome representation (RefSeq)
UP000007463	*Fluviicola taffensis (strain DSM 16823 / NCIMB 13979 / RW262) (Strain: DSM 16823 / RW262 / RW262)*	755732	4016	C:97.1%[S:96.6%,D:0.5%],F:1.6%,M:1.4%,n:443	Close to Standard	full
UP000000584	*Vibrio cholerae serotype O1 (strain ATCC 39315 / El Tor Inaba N16961) (Strain: ATCC 39315 / El Tor Inaba N16961)*	243277	3782	C:98.9%[S:98.5%,D:0.4%],F:0%,M:1.1%,n:452	Standard	full
UP000000537	*Aliivibrio fischeri (strain ATCC 700601 / ES114) (Vibrio fischeri) (Strain: ATCC 700601 / ES114)*	312309	3813	C:99.3%[S:99.1%,D:0.2%],F:0.7%,M:0%,n:452	Standard	full
UP000232179	*Vibrio sp. HA2012 (Strain: HA2012)*	1971595	3382	C:99.8%[S:99.6%,D:0.2%],F:0%,M:0.2%,n:452	Standard	full
UP000235640	*Vibrio sp. 10N.286.49.C2 (Strain: 10N.286.49.C2)*	1880856	4338	C:99.8%[S:98.7%,D:1.1%],F:0%,M:0.2%,n:452	Standard	full
UP000009100	*Vibrio tasmaniensis (strain LGP32) (Vibrio splendidus (strain Mel32)) (Strain: LGP32)*	575788	4420	C:99.1%[S:97.6%,D:1.5%],F:0.9%,M:0%,n:452	Standard	full
UP000094165	*Vibrio genomosp. F6 str. FF-238 (Strain: FF-238)*	1191298	3650	C:98.7%[S:98.5%,D:0.2%],F:0.4%,M:0.9%,n:452	Standard	full
UP000094070	*Vibrio rumoiensis 1S-45 (Strain: 1S-45)*	1188252	2981	C:99.1%[S:99.1%,D:0%],F:0%,M:0.9%,n:452	Close to Standard	full
UP000002943	*Vibrio caribbeanicus ATCC BAA-2122 (Strain: ATCC BAA-2122)*	796620	4023	C:99.3%[S:99.3%,D:0%],F:0.2%,M:0.4%,n:452	Standard	full
UP000184608	*Vibrio aerogenes CECT 7868 (Strain: CECT 7868)*	1216006	4565	C:99.3%[S:99.1%,D:0.2%],F:0.7%,M:0%,n:452	Standard	full
UP000281112	*Vibrio sp. LJC006 (Strain: LJC006)*	2487322	4277	C:100%[S:99.3%,D:0.7%],F:0%,M:0%,n:452	Standard	full
UP000016567	*Vibrio azureus NBRC 104587 (Strain: NBRC 104587)*	1219077	4147	C:99.6%[S:98.9%,D:0.7%],F:0.4%,M:0%,n:452	Standard	full
UP000002493	*Vibrio parahaemolyticus serotype O3:K6 (strain RIMD 2210633) (Strain: RIMD 2210633)*	223926	4821	C:99.6%[S:98.7%,D:0.9%],F:0.2%,M:0.2%,n:452	Standard	full
UP000193432	*Vibrio sp. qd031 (Strain: qd031)*	1603038	3553	C:98.5%[S:98%,D:0.4%],F:0%,M:1.5%,n:452	Standard	full
UP000269041	*Vibrio pectenicida (Strain: CAIM 594)*	62763	3823	C:99.1%[S:94.7%,D:4.4%],F:0%,M:0.9%,n:452	Standard	full

Proteome ID	Organism	Organism ID	Protein count	BUSCO	CPD	Genome representation (RefSeq)
UP000037515	*Vibrio nereis (Strain: DSM 19584)*	693	3660	C:100%[S:99.6%,D:0.4%],F:0%,M:0%,n:452	Standard	full
UP000189475	*Vibrio palustris (Strain: CECT 9027)*	1918946	3328	C:99.6%[S:98.2%,D:1.3%],F:0.2%,M:0.2%,n:452	Standard	full
UP000029994	*Vibrio navarrensis (Strain: ATCC 51183)*	29495	3643	C:99.8%[S:99.1%,D:0.7%],F:0%,M:0.2%,n:452	Standard	full
UP000016895	*Vibrio nigripulchritudo (Strain: SnF1)*	28173	5540	C:100%[S:99.8%,D:0.2%],F:0%,M:0%,n:452	Standard	full
UP000003627	*Vibrio sp. (strain N418) (Strain: N418)*	701176	4028	C:99.6%[S:98.2%,D:1.3%],F:0.4%,M:0%,n:452	Standard	full
UP000198854	*Vibrio xiamenensis (Strain: CGMCC 1.10228)*	861298	4859	C:100%[S:98.9%,D:1.1%],F:0%,M:0%,n:452	Standard	full
BUSCO = Benchmarking Universal Single-Copy Orthologs; CPD = Complete Proteome Detector						

Appendix II: Amoebozoa reference proteome downloaded on the 8th of May 2020 from UniProt.

Proteome ID	Organism	Organism ID	Protein count	BUSCO	CPD	Genome representation (RefSeq)
UP000001064	*Dictyostelium purpureum (Slime mold) (Strain: QSDP1)*	5786	12347	C:92.1%[S:89.1%,D:3%],F:2%,M:5.9%,n:303	Standard	full
UP000002195	*Dictyostelium discoideum (Slime mold) (Strain: AX4)*	44689	12746	C:96%[S:92.4%,D:3.6%],F:0.7%,M:3.3%,n:303	Standard	full
UP000001396	*Polysphondylium pallidum (strain ATCC 26659 / Pp 5 / PN500) (Heterostelium pallidum) (Strain: ATCC 26659 / Pp 5 / PN500)*	670386	12351	C:92.1%[S:92.1%,D:0%],F:3.6%,M:4.3%,n:303	Standard	full
UP000001926	*Entamoeba histolytica (Strain: ATCC 30459 / HM-1:IMSS)*	5759	7959	C:65.7%[S:54.1%,D:11.6%],F:6.9%,M:27.4%,n:303	Standard	full
UP000241769	*Planoprotostelium fungivorum (Strain: Jena)*	1890364	16856	C:94.4%[S:87.5%,D:6.9%],F:2%,M:3.6%,n:303	Outlier	full
UP000014680	*Entamoeba invadens IP1 (Strain: IP1)*	370355	9857	C:53.8%[S:49.5%,D:4.3%],F:4.3%,M:41.9%,n:303	Close to Standard	full
UP000076078	*Tieghemostelium lacteum (Strain: TK)*	361077	10208	C:93.4%[S:92.4%,D:1%],F:2.3%,M:4.3%,n:303	Outlier	full
UP000011083	*Acanthamoeba castellanii str. Neff (Strain: Neff)*	1257118	14939	C:81.5%[S:77.6%,D:4%],F:5.9%,M:12.5%,n:303	Close to Standard	full
UP000007797	*Cavenderia fasciculata (strain SH3) (Slime mold) (Dictyostelium fasciculatum) (Strain: SH3)*	1054147	12152	C:92.1%[S:90.4%,D:1.7%],F:2.3%,M:5.6%,n:303	Standard	full
BUSCO = Benchmarking Universal Single-Copy Orthologs; CPD = Complete Proteome Detector						

Appendix III: *Paramoeba* taxonomy search on UniProt KB downloaded on 8th of May 2020.

Organism	Total protein count	Taxon ID
Candidatus Syngnamydia salmonis	9	504270
Paramoeba aestuarina	1	180227
Paramoeba aparasomata	39	2583407
Paramoeba branchiphila	1	308475
Paramoeba eilhardi	1	200891
Paramoeba invadens	1	1321612
Paramoeba karteshi	2	2583406
Paramoeba pemaquidensis	62	180228
Paramoeba perurans	2	437603
Perkinsela sp. CCAP 1560/4	4883	1314962

Appendix IV: 2D spots with significant ($p \leq 0.05$) fold changes from the virulent-2 *Neoparamoeba perurans* culture identified by LC-MS/MS. A large collated database containing *N. perurans* microbiome, Amoebozoa and *N. perurans* proteins was used in the database search of MaxQuant. Several proteins that are shared between *N. perurans* and species found in the bacteria and Amoebozoa database are distinguished in the organism header. Genes are related to non-*N. perurans* identifications

Genes	**Spot number**	**Estimated MW kDa**	**Estimated pI**	**Fold change**	***P*-value** ≤0.05	**Fasta header**	**Protein identification**	**Organism**	**No Peptides matched**	**Main Biological function**
-	511	20	5.61	4.6	0.005	PPER_00018276-RA	ATP synthase subunit mitochondrial-like	*N. perurans*	2	Metabolism
ACA1_265860	511	20	5.61	4.6	0.005	PPER_00019391-RA; L8H4F8	Histone H2B	*N. perurans; Acanthamoeba castellanii str. Neff*	2	Protein synthesis
EHI_073470	511	20	5.61	4.6	0.005	PPER_00018720-RA; C4LXL1	ADP-ribosylation factor 4 and 1; ADP-ribosylation factor ,putative	*N. perurans; Entamoeba histolytica*	2	Cellular signalling
XU18_0391	326	40	5.55	3	0.042	PPER_00010306-RA; A0A0L1L0Q5	Heat shock protein 8; Heat shock protein 85	*N. perurans; Perkinsela sp. CCAP 1560/4*	3	Stress response
-	198	70	4.45	2.5	9.55E-05	PPER_00013769-RA	Peptidase C53 family protein	*N. perurans*	4	Immune evasion
-	198	70	4.45	2.5	9.55E-05	PPER_00000416-RA	Carbohydrate ABC transporter substrate-binding protein	*N. perurans*	3	Metabolism
ACA1_219820	599	16	6.12	4.4	1.10E-04	PPER_00017761-RA; L8GT00	Actin, cytoplasmic A3a isoform; Actin-1, putative	*N. perurans; Acanthamoeba castellanii str. Neff*	8	Cytoskeleton
-	487	21	5.95	2.1	0.003	PPER_00012939-RA	Cu-Zn Superoxide dismutase	*N. perurans*	2	Oxidative response

Genes	Spot number	Estimated MW kDa	Estimated pI	Fold change	*P*-value ≤0.05	Fasta header	Protein identification	Organism	No Peptides matched	Main Biological function
-	487	21	5.95	2.1	0.003	PPER_00019832-RA	ADF-like domain-containing protein	*N. perurans*	2	Cytoskeleton
-	487	21	5.95	2.1	0.003	PPER_00020879-RA	Lipoxygenase (lox) homology domain-containing protein 1	*N. perurans*	2	Cellular signalling
-	582	17	6.08	7.2	6.25E-04	PPER_00013951-RA	Profilin conserved site domain-containing protein	*N. perurans*	6	Cytoskeleton, immunomodulation
-	582	17	6.08	7.2	6.25E-04	PPER_00018653-RA	Profilin allergen	*N. perurans*	6	Cytoskeleton, immunomodulation
-	348	36	5.61	5.2	0.007	PPER_00013918-RA	Malate dehydrogenase	*N. perurans*	4	Metabolism
PPL_03556	334	39	6.14	6.2	0.007	D3B545	Hsc70 protein	*Polysphondylium pallidum* (strain ATCC 26659 / Pp 5 / PN500)	4	Stress response
PROFUN_09625	314	41	5.48	3.5	0.041	A0A2P6MNZ1	Tubulin beta chain	*Planoprotostelium fungivorum*	3	Cytoskeleton
A1QC_14775	314	41	5.48	3.5	0.041	A0A1E5E4F2	Glyceraldehyde-3-phosphate dehydrogenase	*Vibrio rumoiensis 1S-45*	2	Metabolism
Tuf	283	45	5.36	1.9	0.033	A0A0A7ECJ2	Elongation factor Tu	*Pseudoalteromonas piratica*	6	Cytoskeleton, immunomodulation
-	283	45	5.36	1.9	0.033	PPER_00019602-RA	Fragmin A	*N. perurans*	3	Cytoskeleton
JCM19233_3548	283	45	5.36	1.9	0.033	A0A090RBI3	Chaperone protein DnaK	*Vibrio sp. C7*	2	Multifunctional, stress response

Genes	Spot number	Estimated MW kDa	Estimated pI	Fold change	*P*-value ≤0.05	Fasta header	Protein identification	Organism	No Peptides matched	Main Biological function
-	283	45	5.36	1.9	0.033	PPER_00020852-RA; W5RWF5	Elongation factor 1; Elongation factor 1-alpha (Fragment)	*N. perurans; Paramoeba pemaquidensis*	2	Cytoskeleton
-	283	45	5.36	1.9	0.033	PPER_00013476-RA	Component of cytosolic 80S ribosome and 60S large subunit	*N. perurans*	2	Translation
-	283	45	5.36	1.9	0.033	PPER_00013717-RA	Citrate synthase active	*N. perurans*	2	Metabolism
TH44_03405	69	142	4.5	3.3	0.018	PPER_00005663-RA; A0A367XHX2	Aconitate hydratase	*N. perurans; Thalassospira xiamenensis*	2	Oxidative stress
PSHAa1352	69	142	4.5	3.3	0.018	Q3IL25	Putative TonB-dependent receptor	*Pseudoalteromonas haloplanktis* (strain TAC 125)	3	Receptor
MW kDa= molecular weight kilodalton; pI = isoelectric point										

Appendix V: MaxQuant identifications of bacterial protein from attenuated and virulent-3 exoproteomes characterised by a two-way Students t- test in Perseus. Statistically significant differential proteins are represented with a + sign based on q-value.

Gene	Protein IDs	Fasta headers	Organism	Mol. weight [kDa]	Fold change[a]	Att. Peptides[b]	Vir. Peptides[b]	T-testc
ST37_14230	A0A1X1MR06	ABC transporter substrate-binding protein	*Vibrio sp. qd031*	47.26	-3.18528	6	0	+
VS_0075	B7VH98	Periplasmic dipeptide transport protein	*Vibrio tasmaniensis (strain LGP32)*	57.321	-5.41027	22	2	+
VS_2212; BCU70_21010	B7VI06; A0A2N7D1D1	Extracellular solute-binding protein, family 7; C4-dicarboxylate ABC transporter	*Vibrio tasmaniensis (strain LGP32); Vibrio sp. 10N.286.49.C2*	36.945	-5.87885	19	8	+
VS_II0220	B7VQI9	Maltodextrin-binding protein	*Vibrio tasmaniensis (strain LGP32*	42.386	-4.90439	13	4	+
VS_0757; ushA	B7VKH8; A0A0M0HJC5	5-nucleotidase	*Vibrio tasmaniensis (strain LGP32); Vibrio nereis*	62.392	-0.414253	7	9	+
PSHAa0782	Q3IDX3	Flagellin	*Pseudoalteromonas haloplanktis (strain TAC 125)*	28.577	-0.683505	13	15	+
PP2015; BET10_08905	A0A0S2JZH9; A0A1S1MTX5	Flagellar hook protein FlgE	*Pseudoalteromonas phenolica; Pseudoalteromonas amylolytica*	47.109	-4.78254	8	6	+

Gene	Protein IDs	Fasta headers	Organism	Mol. weight [kDa]	Fold change[a]	Att. Peptides[b]	Vir. Peptides[b]	T-testc
BET10_13780; PSHAa059	A0A1S1MX56; Q3ILL3	Phosphate-binding protein ; Putative phosphate ABC transporter	*Pseudoalteromonas amylolytica; Pseudoalteromonas haloplanktis (strain TAC 125)*	35.016	-4.4918	9	0	+
VS_0355	B7VIC7	Immunogenic protein	*Vibrio tasmaniensis (strain LGP32)*	34.436	-5.20998	11	1	+
VS_1055; EA26_05975	B7VM14	Lysine-arginine-ornithine-binding periplasmic protein; Nickel transporter	*Vibrio tasmaniensis (strain LGP32); Vibrio navarrensis*	28.109	-6.24674	11	0	+
ahpCB	Q3IE21	Peroxiredoxin 2 (TSA) (PRP)	*Pseudoalteromonas haloplanktis (strain TAC 125)*	22.115	-4.75659	9	0	+
PSHAa2977; B1199_19990	Q3IK06; A0A244CKW5	Secreted alkaline phosphatase; Alkaline phosphatase	*Pseudoalteromonas haloplanktis (strain TAC 125); Pseudoalteromonas ulvae*	56.104	-4.1791	10	2	+
glnA; glnA	A0A0A7EBM5; U1KVQ6	Glutamine synthetase	*Pseudoalteromonas piratica; Pseudoalteromonas citrea DSM 8771*	51.771	-3.10513	5	0	+
PP2015_904	A0A0S2K074	Flagellin	*Pseudoalteromonas phenolica*	33.272	-1.98111	8	6	+
A6E14_15830; aapJ	A0A1B9QUY2; A0A2N8ZA18	Amino acid ABC transporter substrate-binding protein; Amino-acid transporter subunit periplasmic-binding component of ABC superfamily	*Vibrio genomosp. F10; Vibrio tapetis subsp. tapetis*	36.779	-4.40352	9	3	+

Gene	Protein IDs	Fasta headers	Organism	Mol. weight [kDa]	Fold change[a]	Att. Peptides[b]	Vir. Peptides[b]	T-testc
PSHAa1824; SAMN02745724_03050	Q3IHE8; A0A1I1NQX5	Putative ferric enterobactin receptor; Iron complex outer membrane receptor protein	*Pseudoalteromonas haloplanktis (strain TAC 125); Pseudoalteromonas denitrificans DSM 6059*	108.47	-0.134083	1	6	-
BCU70_12530; JCM19236_807	A0A2N7D8W8; A0A0B8PY06	Oligopeptide ABC transporter substrate-binding protein OppA; Oligopeptide ABC transporter	*Vibrio sp. 10N.286.49.C2; Vibrio sp. JCM 19236*	60.845	-4.28191	5	0	+
VS_0814; JCM19238_4633	B7VKN2; A0A090P9S5	Flagellin	*Vibrio tasmaniensis (strain LGP32); Vibrio ponticus*	41.306	2.93242	0	6	+
PSHAa0108	Q3IDJ5	Putative TonB-dependent receptor putative outer membrane bound protein involved in iron chelated transport	*Pseudoalteromonas haloplanktis (strain TAC 125*	77.154	-0.386178	0	6	+
PSHAa0695	Q3IFM9	Putative outer membrane receptor for ferric iron uptake putative TonB-dependent receptor	*Pseudoalteromonas haloplanktis (strain TAC 125)*	78.238	-1.66131	0	5	+
rp1L; rp1L	Q3ILQ0; U1KUY2	50S ribosomal protein L7/L12	*Pseudoalteromonas haloplanktis (strain TAC 125); Pseudoalteromonas citrea DSM 8771*	12.23	-1.66069	6	5	+
PCIT_22080	U1KK96	Peptidase S8/S53 subtilisin kexin sedolisin	*Pseudoalteromonas citrea DSM 8771*	127.37	-3.70559	6	3	+
AKJ17_10950 ;VTAP4600_B0650	A0A0M0HMY3; A0A2N8ZK26	Maltoporin	*Vibrio nereis; Vibrio tapetis subsp. tapetis*	50.515	-0.600403	0	6	-

Gene	Protein IDs	Fasta headers	Organism	Mol. weight [kDa]	Fold change[a]	Att. Peptides[b]	Vir. Peptides[b]	T-testc
VS_II0677	B7VRS9	Ferrichrome-iron receptor	*Vibrio tasmaniensis (strain LGP32)*	78.505	-0.834848	0	5	-
fliC	Q3IDX4	Flagellin	*Pseudoalteromonas haloplanktis (strain TAC 125)*	28.504	0.124568	1	3	-
VPA0179; EA26_13395	Q87JR9;A0A099LYB7	Uncharacterized protein ; dTDP-glucose 4,6-dehydratase	*Vibrio parahaemolyticus serotype O3:K6 (strain RIMD 2210633); Vibrio navarrensis*	73.415	-3.83314	3	0	+
gcvH	B7VSK2	Glycine cleavage system H protein	*Vibrio tasmaniensis (strain LGP32*	13.88	-4.08161	3	0	+

MW kDa= molecular weight kilodalton; [a] Fold change of virulent proteins related to attenuated proteins; [b] Razor and unique peptide counts; [c] Students t- test significance

Appendix VI: MaxQuant identification of bacterial proteins from exoproteomes of pen trep treated cultures based on a razor and unique peptide count ≥2.

Gene	Protein IDs	Fasta headers	Organism	Mol. weight [kDa]	Attenuated peptides[a]	Virulent peptides[a]
PSHAa1824	Q3IHE8	Putative ferric enterobactin receptor	*Pseudoalteromonas haloplanktis (strain TAC 125)*	108.47	7	6
fliC	Q3IDX4	Flagellin	*Pseudoalteromonas haloplanktis (strain TAC 125)*	28.51	6	1
PSHAb0286	Q3IBV3	Putative TonB dependent outer membrane receptor	*Pseudoalteromonas haloplanktis (strain TAC 125)*	77.69	5	4
PP2015; BET10_08905	A0A0S2JZH9; A0A1S1MTX5	Flagellar hook protein FlgE	*Pseudoalteromonas phenolica; Pseudoalteromonas amylolytica*	47.1	4	1
SAMN05428964_1011579	A0A285RMP2	Flagellin	*Thalassospira xiamenensis*	39.9	4	2
PSHAa1271	Q3IKW0	Putative TonB-dependent receptor	*Pseudoalteromonas haloplanktis (strain TAC 125)*	93.36	4	3
BET10_08945; GN=PP2015_904	A0A1S1MVN7;A0A0S2K074	Flagellin	*Pseudoalteromonas amylolytica; Pseudoalteromonas phenolica*	33.19	3	1
TH24_16540; SAMN05428964_102116	A0A367W8E6;A0A154KS33	Branched-chain amino acid ABC transporter substrate-binding protein ;Amino acid/amide ABC transporter substrate-binding protein, HAAT family	*Thalassospira xiamenensis*	46.319	2	0
CWB94_14690; PP2015_2655	A0A0F4NVP2;A0A0S2K4C3	Ligand-gated channel protein; TonB dependent outer membrane receptor	*Pseudoalteromonas piscicida; Pseudoalteromonas phenolica*	78.021	2	0
SAMN05428964_101719; TH24	A0A154KZR4;A0A367WFJ7	Cell envelope biogenesis protein OmpA	*Thalassospira xiamenensis*	32.433	2	0

Gene	Protein IDs	Fasta headers	Organism	Mol. weight [kDa]	Attenuated peptides[a]	Virulent peptides[a]
B1199_00785; CJF42_03840	A0A244CTD6;A0A269PTF6;U1JR59;A0A0A7EFT8;A0A1S1MS49	TonB-dependent receptor	*Pseudoalteromonas ulvae ; Pseudoalteromonas sp. NBT06-2*	101.37	2	0
CJF42_03785	A0A269PT88	TonB-dependent receptor	*Pseudoalteromonas sp. NBT06-2*	116.22	2	2
EJA03_10885; ompU	A0A3R9EGD4;Q87LZ1;F9RAK4;A0A090P242	Porin ; Outer membrane protein	*Vibrio pectenicida; Vibrio parahaemolyticus serotype O3:K6 (strain RIMD 2210633)*	38.053	0	2
PSHAb0113	Q3ICW8	Putative TonB-dependent receptor for Fe	*Pseudoalteromonas haloplanktis (strain TAC 125)*	90.434	2	0
PSHAa0108	Q3IDJ5	Putative TonB-dependent receptor putative outer membrane bound protein involved in iron chelated transport	*Pseudoalteromonas haloplanktis (strain TAC 125)*	77.154	2	2
PSHAa0696; OM33_06375	Q3IFM8;A0A0A7EE45	Uncharacterized protein; Porin	*Pseudoalteromonas haloplanktis (strain TAC 125); Pseudoalteromonas piratica*	42.51	2	2
PSHAa0695	Q3IFM9	Putative outer membrane receptor for ferric iron uptake putative TonB-dependent receptor	*Pseudoalteromonas haloplanktis (strain TAC 125)*	78.238	0	2
PSHAa1628; PCIT_1	Q3IGV8;U1JMT0	Putative Glutamate carboxypeptidase II; Glutamate carboxypeptidase II	*Pseudoalteromonas haloplanktis (strain TAC 125); Pseudoalteromonas citrea DSM 8771*	59.406	2	1
PSHAa1840	Q3IHF7	Putative TonB-dependent receptor	*Pseudoalteromonas haloplanktis (strain TAC 125)*	88.12	2	1
pal; pal	Q3IJ17;A0A269PMH8	Peptidoglycan-associated protein	*Pseudoalteromonas haloplanktis (strain TAC 125); Pseudoalteromonas sp. NBT06-2*	19.857	2	1

Gene	Protein IDs	Fasta headers	Organism	Mol. weight [kDa]	Attenuated peptides[a]	Virulent peptides[a]
PSHAa0183	Q3IJ31	Putative outer membrane protein signal peptide	*Pseudoalteromonas haloplanktis (strain TAC 125)*	38.832	0	2
PSHAa1187; PCIT_15765	Q3IKM2;U1J8M1	Uncharacterized protein	*Pseudoalteromonas haloplanktis (strain TAC 125);Pseudoalteromonas citrea DSM 8771*	28.883	2	1
MW kDa= molecular weight kilodalton; [a] razor and unique peptide counts						