The effect of diet and seasonal variations on the gut microbiota of Tasmanian Atlantic Salmon

(*Salmo salar* L.)

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In loving memory of my parents
Abstract

The Atlantic salmon aquaculture industry in Australia is of great importance due to the increased demand for fish and/or fish products, however outbreaks of disease, in particular gastrointestinal (GI) disruptions, can result in underweight fish and increased mortalities, leaving the industry with severe financial losses. The gut microbiota of salmon in other countries has been characterised previously revealing a dominance of Gram-negative bacteria such as *Vibrio, Pseudomonas* and *Aeromonas* species as well as Gram-positive bacteria, eg. lactic acid bacteria (LAB) important for GI health. The composition and change in the GI microbiota in Tasmanian Atlantic salmon has not been investigated, particularly in response to seasonal and diet variations. As the study of such changes often requires lengthy and expensive feed trials, the use of *in vitro* fermentation models, in particular a semi-continuous model, may offer an alternative. This thesis examined the changes in the bacterial composition of farmed Tasmanian Atlantic salmon in response to seasonal variations and different commercial diets, as well as varying fishmeal (FM) inclusion levels. This study also investigated the prevalence and persistence of some of the potential fish pathogens, the functional status, overall metabolic capacity (MC) as well as virulence genes (VGs) present in the gut microbiota of these fish. A semi-continuous fermentation model was also established as an *in vitro* tool to assess the impact of diet components on the microbiota of Atlantic salmon. Lastly this thesis identified LAB strains, isolated from healthy salmon, and characterised these against the existing selection criteria of a potential probiotic.

Changes in the cultureable faecal microbiota of salmon, fed two commercial diets (A and B), were assessed between July 2011- May 2012. The number of smaller cultureable bacterial groups, such as *Pseudomonas* spp., *Acinetobacter* spp. and *Plesiomonas shigelloides*, varied slightly between the two diets however the total number of bacteria for both diets increased as water temperature peaked at 18.5°C during summer. This however, was associated with an increase in the number of *Vibrio* spp. and a decrease in the number of LAB. A shift in the functional status of the gut microbiota was observed as temperature increased, which was coupled with a decrease in the MC-value regardless of diet. Of the 35 VGs tested only *cdt* (cytotolethal distending toxin) and *east1* (heat stable enterotoxin) were detected in the microbiota.
Identification of *Vibrio* and *Pseudomonas* spp. showed little diversity with several bacterial species, namely *V. ichthyoenteri/scophthalmi, V. crassostreae, Aliivibrio finisterrensis, Photobacterium phosphoreum* and *Pseudomonas fragi* dominating the faecal community of Atlantic salmon. Typing of these bacterial species revealed some common types (CT) of *V. ichthyoenteri* persisted for longer periods in the salmon gut, particularly during the warmer months. During the colder months of the year, i.e. temperatures below 13°C, most CT of *P. fragi* were present in the salmon gut, disappearing during the months that *Vibrio* spp. dominated the gut.

The effect of high and low fishmeal (FM) diets, with or without a prebiotic supplement, on the gut microbiota of farmed Atlantic salmon was also investigated between December 2011 and March 2012. There was little variability in regards to the number and types of cultureable bacteria between the diets over the sampling period tested; however, the high FM diet with the supplement was the only diet capable of sustaining LAB populations over the higher water temperatures. A shift in the functional status of the gut microbiota was observed in all diets as temperature increased, which was coupled with a decrease in the MC-value of the microbiota receiving for the low FM diets and an increase for the high FM diets. Of the 35 VGs tested, *cdt* was present in all samples whilst *east1* was only detected in microbiota samples from the high FM diet only.

Characterisation of 160 LAB, isolated from salmon over one year of sampling, identified four strains that show potential as a candidate probiotic. These strains displayed no cytotoxicity, high adherence to Atlantic salmon kidney cells (ASK), low antibiotic resistance, bacteriocin production as well as the ability to exclude up to 16 pathogens *in vitro*.

A semi-continuous fermentation model was developed to assess whether shifts in the gut microbiota occurred in response to diet and temperature. This model demonstrated a shift in the microbiota, their functional status and MC-value similar to what was experienced by the fish *in vivo* in response to diet and temperature.

It has been concluded that temperature, and to a lesser extent diet, were associated with shifts in the gut microbial populations of farmed Tasmanian salmon. It was also concluded that measurement of the functional status of the gut microbiota in combination with their MC-value, as presented in this thesis, could be used as a
potential tool to assess the impact of both temperature and diet on the function of gut microbiota especially. This can be of use in the testing of new diets and diet components. This study also identified some LAB that can be used as potential probiotic candidates, although their use in the salmon industry to improve the health of the gut microbiota and through that also the health of the fish, has yet to be evaluated \textit{in vivo}.
Declaration of Originality

This thesis has not been previously submitted for a degree or diploma at this or any other university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

Christina Neuman

April 2014
Publications related to this thesis


Other publications


Peer reviewed conference proceedings


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I would like to thank my sister Jennifer for her friendship and all the support and laughs you have provided over the years. I love you and couldn’t imagine my life without you.

Finally I would like to thank my father who taught me that with dedication and hard work anything is possible if you believe in yourself and my mother who was always a role model to aspire to be.
## Table of Contents

Abstract ........................................................................................................................... i
Publications related to this thesis ................................................................................ v
Other publications ......................................................................................................... vi
Peer reviewed conference proceedings ..................................................................... vii
Acknowledgements ...................................................................................................... ix
Table of Contents .......................................................................................................... x
List of Figures ................................................................................................................ xiv
List of Figures ................................................................................................................ xv
List of Abbreviations ..................................................................................................... xvii

### CHAPTER 1 ..................................................................................................................... 1

#### 1.0 General introduction and literature review ....................................................... 1
   1.1 Overview of this thesis .......................................................................................... 1
   1.2 Aquaculture worldwide and in Australia ............................................................. 2
   1.3 Atlantic salmon and Rainbow trout ..................................................................... 3
      1.3.1 Atlantic salmon life cycle ........................................................................... 3
      1.3.2 GI tract of salmon ...................................................................................... 4
      1.3.3 GI microbiota of Atlantic salmon ............................................................... 4
   1.4 Diet ....................................................................................................................... 6
      1.4.1 Effect of diet on Atlantic salmon health ..................................................... 6
      1.4.2 Effect of diet on the GI microbiota ............................................................. 7
   1.5 Modelling of the GI tract using fermenters ......................................................... 7
   1.6 Disease of fish ..................................................................................................... 8
      1.6.1 Environmental factors ................................................................................ 8
      1.6.2 Diseases affecting Atlantic salmon ............................................................. 9
         1.6.2.1 Pathogens associated with the salmon diseases .................................... 11
         1.6.2.2 Aeromonas .......................................................................................... 11
         1.6.2.3 Acinetobacter ...................................................................................... 11
         1.6.2.4 Plesiomonas ....................................................................................... 12
         1.6.2.5 Pseudomonas ..................................................................................... 13
         1.6.2.6 Vibrio .................................................................................................. 13
      1.6.3 Virulence factors associated with disease causing bacteria ....................... 14
      1.6.4 Diseases prevention and treatment .............................................................. 15
         1.6.4.1 Antibiotic treatment ............................................................................ 15
         1.6.4.2 Effects of antibiotics ............................................................................ 16


CHAPTER 4................................................................................................................................. 86

4.0 The effect of fish meal composition on the faecal microbiota of farmed Tasmanian Atlantic Salmon (Salmo salar L.) ................................................................. 86
  4.1 Introduction................................................................................................................................. 87
  4.2 Aims of the study.......................................................................................................................... 87
  4.3 Methods..................................................................................................................................... 88
    4.3.1 Sampling................................................................................................................................. 88
    4.3.2 Bacterial enumeration, functional status and VG profiles................................................. 89
    4.3.3 Statistics methods ................................................................................................................ 89
  4.4 Results...................................................................................................................................... 90
    4.4.1 Bacterial enumeration.......................................................................................................... 90
    4.4.2 Functional status and metabolic capacity ........................................................................... 91
  4.5 Discussion ................................................................................................................................. 93

CHAPTER 5..................................................................................................................................... 101

5.0 Characterisation of lactic acid bacteria isolated from the hindgut of farmed Tasmanian Atlantic salmon (Salmo salar L.) ........................................................... 101
  5.2 Aims of this study....................................................................................................................... 102
  5.3 Methods................................................................................................................................... 103
    5.3.1 Sampling and LAB isolation .............................................................................................. 103
    5.3.2 Biochemical fingerprinting of LAB ................................................................................... 103
    5.3.3 Identification of LAB ......................................................................................................... 103
    5.3.4 Cytotoxicity of LAB strains ............................................................................................... 104
    5.3.5 Adhesion capability ............................................................................................................ 105
    5.3.6 Antibiotic resistance ........................................................................................................... 105
    5.3.7 Bacteriocin production by the LAB isolates...................................................................... 106
    5.3.8 Survival through the simulated GI tract ............................................................................ 107
    5.3.9 Competitive adhesion capability ....................................................................................... 107
  5.4 Results..................................................................................................................................... 107
    5.4.1 Identification of Lactic acid bacteria (LAB) from Atlantic salmon ................................... 107
    5.4.2 Cytotoxicity assay of LAB strains ...................................................................................... 108
    5.4.3 Adhesion capability of LAB to Atlantic salmon cells (ASK) ........................................... 108
    5.4.4 Antibiotic resistance of LAB strains ................................................................................ 110
    5.4.5 Bacteriocin production by the LAB isolates ..................................................................... 112
    5.4.6 Survival through simulated environment of the stomach ................................................. 114
    5.4.7 Competitive adhesion assay of the LAB bacteria ............................................................. 114
  5.5 Discussion ................................................................................................................................. 116
  5.6 References................................................................................................................................. 119
CHAPTER 6

6.0 Assessing the impact of fish feeds on the gut microbiota of Tasmanian Atlantic salmon (Salmo salar L.) using a semi-continuous culture

6.1 Introduction

6.2 Aims of the study

6.3 Methods

6.3.1 Fermenter media and gut microbiota inocula

6.3.2 Feeding regime and sampling

6.3.3 Bacterial enumeration

6.3.4 Functional status and metabolic capacity of the faecal biota

6.4 Results

6.4.1 Bacteriological analysis

6.4.2 Substrate utilisation and MC value

6.5 Discussion

6.6 References

CHAPTER 7

7.0 General Discussion and future directions
List of Figures

**Figure 1.1**: The life cycle of the Atlantic salmon (*Salmo salar* L.)

**Figure 2.1**: The MC values of the gut microbiota of Tasmanian Atlantic salmon fed two different diets over 10 months sampling between July 2011 and May 2012.

**Figure 2.2**: Similarity between the patterns of substrate utilisation by the gut microbiota of Tasmanian Atlantic salmon fed two different diets over 10 months sampling between July 2011 and May 2012.

**Figure 3.1**: UPGMA clustering of *Vibrio* (a) and *Pseudomonas* (b) strains showing genetic relatedness of the strains.

**Figure 4.1**: Similarity between the patterns of substrate utilisation by the gut microbiota of Tasmanian Atlantic salmon fed one of four trial diets.

**Figure 4.2**: The MC value of the gut microbiota of salmon fed one of four trial diets, over 4 months of sampling from 2011 to 2012.

**Figure 6.1**: Number of bacterial species present in the faecal samples of Atlantic salmon and per mL of the fermenter culture as well as their functional status measured over 12 days using two commercial feeds.

**Figure 6.2**: Changes in the metabolic capacity value of the gut microbiota of Tasmanian Atlantic salmon fed feed A or B over 12 days of fermentation measured using PhPlate-48 to determine their degree of substrate utilisation.
List of Tables

Table 1.1: Diseases of major significance affecting wild and farmed Atlantic salmon worldwide.

Table 2.1: Composition of two commercially available diets (i.e. A and B) fed to Atlantic salmon during the various stages of their farming cycle.

Table 2.2: List of primers used for identification of bacteria to the genus or species level.

Table 2.3: Substrates used in PhPlate-48 plates for testing functional status of the hindgut microbiota of salmon and for measuring their metabolic capacity value.

Table 2.4: List of 35 virulence genes tested and their function.

Table 2.5: Number of bacteria (CFU/g of faecal material) detected in the hindgut of Atlantic salmon fed different commercial diets over 10 months of sampling.

Table 3.1: List of Vibrio and Pseudomonas common and singles types isolated from farmed Tasmanian Atlantic salmon fed two commercial diets between July 2011 and May 2012.

Table 3.2: Prevalence and persistence of Vibrio common and single types isolated from farmed Tasmanian Atlantic salmon fed two commercial diets between July 2011 and May 2012.

Table 3.3: Prevalence and persistence of Pseudomonas common and single types isolated from farmed Tasmanian Atlantic salmon fed two commercial diets between July 2011 and May 2012.
Table 4.1: Composition of the commercially available diet and subsequent trial diets (i.e. Diet 1 - Diet 4) fed to Atlantic salmon during the feed trial conducted from December 2011- March 2013.

Table 4.2: Number of bacteria (CFU/g of faecal material) detected in the hindgut of Atlantic salmon fed one of four trial diets.

Table 5.1: Number of strains belonging to different species and common types (CT) showing cytotoxic effect on Atlantic salmon kidney (ASK) cells.

Table 5.2: The antibiotic resistance patterns of lactic acid bacterial strains belonging to ten different common types (CTs).

Table 5.3: Percentage of strains from each CT showing bacteriocin activity against 10 selected ATCC strains.

Table 5.4: Adhesion capability of 16 selected pathogenic strains to ASK cells alone and in the presence of four LAB strains.

Table 6.1: Composition (expressed as percentage) of the fish feeds A (summer feed) and B (winter feed) used in this study. Digestible energy (DE) is provided.

Table 6.2: Degree of substrate utilisation by the gut microbiota subjected to two different commercial feed over the course of 12 days of fermentation.
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AA</td>
<td>Aeromonas agar</td>
</tr>
<tr>
<td>AGD</td>
<td>Amoebic gill disease</td>
</tr>
<tr>
<td>ASK</td>
<td>Atlantic salmon kidney</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>BPT</td>
<td>Biochemical phenotype</td>
</tr>
<tr>
<td>cdt</td>
<td>Cytolethal distending toxin</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>CLSI</td>
<td>Clinical laboratory standards institute</td>
</tr>
<tr>
<td>CT</td>
<td>Common type</td>
</tr>
<tr>
<td>DE</td>
<td>Digestible energy</td>
</tr>
<tr>
<td>DO</td>
<td>Dissolved Oxygen</td>
</tr>
<tr>
<td>DP</td>
<td>Digestible protein</td>
</tr>
<tr>
<td>east1</td>
<td>Heat stable enterotoxin</td>
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<tr>
<td>ERM</td>
<td>Enteric red mouth</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
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<tr>
<td>FM</td>
<td>Fishmeal</td>
</tr>
<tr>
<td>GI</td>
<td>Gastro intestinal</td>
</tr>
<tr>
<td>h</td>
<td>Hours</td>
</tr>
<tr>
<td>HA</td>
<td>Herellea agar</td>
</tr>
<tr>
<td>IBBS</td>
<td>Inositol-brilliant-green-bile-salts</td>
</tr>
<tr>
<td>L-15</td>
<td>Leibovitz-15</td>
</tr>
<tr>
<td>LAB</td>
<td>Lactic acid bacteria</td>
</tr>
<tr>
<td>MA</td>
<td>Marine agar</td>
</tr>
<tr>
<td>MAS</td>
<td>Motile Aeromonas septicaemia</td>
</tr>
<tr>
<td>MC</td>
<td>Metabolic capacity</td>
</tr>
<tr>
<td>min</td>
<td>Minutes</td>
</tr>
<tr>
<td>MRS</td>
<td>de Man Rogosa Sharpe</td>
</tr>
<tr>
<td>No.</td>
<td>Number</td>
</tr>
<tr>
<td>PA</td>
<td>Pseudomonas agar</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PKD</td>
<td>Proliferative kidney disease</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SBM</td>
<td>Soy bean meal</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SGS</td>
<td>Summer gut syndrome</td>
</tr>
<tr>
<td>ST</td>
<td>Single type</td>
</tr>
<tr>
<td>sp.</td>
<td>Species (singular)</td>
</tr>
<tr>
<td>spp.</td>
<td>Species (plural)</td>
</tr>
<tr>
<td>TCBS</td>
<td>Thiosulfate-citrate-bile salts-sucrose</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>TSA</td>
<td>Tryptone soya agar</td>
</tr>
<tr>
<td>TSB</td>
<td>Tryptone soya broth</td>
</tr>
<tr>
<td>UPGMA</td>
<td>Unweighted pair group method with arithmetic averages</td>
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<tr>
<td>UTI</td>
<td>Urinary tract infection</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
</tr>
<tr>
<td>VGs</td>
<td>Virulence genes</td>
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CHAPTER 1
1.0 General introduction and literature review

1.1 Overview of this thesis

Aquaculture has seen a dramatic increase within the last few decades due to the decline in global fish stocks and a simultaneous increased demand for fish products (1, 2). Since the start of the Atlantic salmon aquaculture in Australia, this industry continues to grow each year (3), as fish are cultivated towards their upper thermal limit. This increased stress experienced by the fish often results in revenue loss due to underweight fish or stock losses due to disease outbreaks (4). The gut microbiota of fish has only been studied in depth within the last decades, however geographical location affects its bacterial composition. Due to infancy of Australia’s aquaculture system, the gut microbiota of Atlantic salmon has not been characterised and the effect of temperature variations on this microbiota has not been explored. Causative agents of common Atlantic salmon diseases are of viral (5), amoebic (6) or more commonly of bacterial origin (7, 8, 9). The use of antifoulants (10) and antibiotics (11) is often necessary to ensure the health of the fish during disease outbreaks and a wide range of antibiotic classes are used worldwide in the aquaculture industry (12). As the industry is trying to find more sustainable practices, the use of prophylactic treatments such as vaccination and probiotics offer a suitable alternative (13). Probiotics are of particular interest as the use of antibiotics for gastrointestinal (GI) diseases further disrupts the microbiota, potentially exacerbating the condition (14). Probiotic supplementation can improve GI health of the fish and competitively exclude pathogens before disease occurs, however, extensive testing is necessary to ensure their safety in the target organism (15). The use of in vitro modeling using fermentation systems is an effective way to test the effect of diet components (16) or pre- and probiotic supplementation (17) on the gut microbiota of mammals and fish.

The following literature review will provide an overview of the GI tract microbiota of Salmonids and how diet can affect the composition. A particular emphasis is placed on some of the common Gram-negative bacterial species present in the microbiota of Salmonids and the diseases they can cause in the fish. Focus is also made to the
treatment of GI diseases and prophylactic use of lactic acid bacteria (LAB) as probiotics. Effective *in vitro* modeling systems will also be discussed.

### 1.2 Aquaculture worldwide and in Australia

Aquaculture began as early as 475 B.C. in China (18), however it was established as an industry, particularly involving marine fish, prawns and bivalves, within the last 20 years, increasing fourfold worldwide since (1). This increase has resulted mainly as a response to both ocean fishery depletion and increased market demand (1, 2). Farming of Atlantic salmon (*Salmo salar* L.) accounts for roughly 4.5% of the total global seafood production (19). Atlantic salmon aquaculture first began in the 19th century in the UK as a method of restocking fish into waterways for recreational fishers (19), however Norway in the mid-1960s was the first country to employ the use of sea cages as a method of farming fish to market size (20). Due to biological, natural and seawater temperature restraints the only countries employing Atlantic salmon aquaculture are Norway, Chile, UK, North America, Australia (Tasmanian coast only) and to a lesser extent New Zealand, France and Spain (19).

Salmonid aquaculture, specifically Brook Trout (*Salvelinus fontinalis*), developed in Victoria, Australia during the 1970-80’s as a method to continue restocking waterways as well as farming for human consumption (21). Rainbow Trout (*Oncorhynchus mykiss*) were introduced to Tasmania, Australia in 1981 and the industry expanded further to include Atlantic salmon in 1984 with egg stocks exported from Nova Scotia, Canada (19). As of 2012, 98% of Australia’s total salmonid production occurred in Tasmania (22). The Atlantic salmon industry accounts for 90% of fish production in Tasmania and according to the Australian Fisheries Statistics, during the period of 2011-2012; it also accounted for 52% of Australia’s total fish production and grossed a value of more than $348 million in revenue (23, 24). In the period between 2001-2012, the volume of farmed salmon rose to 27,769 tonnes showing a 171% increase in production (22). Salmonid production has experienced a 211% profit increase since the year 2002 making it to date the most profitable aquaculture product in Australia and predictions are that this figure will increase further (22). Tasmania remains one of the few locations in Australia where the use of Aquaculture is employed via the use of sea cages. The Tasmanian climate makes an ideal living and breeding ground for Atlantic salmon, which prefer an optimal water temperature of between 8 and 14°C but are able to
tolerate temperatures varying between 0 to 30°C (25, 26). The relative isolation of this location from natural and other cultured Atlantic salmon stocks provides some protection from the major infectious disease problems encountered by the industry (19), which are discussed in more detail below.

1.3 Atlantic salmon and Rainbow trout

Atlantic salmon (*Salmo salar* L.) are members of the *Salmonidae* family whose relatives include Rainbow trout, Brook trout, Brown trout and Chinook salmon (25). The life cycle and GI tract of salmonids, including the microbial composition of the biota will be discussed below.

1.3.1 Atlantic salmon life cycle

There are seven stages within the life cycle of Atlantic salmon namely alevin, fry, parr, smolt, post-smolt, adult salmon and kelt (Figure 1.1) (20). Wild fish spawn in the same fresh water in which they were spawned (20). After hatching the first three stages of the fishes life are spent only in fresh water, for which development lasts between two to five years (20, 27). After this time the young fish, smolts at this stage, migrate to sea and spend one or more winters in the ocean maturing into adult salmon before returning to the lakes and rivers as kelt to spawn (20). For this reason the last four life stages are found both within fresh and salt water (20).

In contrast, in captivity, fish require only 8-16 months until they reach the fourth stage in their life cycle (i.e. smolt) and are subsequently transferred into sea cages where fish continue to grow for up to two years until harvest size is reached (19).

In addition to the already shortened life cycle length in Tasmanian waters, Atlantic salmon are cultivated towards their upper thermal limits (23). Growth rates of the fish are therefore extremely high resulting in production cycles of roughly 30 months rather than the usual 36 months common in most other salmon producing countries (23).
Figure 1.1: The life cycle of the Atlantic salmon (Salmo salar L.). (Image taken from http://www.asf.ca/life-cycle.html).

1.3.2 GI tract of salmon
The GI system of salmonids, such as Atlantic salmon and Rainbow trout, is quite different to that of humans in that it is composed of a U-shaped stomach, which is followed by the proximal intestine (28, 30). The pyloric caeca protrude from the anterior region of the proximal intestine (30) and functions as a secretory organ to aid in digestion as well as increase the surface area in the intestine for increased absorption (31). The GI tract ends in the distal intestine, also commonly referred to as the ‘hindgut’ (28). The intestines of fish act as a tool for the digestion, absorption and metabolism of food, however, they are also one of the main routes of entry for bacteria including those capable of causing infection (32, 33).

1.3.3 GI microbiota of Atlantic salmon
The varied GI structures between animal genera indicate the diverse nutritional requirements as well as the dissimilar bacterial microbiota, differing in their location within the GI tract (28). Bacterial populations within the GI tract of Atlantic salmon are spread fairly evenly throughout the entire length and exhibit a lower density, compared
to homoeothermic animals, where bacterial populations are dense and concentrated to specific areas (28). A possible explanation may lie in the low body temperature and the low carbohydrate, highly digestible diet, which results in a lower need for bacterial fermentation as well as the production of lactic and acetic acids (28). The gut microbiota in warm-blooded animals has been well studied. This is not the case for fish and up until 20 years ago the idea of an established microbiota had been disputed (29, 34). Since this time, studies have focused on the intestinal microbiota of numerous fresh and salt water species (35 - 42) of which a limited number have focused on the larval or juvenile stages (43 - 51).

In fish, the microbiota have been shown to contribute to the proliferation of the gut epithelium (52), nutrient breakdown (53, 54), physiological development (55) and immune responses (56) (57). Despite these studies, the microbiota of fish has not been studied as extensively as that of humans and other mammals however as the aquaculture industry continues to grow, there is a need for further research to improve fish GI health (58 - 61).

There is still little known about the initial colonisation of the fish gut by bacteria, however it is believed that the microbiota originates from the surrounding water after hatching and develop into an initial transient microbiota which evolve into a stable gut community usually after the juvenile stages, depending on the fish species (62). It has been suggested that the composition of the stable gut microbiota is formed as a result of initial colonisation, gut structure, environmental conditions (i.e. water temperature) (63) and are affected by the age of the fish, as well as dietary changes (64) and nutritional status (58, 61, 65 - 67).

In Atlantic salmon fry, LAB constitute the major component of the intestinal microbiota compared to adult fish where LAB are only present in small numbers (68) while the gut of juvenile Atlantic salmon is dominated by *Pseudomonas* spp. (69). Whether this is indeed the case due to the transient biota seen in young fish, or if these bacteria are favoured by the geographical location (70) remains to be elucidated. The gut microbiota of adult Atlantic salmon has been well studied.

In adult Atlantic salmon, the use of culture-based identification has found two phyla, which predominate within the gut, *Gammaproteobacteria* and *Firmicutes* (58). Bacterial members from families belonging to the phyla *Gammaproteobacteria* include *Moraxellaceae* (Acinetobacter spp., Psychrobacter spp.), *Enterobacteriaceae* (*Plesiomonas shigelloides*, *Enterobacter* spp.), *Pseudomonadaceae* (*Pseudomonas*
(58). The Firmicutes phylum comprises mainly Gram-positive bacterial families such as Bacillus and relatives, Carnobacterium, Clostridium, Staphylococcus and Streptococcus spp. (58, 71).

Since the introduction of molecular methods, other species such as Lactococcus spp., Weissella spp., Shewanella spp., Citrobacter spp., and Mycoplasma spp. have also been identified (28, 63, 72 - 74). These advances in identification and detection techniques have made it possible to conclude that amongst healthy farmed Atlantic salmon reared in Norwegian waters, LAB, namely Lactobacillus and Lactococcus, as well as Photobacterium phosphoreum, Acinetobacter, Pseudomonas spp. and Vibrio spp. are the dominant groups of bacteria (63).

Although the bacterial species present in the gut have been identified, these studies have focused on samples collected on only a single or relatively few occasions and to date only one study has addressed the impact of seasonal temperature on gut microbiota of sea-reared Atlantic salmon (63). The composition of the microbiota, as well as the pathogens encountered, appears to be location specific (75, 76). Among the bacterial species mentioned above, a few have been commonly associated with diseases of fish as well as humans. These bacteria will be briefly discussed later in this literature review. Greater knowledge of the composition and role of the intestinal microbiota in marine species can help improve diets and husbandry to ensure the rearing of healthy fish in aquaculture systems.

1.4 Diet

1.4.1 Effect of diet on Atlantic salmon health

The continual growth of the aquaculture industry has caused issues regarding the cost and sustainability of fishmeal (FM) and other raw materials for use in fish feeds. Over the past several years, there has been a push to find suitable protein and oil alternatives for carnivorous fish to avoid the use of fish products in the feed (77). Inclusion of plant-based proteins over fishmeal does not seem to affect the overall health of fish where no increased mortalities have been recorded, however the growth rate of fish and their GI health is negatively impacted (78). Studies focused on the growth rate and histological effects of diet components on the fish, have shown that diets containing de-shelled krill-meal can cause nephrosis (79) and the inclusion of pea-protein leads to enteropathy.
Soybean meal (SBM) is now widely substituted as the major protein source however soy-based diets cause changes in the gut structure and function of Atlantic salmon leading to enteritis, the severity of which depends on the source (81) and inclusion level of the SBM (82). Research suggests the addition of raw materials such as mannan oligosaccharide may overcome these associated problems by acting in a protective manner on the gut epithelium (83). More research is necessary to ensure the correct balance of suitable alternatives to ensure adequate nutrition and health for aquaculture species.

1.4.2 Effect of diet on the GI microbiota
Diet components not only affect the overall health and growth of the fish, but are also an important factor for the development and/or shift in the gut microbiota (64, 84, 85). Changes in the number of *Vibrio* spp. in response to diet changes such as variations in carbohydrate levels have been observed in European Sea bass (86) and shifts in the microbiota are seen in Artic charr (84). Limited studies have assessed the effect of diet on the gut microbiota of salmonids however it has been found that the use of these alternate dietary protein sources greatly influence the smaller subsets of bacteria in the GI tract of Atlantic salmon (87), a result which was not observed in Rainbow trout (88). The abundance of LAB within the GI tract of Rainbow trout is however greatly affected by diet with an increase in these species observed in fish fed plant-based food (89, 90). With limited data, further research is needed to elucidate the impact that diet has on the microbiota of fish.

1.5 Modelling of the GI tract using fermenters
Often there are logistical, financial or ethical constraints which make it difficult to use live models for GI experiments. *In vitro* models of the GI tract using a fermenter offer a suitable alternative and have successfully been used to simulate the gut microbiota of humans to assess the effect of dietary components (16, 91), probiotics (92), or the synergistic effect of both pre- and probiotics (17). There are several different established fermentation models to study the gut microbiota, however, the use of either a semi-continuous or a continuous culture is shown to better simulate the GI system (93).

As with any *in vitro* model there are some limitations to the system and one is that the fermenter model itself can lead to the rapid loss of less competitive bacteria due to the
presence of free cells in the microbiota rather than their natural protected state within the faecal material in the gut (94 - 97). Another is that often to ensure enough inocula are present, and to eliminate differences between the microbiota of individuals, pooled faecal samples are used. This however may lead to inter-microbiota interactions favoring the growth of certain bacterial species and therefore leading to an unrepresentative sample (98).

Studies have not only focused on humans but have also included other mammals such as rabbits (17) and cows (99), however, no studies to date have used a fermenter model to investigated the impact of diet or diet components on the gut microbiota of fish.

1.6 Infections associated with fish and their consequences for fish and humans

1.6.1 Environmental factors
Seawater functions as a medium for both the growth and transportation of bacteria. To date over a hundred fish and shellfish pathogens have been reported, however the majority of fish pathogens are opportunists or facultative pathogens present as part of the microbiota or in the surrounding water (4, 100, 101, 102). Infections occur mainly due to immune suppression within the host or in response to environmental conditions, such as changes in temperature, oxygen saturation or pollution (4, 101, 102) which stress the fish, further weakening their defenses and allowing colonisation and invasion by bacteria (101, 102). Changes to environmental factors such as temperature, oxygen saturation, osmotic strength and iron availability have been shown to allow the expression of virulence capabilities in certain bacteria (103) but can also lead to bio-fouling. Bio-fouling, as well as increased handling practices, can stress the fish, which results in decreased function of the immune system and contributes to greater disease susceptibility (10, 104, 105). Among the causes of disease outbreaks, temperature has one of the greatest effects, where the summer months reflect the higher rates of infection, disease development and subsequent mortalities (23). Whilst some bacteria cannot tolerate higher water temperatures, others flourish and establish dominance during this time (23). A reduction in rainfall, which often coincides with increased temperature and a decreased water flow rate, in turn decreases the availability of fresh water within the cages, exacerbating disease
development, duration and severity (23). Although stock density itself is not the cause of disease, its contribution to fish stress as mentioned above, can exacerbate disease.

1.6.2 Diseases affecting Atlantic salmon

Environmental locations, as well as environmental factors, have a major impact on the types of diseases manifesting in Atlantic salmon. There are many diseases that can affect farmed Atlantic salmon some of which occur worldwide such as amoebic gill disease, furunculosis, vibriosis and enteric redmouth disease whilst others are present only in specific geographical regions (Table 1.1).

An example of this is the disease “cold-water vibriosis”, which much like vibriosis in fish presents as haemorrhagic septicaemia, however the main difference between these diseases is that cold-water vibriosis is specifically caused by V. salmonicida, and only occurs in the North Atlantic region (106). Cold-water vibriosis can result from environmental stress as well as poor nutrition (106).

Another more recent disease, which has been identified in Tasmania, Australia, is Summer Gut Syndrome (SGS). As the water temperature increases over summer, the farmed Atlantic salmon have been shown to exhibit loss of appetite and the production of diarrhoeal like faeces (4, 107). These symptoms collectively have been named “irritable gut syndrome”, or as it is more commonly known, “SGS” and were only observed in fish receiving a diet high in non-fish sourced protein. Due to the nature of the condition it results in underweight fish and often can cause 100% mortality, resulting in significant economic losses (4). The causative agent is to date still unknown, however SGS, does not present in fish fed FM diets (107). Treatment of fish afflicted with SGS with oxytetracycline or tylosin has shown temporary improvement suggesting one or more bacterial agents may be involved in the aetiology (4).
# Table 1.1: Diseases of major significance affecting wild and farmed Atlantic salmon worldwide

<table>
<thead>
<tr>
<th>Disease</th>
<th>Causative agent</th>
<th>Symptoms</th>
<th>Effected areas; conditions</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferative kidney disease</td>
<td><em>Tetracapsuloides bryosalmonae</em></td>
<td>Abdominal swelling, equilibrium loss, respiratory distress</td>
<td>Western Europe &amp; North America water; temperatures above 15°C</td>
<td>108-110</td>
</tr>
<tr>
<td>(PKD)</td>
<td>(Myxozoa)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amoebic gill disease (AGD)</td>
<td><em>Neoparamoeba perurans</em></td>
<td>Gill epithelium proliferation, inhibited oxygen diffusion, mortality</td>
<td>Ireland, Japan, Norway, US, Australia; salinity increased water temperatures</td>
<td>20, 111</td>
</tr>
<tr>
<td>Furunculosis</td>
<td><em>Aeromonas salmonicida</em></td>
<td>Ulcers and systemic infection</td>
<td>Worldwide</td>
<td>112, 113</td>
</tr>
</tbody>
</table>
| Motile *Aeromonas hydrophila* |                                   | Reddening of the skin, fluid accumulation in the scale pockets, exophthalmia, 100% fatal septicaemia | Higher prevalence during cage overcrowding and increased water temperatures of 24°C and above | 7, 114-
| *Aeromonas septicaemia*       |                                   |                                               |                                           | 116   |
| (MAS)                         | *Vibrio spp. but mainly* V.      | Haemorrhagic septicaemia, 100% mortality       | Worldwide; increased water temperature, stress | 8, 117-
|                               | *salmonicida*, V. *anguillarum*, V. *ordalii*, and V. *harveyi* |                                               |                                           | 119   |
| Enteric redmouth disease (ERM)| *Yersinia ruckeri*               | Reddening of the mouth and throat due to subcutaneous haemorrhaging | Worldwide                                  | 120, 121 |
| Winter ulcers                 | *Moritella viscosa*              | Skin ulcers                                   | Norway, Iceland, Scotland; Temperatures below 10°C | 122   |
1.6.2.1 Pathogens associated with the salmon diseases
The gut microbiota is important to ensure the health of fish however some bacterial species in the gut of Atlantic salmon have the potential to cause disease in both humans and fish. The importance of these will be discussed below. Although LAB are also an important component of the microbiota of salmon, and a particular focus has been placed on these bacteria in this thesis and they will be described in depth later in this literature review.

1.6.2.2 Aeromonas
The genus *Aeromonas* belongs to the family *Aeromonadaceae*, and currently consists of 28 validated species, and several more proposed species, classified based on their DNA hybridisation profiles (123-125). Widespread throughout the environment these Gram-negative rods are found in places such as water and soil, and are ubiquitous to aquatic environments and are pathogens of both cold and warm blooded animals alike (114, 126, 127).

As human pathogens, *Aeromonas hydrophila*, *A. caviae*, *A. veronii* biovar sobria and *A. veronii* biovar veronii are the most recognised of the *Aeromonas* species (128, 129) however *A. jandaei*, *A. schubertti* and *A. trota* have additionally been classified as human pathogens (7, 130). *Aeromonas* species not only pose a threat to human health but also cause serious disease in many wild and farmed fish species and other amphibians (131-133) affecting species such as salmon, bream, eel, goldfish and carp (134-137). Infections with *A. sobria*, *A. hydrophila*, *A. caviae*, *A. jandaei* and *A. salmonicida* are some of the most common bacterial infections in farmed fish, including Atlantic salmon, and can cause motile *Aeromonas* septicaemia (MAS), furunculosis and tail/fur rot. The mortality rate caused by MAS is roughly 10 % but can reach up to 100 % (136, 138). *Aeromonas* species can also cause local inflammation and necrosis of the skin, soft tissues and muscles in salmonids (138).

1.6.2.3 Acinetobacter
The family *Neisseriaceae*, genus *Acinetobacter* has undergone significant taxonomic revision since 1986 and to date consists of a total of 32 genomospecies, 17 with validated names and 10 of which have been isolated from clinical specimens (139-141). The remaining seven named species (*Acinetobacter baylyi*, *A. bouvetii*, *A. gerneri*,
A. grimmontii, A. tandoii, A. tjernbergiae, and A. towneri) have been isolated within the past several years from activated sludge sewage treatment plants (141). Bacteria of the Acinetobacter genus are Gram-negative coccobacilli, which are ubiquitous to dry environments, such as inanimate objects, soil and are often isolated from aquatic environments, including treated drinking water and sewage (140, 141). Acinetobacter are also commonly isolated from hospital environments where they colonise the skin of both patients and staff (142). Acinetobacter, namely the species A. johnsonii, A. iwoffii and A. radioresistens, are also a natural inhabitant of the human skin microbiota and occasionally can be found as commensal bacteria within the respiratory tract and vagina of healthy individuals (140, 143). A. baumannii is most commonly associated with nosocomial infections in humans causing bacteremia, urinary tract infections (UTIs), secondary meningitis and pneumonia (140, 143) whilst A. iwoffii and A. ursingii have been isolated as the cause of meningitis and bacteraemia (143). A. shindleri is another species which has been recovered from the cervix, nose, ear, conjunctiva and urine of humans (143).

Bacteria from the genus Acinetobacter show universal resistance to penicillin, ampicillin, cephalothin and also commonly to chloramphenicol. Although bacteria from this genus do not seem to cause disease in aquatic animals, due to their ability to easily acquire multiple antimicrobial resistances, Acinetobacter are often used as antimicrobial resistance indicators within aquatic environments (144-146).

1.6.2.4 Plesiomonas

The family Pseudomonadaceae, genus Plesiomonas currently only contains one known species namely P. shigelloides, however the species has many subtypes and serovars (147). P. shigelloides is a Gram-negative, straight rounded short bacillus, which has been reported as the cause of gastroenteritis in humans, presenting usually as mild watery diarrhoea. This bacterium is found in a range of natural environments, such as water and soil, however, is more commonly associated with aquatic environments and therefore commonly inhabits cold-blooded animals (148).

There does not seem to be any indication that this bacterium causes disease in aquatic animals, as P. shigelloides is typically isolated from healthy fish and seafood products (149). Although not associated with disease in fish, contact with environmental sources or the ingestion of contaminated or unwashed food are the main routes of infection in humans (148, 149). P. shigelloides cause GI diseases in humans which usually present
as mild-watery diarrhoea however rare case reports describe extra-intestinal infections including septicaemia, cellulitis, septic arthritis and acute cholecystitis (149). Infections with *P. shigelloides* occur more frequently in the tropics or during the warmer summer months (149).

1.6.2.5 *Pseudomonas*

The family *Enterobacteriaceae*, genus *Pseudomonas* was described for the first time in 1894 and is one of the most diverse genera consisting of 192 species (150). Bacteria from this genus are Gram-negative rods (143) that are ubiquitous to a variety of habitats including soil, but are commonly associated with aquatic sources including fresh water, marine environments and plant roots worldwide (150).

In humans, *P. aeruginosa* is the species most often isolated as a clinical specimen from a range of infection such as UTIs, endocarditis and secondary infections in immune-compromised patients or cystic fibrosis sufferers (143, 151). There are several other *Pseudomonas* species that have been described as opportunistic pathogens in humans (143). In fish, *P. anguilliseptica* has been shown to be the causative agent of disease where disease presents as petechial haemorrhages in the mouth, anal region and on the skin while *P. plecoglossicida* has caused mortalities in salmonids (152) and in Ayu (153) respectively. *P. putida* (154) and *P. fluorescens* (155) as well as *P. aeruginosa* (156) have also been implicated in disease outbreaks in fish. There are a number of *Pseudomonas* species, such as *P. chlororaphis* subsp. *piscium*, that have been isolated from fresh water fish, however to date, have not been shown to cause disease (157).

1.6.2.6 *Vibrio*

The new family *Vibrionaceae*, genus *Vibrio*, previously consisting of 63 species (158), now contains 100 species (159) some of which were recently identified (160-164). *Vibrio* bacteria are Gram-negative rods (158) whose natural habitat is water; fresh, brackish or salt water (143).

In humans, infections typically occur after exposure to contaminated aquatic sources and there are numerous *Vibrio* spp. which cause disease, with the most widely known strain being *V. cholerae* (serotypes O1 and O139) (165). *V. cholerae* (O1 and O139) exhibits high pathogenicity where disease manifests as severe watery diarrhoea (166).
Since the first pandemic in 1817, all have been associated with poverty stricken countries and poor sanitation (167-168).

In fish, *V. harveyi* has been shown to be the cause of dermal lesions (169) and eye lesions (170) in various species, as well as mortalities in several other marine animals (171-173). *V. harveyi* has also been identified as the pathogen that causes luminous vibriosis in a number of aquatic animals including Atlantic salmon (174-176). Any *Vibrio* spp. but mainly *V. salmonicida, V. anguillarum, V. ordalii, and V. harveyi* are capable of causing the disease Vibriosis (8, 118, 119, 120) and other species such as *V. parahaemolyticus, V. campbellii, V. carchariae* and *V. alginolyticus* are also significant pathogens of marine organisms (118, 119, 177-181).

1.6.3 Virulence factors associated with disease causing bacteria

Despite the fact that some of these bacteria are significant pathogens their virulence factors and disease mechanisms remain to be fully elucidated. There are many virulence factors considered to be important in disease however the exact role these virulence factors play remains to be elucidated (182). For instance, capsules synthesis allows the bacteria to evade the immune system and also provides protection from hydrophobic toxins (183).

Although the secretion of extracellular enzymes such as, proteases (184, 185) and phospholipase (186) do not directly contribute to bacterial pathogenicity, the resulting damage to host tissue increases the invasion potential (187). However, the production and secretion of cytotoxic enterotoxins by these bacteria, is directly related to pathogenicity and can be quite damaging to host tissues (188, 189) examples of which include *V. harveyi* specific haemolysin (190), *Aeromonas* haemolysin (191), *Aeromonas* aerolysin (191) and *P. fluorescens* heat labile toxin (192, 193).

Adhesion to host cells is is important for the initial stages of invasion and therefore the ability to attach to chitin (194, 195) and other host cell structures enhances the capability of colonisation and infection by the bacteria. Attachment can be mediated via fimbriae/pili (196) or through non-filamentous adhesins such as outer membrane proteins (197).

Lastly, the formation of biofilms on surfaces allows bacteria to persist in the environment (198), at times during unfavourable conditions such as low nutrient availability (199).
1.6.4 Diseases prevention and treatment

Aquaculture practices employ the use of both antibiotic and antifouling agents as part of a regime to ensure the health and growth of fish stocks and to prevent colonisation by pathogenic bacteria and parasites (10, 11).

1.6.4.1 Antibiotic treatment

Antifoulants are directly supplemented into the water in response to bio-fouling, which reduces water flow and subsequently oxygen supply within the fish cages (10) whilst antibiotics are conventionally either added into the fish feed or water either as a prophylactic or treatment method in response to infection (11). In the global Atlantic salmon aquaculture industry, a variety of antibiotic drugs are currently utilised, some of which include folate inhibitors (eg. sulphonamide, trimethoprim), tetracyclines (eg. tetracycline, oxytetracycline), amphenicols (eg. chloramphenicol), quinolones (eg. oxolinic acid), macrolides (eg. erythromycin) and β-lactams (eg. amoxicillin) (12). This can be problematic as quinolones, tetracylines, β-lactams and macrolides are used in the treatment of human infections and bacterial resistance to these classes of antimicrobials have been increasing over time (200, 201). The use of these antibiotics varies widely between countries due to differences in approved use regulations (202). Norway, but not the UK, for example, allows the use of amphenicols, and whilst both these countries allow roughly 6-7 drug classes for use in aquaculture, Japan allows 24 classes of drugs (202). The choice of drug utilised is dependent on the disease or symptoms present, however at times the aetiological agents of some diseases are unknown. This in particular is the case in Tasmania, where SGS is caused by an unknown pathogen, and therefore broad-spectrum antibiotics, such as oxytetracycline, are used to alleviate the disease and it’s associated symptoms (4). Administration most commonly occurs as a constituent within or surface coated onto food pellets, however occasionally antibiotics are administered as injections or baths (4, 203, 204). Unconsumed food and faeces fall through the nets to the bottom of the holding cages. From there pellets, are washed away and the antibiotics are either ingested by wild fish and invertebrates or they leach into the surrounding environment, leaving residual antibiotics within the sediment and creating selective pressure for environmental bacteria (202, 205- 208).
1.6.4.2 Effects of antibiotics
The large-scale and oftentimes unregulated use of antibiotics for disease control and growth promotion is of major concern as there is evidence that the selective pressure has led to the development of antibiotic resistance in fish pathogens over the period of time in which antibiotics have been used to control aquacultural diseases (4, 209). Antimicrobial resistance also occurs in the commensal microbiota of treated fish and within the surrounding environment, which have the ability to harbour new and previously uncharacterised resistance determinants (11, 210-215). Antibiotic resistance in potential fish pathogens not only renders the future prophylactic use of antibiotics ineffective, but can also lead to the transfer of resistance genes via plasmids, not merely between other fish pathogens, but onto the bacteria infecting terrestrial animals and humans (4, 216-218). This can have severe public health consequences due to either the consumption of food contaminated with antibiotic resistant bacteria or exposure to animals fed antibiotics causing increased risk of colonisation or infection of humans with resistant bacteria (219). Another side effect of the excessive use of antibiotics in aquaculture is the presence of residual antibiotics in fish and shellfish products, leading to undetected consumption by the consumer which has been shown to produce allergies and toxicity (4, 103, 203, 220-222) and can induce antimicrobial resistance in the normal microbiota (219). Increased sanitary measures, vaccination and an increased control of antibiotic prescriptions can drastically reduce antibiotic use and the surmounting of after effects that ensue (203, 223). The use of antibiotics in fish affected by GI tract infections is not advisable as this further disrupts the normal microbiota causing exacerbated infection (14). In view of the problems associated with the use of antibiotics, the use of vaccines and the introduction of probiotics into fish feed offer an attractive alternative as both a prophylactic and treatment method for current GI diseases afflicting Atlantic salmon.

1.6.4.3 Vaccination, prophylactics and other treatments
In the last few decades several injectable vaccines have been develop to target the aetiological agents of cold-water vibriosis (224), furunculosis (119) and winter ulcer disease (122). Immersion vaccines have also been developed for vibriosis (119) and enteric red mouth disease (ERM) (225, 226)
There are, however, several diseases for which there is no prevention or vaccine, one of these being proliferative kidney disease (PKD). There is no vaccine required for amoebic gill disease (AMG), as fresh water bathing is an effective treatment method (111). Many approaches have been used to develop a suitable and safe vaccine for furunculosis, but none of these have been approved to date (227-230). It has been proposed that the use of *A. hydrophila* in a vaccine stimulates non-specific immunity, with the result being cross-over protection to various *Aeromonas* spp. However, the large number of serotypes has hampered the availability of a commercial vaccine for motile *Aeromonas septicaemia* (MAS) (13, 132, 231, 232). Prophylactic measures, such as low stocking density and good hygiene are the best prevention methods.

1.6.4.4 Probiotics

Pasteur and his colleague Joubert observed the first antagonistic bacterial interactions in 1877, and in 1908 pathogen displacement capability and the resulting health benefits from the ingestion of lactobacilli species was proposed (233). Between 1965 and the present, the description of the term probiotic has varied, however at present the term probiotic denotes ‘for life’ and refers to a non-pathogenic microorganism which by direct or indirect action improves the health of the host beyond the merely the nutritional status (108, 233-236).

1.6.4.1 Probiotics and their selection

To ensure the safety of the host and suitability of the strain, the bacteria intended for probiotic use must be tested against a number of criteria. The classic human criteria for probiotic candidates may not apply for aquatic application (237) nonetheless there are three desirable criteria for these probiotics:

1. The ability to colonise and prevent the adherence of pathogens within the GI tract.
2. Beneficial effects from their use, such as increased nutrition and an enhanced immune system.
3. Commercial longevity and viability (234, 238).

The ability to survive and colonise the GI tract, as well as a lack of pathogenicity or toxicity towards the host, are other preferred qualities of probiotics (233). In aquaculture the main focus of probiotic use is to decrease mortality rate, thereby decreasing financial losses (15, 239-241). Preliminary results have shown probiotics to
be very pathogen specific. LAB are naturally occurring in the gut of most fish species and therefore these commensal bacteria are the best choice as potential probiotics (210). The use of probiotics has been shown to improve the host’s intestinal microbiota and provides a source of nutrients and enzymes, which aids the digestion process (234, 242). This is considered to be one of the most important gut microbiota functions, which bacteria can exert either locally or transiently, as some probiotic strains do not necessarily colonise the GI tract (243, 244, 245). Possible mechanisms for the improvement within the host gut is that probiotics compete with pathogens for the adhesion to binding sites on the GI epithelial layer, which is entirely strain dependent (246), or that these bacteria produce environmental factors known to influence colonisation (244). Interestingly, the adhesion capability of a probiotic strain does not correlate with the degree of pathogen inhibition (247). Austin et al. (1995) showed that use of the strain *V. alginolyticus* as a probiotic against the fish pathogens *A. salmonicida, V. anguillarum* and *V. ordalii* significantly decreasing mortality rates (13). Research however suggests that some probiotics such as the genus *Lactobacillus* do not affect pathogen population or the composition of the host microbiota, but alternatively interact directly with the host GI tract epithelial cells bringing about immunomodulation (10, 247). The result of *L. rhamnous GG* ingestion in a rat model promoted positive changes to the permeability of the GI epithelial cells and increased the mitotic rate (248). Sakai et al. (1995) and Balcazar (2003) both demonstrated after probiotic use, the stimulation of the non-specific immune system in fish, through the enhancement of phagocytic and antibacterial activity of leukocytes (233). *P. acidilactici* strains have proved potentially beneficial to the gut microbiota of Tilapia (246), improving both microvilli morphology (250, 251) and reducing spinal deformities in Rainbow trout (252) and one strain is currently approved for inclusion into fish feed (253).

Some bacterial species, mainly probiotics, may exhibit the capability to produce antimicrobial compounds such as bacteriocins, or to exclude pathogens from colonising the gut epithelium (248, 254). Some bacteriocins only effectively target bacteria, which are close relatives of the producer strain therefore, most lactobacilli produce compounds that are only active against other LAB (255). There have been compounds isolated from lactobacilli however that demonstrate inhibition of other Gram-positive and even selected Gram-negative species (252). For instance, *P. acidilactici* (236, 256), *W. hellenica* (238) and *E. faecium* (257-259) isolates produce bacteriocins that are
particularly active against Gram-negative bacteria. Bacteriocins produced by some Gram-negative species show much greater bacterial species specificity than those produced by Gram-positive bacteria where the compounds are generally small and act by increasing the permeability of the outer membrane of the target (235). Many LAB are also capable of organic acid production which cause pH changes in the surrounding environment and inhibit the growth of other bacteria (233).

Another proposed mode of probiotic action is the acquisition of nutrients, which is considered one of the most important functions of the gut microbiota (244). This effect can be exerted either by the species established in the microbiota or transiently, as it is not necessary to colonise the GI tract for benefits to be observed (243, 245). There are many other effects that have been attributed to probiotic strains in humans such as the maturation and modulation of the mucosal immune system, improvement of the cell barrier function and the prevention of allergy development during infancy (236, (260, 261). Most of the studies involving the effects of probiotics have used in vitro models which have been shown to differ significantly in respect to their anti-inflammatory and cytokine response in vivo presumably due to variability in the strains and methods of induction for cytokine response (262-264).

1.6.4.4.2 Bacterial species used as probiotics in aquaculture

Generally probiotics are Gram-positive non-sporulating rods or cocci that produce lactic acid through the fermentation process. This metabolic process of fermentation is the reason that these bacteria are also known as LAB. They are natural inhabitants of the GI tract of humans, animals and many fish species, due to a natural diet that includes fermented foods (236, 265). There are currently more than 60 strains of LAB used by both researchers and the commercial sector and the three most commonly used genera are Lactobacillus, Bifidobacterium and Propionibacterium (236). Since the first application of a probiotic in aquaculture in 1986 (266), commonly used probiotics in fish aquaculture tend to be human probiotic strains from the genera Lactobacillus, Bifidobacterium and Bacillus (267) as these have also been shown to benefit fish species (268). Other probiotics include Pediococcus acidilactici, W. hellenica, Enterococcus faecalis, E. faecium and E. casseliflavus, all of which have been previously isolated from aquaculture sources (269-272). There are also Gram-negative bacteria which are also used as probiotics in the aquaculture industry, namely A. hydrophila, A. sobria, Vibrio fluvialis, Pseudomonas spp. as well as Enterobacter spp.
A range of microorganisms have been evaluated for use including Gram-negative and Gram-positive bacteria, yeasts, bacteriophages and uni-cellular algae. Bacteria also previously used as probiotics in Atlantic salmon include *Carnobacterium inhibens* (273), an unknown LAB strain (274), *P. fluorescens* (275) and the microalgae *Tetraselmis suecica* (276). At the time of writing there is only one commercially registered probiotic for fish feed supplementation (253). Although a number of suitable probiotic candidates have been identified, the use of probiotics is currently still a relatively new tool in aquaculture however it has the potential to be both a preventative and a treatment for many bacterial fish diseases (15).

### 1.6.4.4.3 Administration of probiotics

Unlike the administration of probiotics into the feed of terrestrial animals, encorporation into commercial fish feeds is problematic. The probiotics need to be ‘fixed’ to the pellets rather than just coated, as the organism will wash away before enough can be consumed to be of benefit. Successful probiotic supplementation includes cases where the strains were premixed with feed (151, 152, 154) or alternatively administered via bathing (153) however more research is required in this area as the preferred site of interaction in the gut may vary between probiotic strains and sampling sites may not coincide with population sites all of which, may cause difficulties in the verification of colonisation (277).

### 1.7 Knowledge gaps

With the depletion of wild fish stocks and an increasing demand on fish products the aquaculture industry has experienced rapid growth within the past few years. In any industry there are problems encountered and with the large-scale production of Atlantic salmon in Tasmania, a major issue is the rearing of fish outside of their optimal thermal range and the lack of information on the effect of diet formulations on the health of the fish. This can subsequently effect the composition of the gut microbiota which in turn can lead to overall poor health of the fish. Whilst previous studies have identified the major bacterial populations in the hindgut of Atlantic salmon, geographical location and diet are known to greatly influence the bacterial composition of the gut and to date the gut bacteria of Tasmanian Atlantic salmon remains unstudied. Australia experiences much higher water temperature during summer, the effect of which, on the gut
microbiota, has not been studied before. These varying environmental factors and locations influence the diseases that manifest in fish. For example, the Tasmanian salmon industry has been afflicted with gastrointestinal disturbances associated with diarhoea and loss of appetite which has been named Summer Gut Syndrome (SGS). Outbreaks of SGS have caused significant economic losses for the industry and to date the cause remains unknown however it is suspected to be of bacterial origin. Treatment of bacterial disease in fish, in the absence of a vaccine, requires the use of antibiotics however, the use of these can further exacerbate disruption of the microbiota and therefore there is a need for more sustainable and appropriate practices. Probiotics offer a cheap and effective alternative and are currently used in humans and other mammals. At present there is a lack of suitable probiotics approved for use in the aquaculture industry. Furthermore, ethical, financial and logistical constraints often make the use of \textit{in vivo} trials to test the efficacy of potential probiotic strains difficult. The use of fermentation models to study human and other mammals provides a cheap and effective option to assess the impact of diet and probiotics on the gut microbiota. To date this has not been attempted for the gut environment of fish, however it could be a relatively simple, timely and cost effective method to assess feed formulations and the effects these have on the gut microbiota.
1.8 Aims, hypothesis and structure of this thesis
Although the gut microbiota of Atlantic salmon has been characterised in the past, geographical location and diet are known to greatly influence the bacterial composition of the gut and to date the composition of the gut microbiota of Australian Atlantic salmon remains unknown. The prevalence and persistence of certain bacterial genera and how the overall microbiota fluctuates in response to diet in salmon has also not been assessed in Australia. Feed trials can be quite lengthy and also costly for both the feed company and fish farms involved. Fermentation models have been used in the past to model the gut of humans and other mammals and assess the impact of diet and probiotics on the gut microbiota. There have however been no attempts made to simulate the gut environment of fish, which would be a relatively simple, timely and cost effective method to assess feed formulations and the effects these have on the gut microbiota of fish. Furthermore the lack of suitable probiotics approved for use in fish deems it necessary to find appropriate alternatives that are present in the gut as commensals. In this thesis efforts were made to solve some of these issues.
Specific aims include:
1. To identify changes in the microbial population of the hindgut of Atlantic salmon over the course of 10 months as a result of changes in the water temperature and in response to diet.
   This was based on the hypothesis that changes in water temperature and diet will cause changes in the population structure of the hindgut microbiota of Atlantic salmon.
2. To establish VG profiles for the microbiota of each sample using 35 genes found in potentially pathogenic bacteria present in the gut.
   This was based on the hypothesis that the warmer periods of the year will be associated with an increase in the presence and number of VGs found in the hindgut bacteria.
3. To determine the functional status and metabolic capacity of the gut microbiota of Atlantic salmon in response to diet and temperature.
   This was based on the hypothesis that changes in the bacterial populations within the hindgut, in response to both temperature and diet, will lead to changes in both the metabolic capacity as well as the functional status.
4. To explore the persistence and prevalence of *Vibrio* and *Pseudomonas* species in the gut of Atlantic salmon in relation to temperature and diet changes.
This was based on the hypothesis that certain clones of *Vibrio* and *Pseudomonas* species are able to persist throughout and increase in numbers over the warmer period thereby outcompeting other bacteria.

5. To test whether a semi-continuous fermenter model can be used to simulate the salmon gut microbiota in response to diet and temperature *in vitro.*

This was based on the hypothesis that the use of a semi-continuous fermenter model can simulate changes in the host microbiota in response to diet variations and will therefore be a valuable tool to assess new diet formulations.

6. To characterise LAB strains, isolated from the faecal microbiota of the salmon during sampling and assess whether any strains show the properties necessary for a probiotic candidate.

This was based on the hypothesis that LAB strains isolated from the microbiota of healthy Atlantic salmon could be used as a probiotic supplement in fish feed to improve the health of the fish.
1.9 References


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CHAPTER 2

2.0 The effect of diet and environmental temperature on the faecal microbiota of farmed Tasmanian Atlantic salmon (Salmo salar L.)

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2.1 Introduction

The composition of the gut microbiota of fish has been shown to be dependent on initial colonisation, age, environmental conditions, nutritional status as well as dietary changes (1-4). The gut microbiota originates from the surrounding water, in which the fish hatch, where bacteria colonise and develop into an initial transient microbiota. Depending on the fish species these evolve into a stable community, usually after the juvenile stages (5).

The most common bacteria isolated from the gut of Atlantic salmon *Salmo salar* L. were *Acinetobacter* spp., *Enterobacteriaceae*, *Aeromonas* spp., *Flavobacterium* spp., *Pseudomonas* spp. as well as *Lactobacillus* spp. (2). Since the introduction of molecular methods, other species such as *Vibrio* spp., *Lactococcus* spp., *Bacillus* spp., *Photobacterium* spp., *Weissella* spp., *Shewanella* spp., *Carnobacterium* spp., *Citrobacter* spp., *Clostridium* spp. and *Mycoplasma* spp. have also been identified (6-10). However, all of these studies except one (7), have examined the gut microbiota from single or only several samples and have not addressed the impact of seasonal temperature on gut microbiota of sea-reared Atlantic salmon.

Farmed Atlantic salmon reared in Australia experience higher water temperatures throughout summer, well above the thermal threshold for Atlantic salmon, reaching temperatures of up to 21°C, as compared to countries such as Chile and Norway (11, 12). Intensive farming (i.e. high pen stocking densities and feed input) coupled with temperature increases can lead to conditions which may favour the overgrowth of certain bacterial species, including opportunistic pathogens (13) which can lead to GI disease.

2.2 Aims of this study

In view of the above, this study was undertaken to examine changes in the population structure of the gut microbiota of farmed Tasmanian Atlantic salmon throughout the year in coincidence with periods of dramatic changes in water temperature. To investigate the impact of diet on gut bacterial population of the fish, we expanded the scope of the study by including samples from fish that receive two different commercial diets.
2.3 Materials and methods

2.3.1 Sampling
Between July 2011 and May 2012 a total of 400 faecal samples were collected over eight sampling occasions ranging from 4 week (during warmer months) to eight week intervals (during the cooler period of the year) from Atlantic salmon, reared in sea cages in a commercial fish farm south of Hobart, Tasmania. Fish were farmed according to standard commercial practices, which included feeding to satiation, checking for AGD and subsequent fresh water bathes upon clinical signs of disease. Faecal samples were collected from healthy fish, which showed no signs of clinical infection and had not been recently (within the previous 14 days) subjected to fresh water bathing (14). Fish did not receive antibiotic treatment throughout the course of this study. Atlantic salmon were fed with two commercially available dietary regimes, designated A and B (Table 1.2) neither supplemented with probiotics. From each cage randomly receiving either diet A or B, 40 fish (20 per cage) were caught at random via netting, from a pen stocked with 70,000 female smolt, at each sampling round and anaesthetised using 17ppm isoeugenol (Aqui-S®) according to the commercial fish farms procedure, before being gently squeezed to collect the faecal material by the fish farm veterinarian in sterile 50 mL centrifuge tubes. Average fish weights per pen ranged from 0.4 kg for diet A and 0.4 kg for diet B (in the first sampling round in July 2011) to 2.8 kg for diet A and 2.9 kg for diet B in the last samples obtained (May 2012). At each sampling occasion the water temperature was recorded and ranged from 10.1°C in July 2011, peaking to 21°C in-between two sampling periods (February and March 2012) and falling back to 11.9°C in May 2012. Dissolved oxygen (DO) concentrations were monitored as per standard farming practices and the water within the pens was supplemented with O₂ as required during warmer periods. Salinity did not vary greatly throughout the sampling period. The sampled pens were located within close proximity (roughly 10 m) to each other and the same population of fish were sampled throughout this study. Over the course of the study, fish were switched from the winter Diet A or B to the respective summer diet in September 2011 and from the summer diet A or B back to the respective winter diet in March 2012. The faecal samples (n=20) from each cage were pooled into two groups (n=10) per diet to determine intra assay variations and also to eliminate individual differences between fish so that fluctuations in the overall bacterial
populations could be assessed. The pooled faecal samples were placed on ice, transferred to the laboratory and processed within 2 h.

Table 2.1: Composition of two commercially available diets (i.e. A and B) fed to Atlantic salmon during the various stages of their farming cycle.

<table>
<thead>
<tr>
<th>Composition of Diet (%)</th>
<th>Type of diet and feeding regime</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Input till late October 2011 (smolt diet)</td>
</tr>
<tr>
<td></td>
<td>Late October till mid-March 2012 (summer diet)</td>
</tr>
<tr>
<td></td>
<td>Mid-March till harvest (growing diet)</td>
</tr>
<tr>
<td></td>
<td>A          B          A          B          A          B</td>
</tr>
<tr>
<td>Protein</td>
<td>46-50      47-49     42-46      45         38-41      40</td>
</tr>
<tr>
<td>Lipid</td>
<td>22-28      23-24     28-29      25         33-36      30</td>
</tr>
<tr>
<td>Digestible energy (Mj/kg)</td>
<td>19.8-</td>
</tr>
</tbody>
</table>

2.3.2 Bacterial enumeration
Faecal samples were initially diluted (1:1 w/v) in sterile PBS (pH 7.4). This suspension was used to make a serial dilution of the total faecal microbiota and 100 µL of each dilution was spread onto Marine agar (MA) (Difco, Australia) to obtain the total number of cultureable bacteria, Herellea agar (HA) (15) for isolation of Acinetobacter spp., Pseudomonas agar (Difco) for isolation of Pseudomonas spp., Thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Oxoid, Australia) for isolation of Vibrio spp., MRS agar (Oxoid) for isolation of LAB, Inositol-brilliant-green-bile-salts (IBBS) agar (16) for isolation of Plesiomonas shigelloides and Aeromonas agar (AA) (Oxoid) for isolation of Aeromonas spp.

Water samples from the outside of each pen were also collected in a sterile 500 mL bottle and transported on ice to the laboratory where they were serially diluted and 100 µL of each dilution was spread on MA and together with other plates incubated at room temperature (20-23°C) for one week. For enumeration of bacteria, duplicate pooled samples were used and the results were expressed as the mean of duplicate plates.
Identification of bacterial isolates was undertaken using biochemical tests and genus- or species-specific primers (Table 2.2). PCR protocols for amplification of the genes were followed as previously described (17-20). The bacterial-specific primer 27F and the Universal 16S rRNA gene primer 1492R were used on unidentified isolates. PCR
products from primers 27F and 1492R were sequenced by Macrogen (Korea) and analysed using the NCBI BLAST search program (blastn).

Table 2.2: List of primers used for identification of bacteria to the genus or species level

<table>
<thead>
<tr>
<th>Bacteria genus/species</th>
<th>Primer</th>
<th>Sequence</th>
<th>bp</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vibrio spp. rpoA</td>
<td>F- ATGCAGGGTTCTGTDACAG</td>
<td>242</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R- GGGCCARTTTTTCHARRGC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pseudomonas spp. PA-GS</td>
<td>F- GACGGGTGAGTAATGCCTA</td>
<td>618</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R- CACTGGTGTTTCTTCTATA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Escherichia coli uspA</td>
<td>F- CCGATAACGCTGCCAATCAGT</td>
<td>884</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R- ACGCAGACGTTAGGCCAGAT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. shigelloides hugA</td>
<td>F- GCGAGCGGGGAAGGGAAGACC</td>
<td>435</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R- GTCGCCCAACGCTAACTCATCA</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.3.3 Functional status and metabolic capacity of the faecal microbiota

Twenty-five grams of faecal sample was diluted with sterile PBS (pH 7.4) (1:1 w/v), filtered through glass wool using a suction pump. Bacteria were collected in sterile PBS (pH 7.4) and centrifuged at 2500 rpm for 10 min. For each diet and sampling occasion, the total bacterial microbiota collected were tested for their functional status using the PhPlate-48 generalised microplate system (PhPlate AB, Stockholm, Sweden) as described previously (21). The faecal bacteria were mixed with the PhP-medium comprised of 0.011% w/v bromothymol blue and 1% w/v proteose peptone and 150 µL of the suspension was inoculated into each of the 48 wells of PhP-48 plates. The substrates used in the PhP-48 plates are provided (Table 2.3). Plates were incubated at 20°C and images of plates at 4, 16, 24, 48 and 72 h were scanned using a HP Scanjet 4890 desktop scanner. All images were imported into the PhP software where the absorbance of each reagent was converted to numerical values ranging from 0-30. After the final reading of plate images, the mean absorbance value from all individual readings was calculated for each reagent. Similarity among substrate utilisation of each sample was calculated after pair-wise comparison and expressed as the correlation coefficient using the PhP-Win 4 software as previously described (21) and clustered into a dendrogram according to the unweighted pair group method with arithmetic averages (UPGMA) (22). The final value for each substrate utilisation depends on the kinetics of bacterial population using that substrate as well as the intensity of that reaction, and therefore rather than an absolute ‘positive’ or ‘negative’ value as is...
obtained in conventional assays, the kinetics of substrate utilisation is instead obtained, which reflects the overall functional status of the microbiota (21).

The ability of the gut bacteria to metabolise various carbon sources was referred to as the metabolic capacity (MC) as described before (21, 23). This was measured by dividing the sum of the positive test results by the total number of substrates tested using the PhP-Win 4 software. A high MC-value (maximum =1) for a gut microbiota indicates that most carbon sources have been metabolised by members of the gut microbiota. In contrast a low MC value (minimum =0) is an indication that low numbers of substrates have been utilised (21, 23).
Table 2.3: Substrates used in PhPlate-48 plates for testing functional status of the hindgut microbiota of salmon and for measuring their MC value

<table>
<thead>
<tr>
<th></th>
<th>Mannonic acid lactone</th>
<th>L- Arabinose</th>
<th>D- Xylose</th>
<th>Galactose</th>
<th>Maltose</th>
<th>Celllobiose</th>
<th>Trehalose</th>
<th>Palatinose</th>
<th>Sucrose</th>
<th>Lactose</th>
<th>Melibiose</th>
<th>Lactulose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Gentobiose</td>
<td>14 Melezitose</td>
<td>15 Raffinose</td>
<td>16 Inosine</td>
<td>17 Adonitol</td>
<td>18 Inositol</td>
<td>19 D-Arabitol</td>
<td>20 Glycerol</td>
<td>21 Maltitol</td>
<td>22 Sorbitol</td>
<td>23 Dulcitol</td>
<td>24 pH 7.4 Control **</td>
</tr>
<tr>
<td>25</td>
<td>Sorbose</td>
<td>26 Deoxy- glucose</td>
<td>27 Deoxy- ribose</td>
<td>28 Rhamnose</td>
<td>29 D-Fucose</td>
<td>30 L-Fucose</td>
<td>31 Tagatose</td>
<td>32 Amygdalin</td>
<td>33 Arbutin</td>
<td>34 b-Methylglucoside</td>
<td>35 5-Keto- gluconate</td>
<td>36 Gluconate</td>
</tr>
<tr>
<td>38</td>
<td>Galacturo-nic lacton</td>
<td>39 Salicine</td>
<td>40 pH 5.5 Control *</td>
<td>41 Citrate *</td>
<td>42 Fumarate *</td>
<td>43 Malinate *</td>
<td>44 Malonate *</td>
<td>45 Pyruvate *</td>
<td>46 L-Tartarate *</td>
<td>47 Urea *</td>
<td>48 Ornithine *</td>
<td></td>
</tr>
</tbody>
</table>

*Control test for alkaline reactions (wells 41-48), **Control test for acidic reactions (wells 1-39); substrates never utilised are represented in shading
2.3.4 DNA extraction, species-specific primers and virulence gene profiles

The genomic DNA of the whole hindgut biota at each sampling occasion was extracted from the faecal material using a QIAGEN Stool and Tissue kit (Qiagen) according to manufacturer’s instructions and stored until further use at -20°C. DNA was visualised on a 1% (w/v) agarose gel run at 100 V for 40 min, pre-stained with ethidium bromide, and viewed under UV light using a Gel Doc System (BioRad).

Bacterial identification within the total DNA extract was confirmed using genus- or species-specific primers (Table 2.2). PCR protocols for amplification of the genes were followed as previously described (17-20). DNA was diluted to a final concentration of 1:10 with sterile water, to decrease potential inhibitors in the DNA extraction (24). All PCRs using the genus- or species-specific primers were repeated with the diluted DNA to assess whether potential inhibitors may have affected the previously obtained results. Single and multiplex PCR’s were used to screen for the presence of 35 VGs associated with Gram-negative pathogenic bacteria in the total DNA extract of hindgut microbiota of salmon (Table 2.4). The function, source and protocols for the listed multiplex and uniplex PCRs were performed according to the references provided for each multiplex or uniplex PCR (Table 2.4). Positive controls were used for the detection of VGs and negative controls were samples without templates.

2.3.5 Statistical analysis

Student’s t-test was used to compare the number of bacteria belonging to *Vibrio* and *Pseudomonas* spp. to the other bacterial populations. Significance was established as P<0.05. This test was also used to compare the MC-value obtained at each sampling occasion between two diets. Significance is established as P < 0.05.
Table 2.4: List of 35 VGs tested and their function. Protocols for each multiplex (MP) and uniplex PCR was preformed according to the references given and product sizes are presented in base pair (bp) numbers.

<table>
<thead>
<tr>
<th>VGs</th>
<th>Target species</th>
<th>Function of the gene</th>
<th>Sequence 5'-3'</th>
<th>bp</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MP - 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>finH</td>
<td>E. coli</td>
<td>D-mannose specific adhesin</td>
<td>F: TOCGAGACCGATAAGGCCTG&lt;br&gt;R: GCCGCTACCTGCTCCCGGTA</td>
<td>508</td>
<td>25</td>
</tr>
<tr>
<td>kpsII</td>
<td>E. coli</td>
<td>Group II capsular polysaccharide synthesis</td>
<td>F: TCCCTTGGCTACTATTTCCCCCT&lt;br&gt;R: ACGGCTATCCATCTGGTTAAC</td>
<td>392</td>
<td></td>
</tr>
<tr>
<td>ibeA</td>
<td>E. coli</td>
<td>Invasion of brain endothelium</td>
<td>F: AGGCAGGTGCGGCGCGGTAC&lt;br&gt;R: TGGTGCTGGCGAACACATCG</td>
<td>170</td>
<td></td>
</tr>
<tr>
<td><strong>MP - 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fyuA</td>
<td>Yersinia spp.</td>
<td>Sidephore receptor</td>
<td>F: TGATTACCGCGGCGGCCAAG&lt;br&gt;R: CGCAGATGGCTGCCGTCGTTA</td>
<td>880</td>
<td>25</td>
</tr>
<tr>
<td>bmaE</td>
<td>E. coli</td>
<td>M-agglutinin subunit</td>
<td>F: ATGCGCTAATGCGATCTGCT&lt;br&gt;R: ACGGGGACATAGGCCCCCGTGC</td>
<td>507</td>
<td></td>
</tr>
<tr>
<td>safCDE</td>
<td>E. coli</td>
<td>Region of S fimbiae/ FIC fimbiae operon</td>
<td>F: CTCCGGGAAGACTGGTCGACATCTTAC&lt;br&gt;R: CGCAAGGAAGTAATTTAACCTGGCGA</td>
<td>410</td>
<td></td>
</tr>
<tr>
<td>iutA</td>
<td>E. coli</td>
<td>Ferric aerobactin receptor</td>
<td>F: GCCTGGACATCATGGGAAACTTG&lt;br&gt;R: CGTCCGGGACGGTTAAGATC</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td>kpsMT K1</td>
<td>E. coli</td>
<td>Capsular polysaccharide synthesis</td>
<td>F: TACAGACCGTGAAGCAGTAC&lt;br&gt;R: CATCCGACGATGGCATGACGCA</td>
<td>153</td>
<td></td>
</tr>
<tr>
<td><strong>MP - 3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>exbA</td>
<td>E. coli</td>
<td>Enterohaemolysin</td>
<td>F: GCACTATACGGGATGTCTGC&lt;br&gt;R: AATAGGCGCAATGTTAAGCT&lt;br&gt;GA</td>
<td>534</td>
<td>26</td>
</tr>
<tr>
<td>eaeA</td>
<td>E. coli</td>
<td>Intimin</td>
<td>F: GACCCCGGACAGCATGAC&lt;br&gt;R: CCAGATTATTGCATTAATGTC</td>
<td>384</td>
<td></td>
</tr>
<tr>
<td>stx2</td>
<td>Shigella spp.</td>
<td>Shiga toxin 2</td>
<td>F: GCACCGCTTGGGCGAC&lt;br&gt;R: TGCGACATTTATCATGCC</td>
<td>255</td>
<td></td>
</tr>
<tr>
<td>stx1</td>
<td>Shigella spp.</td>
<td>Shiga toxin 1</td>
<td>F: ATAAATGCCTTATGCTTAC&lt;br&gt;R: AGAGCCGCCCAGATGAC</td>
<td>180</td>
<td></td>
</tr>
<tr>
<td><strong>MP - 4</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eltA</td>
<td>E. coli</td>
<td>Heat-labile toxin</td>
<td>F: GGCGGAGATACCATAC&lt;br&gt;R: CCAGATTATTGCATTAATGTC&lt;br&gt;GA</td>
<td>696</td>
<td>27</td>
</tr>
<tr>
<td>fasA</td>
<td>E. coli</td>
<td>Fimbrial Adhesin</td>
<td>F: AAACCAATTCGTTGAT&lt;br&gt;R: GGGGCCGACGCTGAC</td>
<td>333</td>
<td></td>
</tr>
<tr>
<td>evlII</td>
<td>E. coli</td>
<td>Heat-stable enterotoxin b</td>
<td>F: GCTTCTCCCTCTGCT&lt;br&gt;R: GGGGCCGACGCTGAC</td>
<td>172</td>
<td></td>
</tr>
<tr>
<td><strong>MP - 5</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>farG</td>
<td>E. coli</td>
<td>Fimbrial Adhesin</td>
<td>F: GGATGGTCTACATGGTTGC&lt;br&gt;R: ATGGCAATGCTGACGAC&lt;br&gt;CG</td>
<td>764</td>
<td>27</td>
</tr>
<tr>
<td>fanC</td>
<td>E. coli</td>
<td>Fimbrial Adhesin</td>
<td>F: TCTGTCTTTACTGAT&lt;br&gt;R: TATCCCACATTGAGGAC&lt;br&gt;GC</td>
<td>450</td>
<td></td>
</tr>
<tr>
<td>esp1</td>
<td>E. coli</td>
<td>Heat-stable enterotoxin a</td>
<td>F: CTTTCCGCTTTTTATCG&lt;br&gt;R: GGGGCCCCGACGCTGAC</td>
<td>166</td>
<td></td>
</tr>
<tr>
<td><strong>MP - 6</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aut</td>
<td>Aeromonas spp.</td>
<td>Cytolytic enterotoxin</td>
<td>F: TCCCTGCGCGCTTGGCGCG&lt;br&gt;R: AGGACATGCTGCGACG&lt;br&gt;CG</td>
<td>361</td>
<td>28, 29</td>
</tr>
<tr>
<td>ast</td>
<td>Aeromonas spp.</td>
<td>Cytolytic enterotoxin</td>
<td>F: GACTCTCACGGCTCCT&lt;br&gt;R: GCATCGAATGCTGCTGGA&lt;br&gt;GC</td>
<td>536</td>
<td></td>
</tr>
<tr>
<td>act</td>
<td>Aeromonas spp.</td>
<td>Cytolytic enterotoxin</td>
<td>F: GACACGATGCTGCGACG&lt;br&gt;CG</td>
<td>232</td>
<td></td>
</tr>
<tr>
<td><strong>MP - 7</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ctxA</td>
<td>Vibrio spp.</td>
<td>CT Subunit A</td>
<td>F: CGCGAACGCTTCTGCT&lt;br&gt;R: CGATGATCTGCGACG&lt;br&gt;GA</td>
<td>564</td>
<td>30</td>
</tr>
<tr>
<td>ompU</td>
<td>Vibrio spp.</td>
<td>Outer membrane protein</td>
<td>F: ACGGATGCGACGCGAC&lt;br&gt;GA</td>
<td>869</td>
<td></td>
</tr>
<tr>
<td><strong>MP - 8</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>exoA</td>
<td>P. aeruginosa</td>
<td>Exotoxin</td>
<td>F: GCAAGGACGCGACG&lt;br&gt;GA</td>
<td>396</td>
<td>36</td>
</tr>
<tr>
<td>pilA</td>
<td>P. aeruginosa</td>
<td>Type IV pilin</td>
<td>F: ATGAAAATGCTGACCTACG&lt;br&gt;R: TTTTTTGT&lt;br&gt;GAC&lt;br&gt;CG</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>pilA/B</td>
<td>P. aeruginosa</td>
<td>Type IV pilin</td>
<td>F: ATGAAAATGCTGACCTACG&lt;br&gt;R: TTTTTTGT&lt;br&gt;GAC&lt;br&gt;CG</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>pilB2</td>
<td>P. aeruginosa</td>
<td>Type IV pilin</td>
<td>F: ATGAAAATGCTGACCTACG&lt;br&gt;R: TTTTTTGT&lt;br&gt;GAC&lt;br&gt;CG</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td><strong>Uniplex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eae1</td>
<td>E. coli</td>
<td>Heat-stable enterotoxin</td>
<td>F: CCACTACACACATGATAC&lt;br&gt;R: GGTCCGGAGTGACG&lt;br&gt;CG</td>
<td>111</td>
<td>38</td>
</tr>
<tr>
<td>cdt</td>
<td>E. coli</td>
<td>Cytolath distending toxin</td>
<td>F: GAGTATGCTCCTCCTAC&lt;br&gt;R: CAAAGGCAACAGAG&lt;br&gt;GA</td>
<td>108</td>
<td>39</td>
</tr>
<tr>
<td>ipah</td>
<td>Shigella flexneri</td>
<td>Invasion plasmid antigen</td>
<td>F: GTTCCTGGCGACG&lt;br&gt;R: GCCGACGACG&lt;br&gt;GA</td>
<td>600</td>
<td>40</td>
</tr>
<tr>
<td>axT</td>
<td>Aeromonas spp.</td>
<td>ADP-ribosyltransferase toxin</td>
<td>F: GCGCATGCTGAC&lt;br&gt;R: GACG&lt;br&gt;CG</td>
<td>535</td>
<td>41</td>
</tr>
<tr>
<td>tapA</td>
<td>Aeromonas spp.</td>
<td>Type IV pilin</td>
<td>F: ATGAAAATGCTGACCTACG&lt;br&gt;R: TTTTTTGT&lt;br&gt;GAC&lt;br&gt;CG</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>aerA</td>
<td>Aeromonas spp.</td>
<td>Aerolysin</td>
<td>F: GCCTGAGGAGG&lt;br&gt;R: CAGTCC&lt;br&gt;CA</td>
<td>416</td>
<td>43</td>
</tr>
<tr>
<td>hylA</td>
<td>Aeromonas spp.</td>
<td>Haemolysin</td>
<td>F: GCCGAG&lt;br&gt;R: GCCGAG&lt;br&gt;GA</td>
<td>597</td>
<td>43</td>
</tr>
</tbody>
</table>
2.4 Results

2.4.1 Bacterial enumeration
There were some variations (up to 1 log) in the number of bacteria belonging to different groups within two diets at each sampling round. There were however no significant differences in the bacterial populations within the same diet or between the two diets. There was also a difference in the total number of culturable bacteria as well as the number of bacteria belonging to different species in the samples exposed to the two diets (Table 2.5). During the cooler months (i.e. June), the total bacteria in the gut of fish fed diet A were slightly lower than those fed diet B ($1.1 \times 10^6$ and $4.5 \times 10^6$ colony forming units (CFU)/gram faecal sample respectively) (Table 2.5). The total number of bacteria peaked shortly after the highest temperature (i.e. 21°C between samples collected in February and March) for both diet A and B ($3 \times 10^8$ CFU/g of faecal samples) (Table 2.5). Regardless of the diet, generally there was an increase in the number of bacteria (except LAB) as the water temperature increased. This however was not consistent with some bacterial species. For instance, *Acinetobacter* strains were undetectable in two samples collected during the warmest water temperature (Table 2.5). No LAB were detected in fish receiving diet A once the temperature reach >13°C. This was not the case however, for diet B where LAB were sporadically detected throughout the sampling period. There were also slight differences between the numbers of bacteria in hindgut of fish receiving two diets different (Table 2.5).

2.4.2 Functional status and metabolic capacity of microbiota
There was a temperature related change in the pattern of substrate utilisation by the gut microbiota of salmon. Both diet A and diet B did not cause major changes in the capability of the gut bacteria to utilise substrates during the cooler months of the year (May through to November), with a similarity of 85% for fish fed diet A and 75% for fish fed diet B during this period (Figure 2.2). An increase in the water temperature was associated with an abrupt shift in the pattern of substrate utilisation with a decrease in the similarity between the functions of the gut biota of fish in both diets (Figure 2.2). The decrease between similarity and the pattern of substrate utilisation of gut
Table 2.5: Number of bacteria (CFU/g of faecal material) detected in the hindgut of Atlantic salmon fed diets A and B over 10 months of sampling. There were no significant differences in the number of the same bacterial populations between the two diets.

<table>
<thead>
<tr>
<th></th>
<th>July</th>
<th>September</th>
<th>November</th>
<th>December</th>
<th>January</th>
<th>February</th>
<th>March</th>
<th>May</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water temperature (°C)</td>
<td>10.1</td>
<td>12.3</td>
<td>13</td>
<td>16.2</td>
<td>18</td>
<td>18.5</td>
<td>15.6</td>
<td>11.9</td>
</tr>
<tr>
<td>Fish weight (g)</td>
<td>390</td>
<td>761</td>
<td>793</td>
<td>1074</td>
<td>1131</td>
<td>1545</td>
<td>1741</td>
<td>2882</td>
</tr>
<tr>
<td>Water bacterial count</td>
<td>(7.8 \times 10^2)</td>
<td>(4 \times 10^3)</td>
<td>(3 \times 10^3)</td>
<td>(1.7 \times 10^3)</td>
<td>(3 \times 10^3)</td>
<td>(3 \times 10^3)</td>
<td>(3.9 \times 10^3)</td>
<td></td>
</tr>
<tr>
<td>Total bacterial count</td>
<td>(1.1 \times 10^6)</td>
<td>(9 \times 10^4)</td>
<td>(8.1 \times 10^6)</td>
<td>(4 \times 10^6)</td>
<td>(1.8 \times 10^7)</td>
<td>(2.2 \times 10^7)</td>
<td>(3 \times 10^7)</td>
<td>(1.3 \times 10^7)</td>
</tr>
<tr>
<td>Vibrio sp.</td>
<td>(1.7 \times 10^3)</td>
<td>(2 \times 10^2)</td>
<td>(2.4 \times 10^3)</td>
<td>(5.4 \times 10^3)</td>
<td>(1.3 \times 10^6)</td>
<td>(1.2 \times 10^6)</td>
<td>(3 \times 10^7)</td>
<td>(1.3 \times 10^6)</td>
</tr>
<tr>
<td>Aeromonas sp.</td>
<td>(6 \times 10^8)</td>
<td>(1.2 \times 10^8)</td>
<td>(8.7 \times 10^8)</td>
<td>(1.9 \times 10^9)</td>
<td>(4 \times 10^9)</td>
<td>(3 \times 10^9)</td>
<td>(3 \times 10^9)</td>
<td>(1.7 \times 10^9)</td>
</tr>
<tr>
<td>Pseudomonas sp.</td>
<td>(4 \times 10^8)</td>
<td>0</td>
<td>0</td>
<td>(5 \times 10^2)</td>
<td>(5 \times 10^3)</td>
<td>(1.1 \times 10^2)</td>
<td>(1.2 \times 10^3)</td>
<td>(1.5 \times 10^3)</td>
</tr>
<tr>
<td>Plesiomonas sp.</td>
<td>(9 \times 10^4)</td>
<td>(7.5 \times 10^2)</td>
<td>0</td>
<td>(2.2 \times 10^3)</td>
<td>(3 \times 10^3)</td>
<td>(3 \times 10^3)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Acinetobacter sp.</td>
<td>(8 \times 10^5)</td>
<td>(1.1 \times 10^5)</td>
<td>(5.5 \times 10^5)</td>
<td>(3 \times 10^5)</td>
<td>0</td>
<td>0</td>
<td>(3 \times 10^5)</td>
<td>(2.5 \times 10^5)</td>
</tr>
<tr>
<td>Lactic acid bacteria</td>
<td>(3 \times 10^4)</td>
<td>(6.5 \times 10^4)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>July</th>
<th>September</th>
<th>November</th>
<th>December</th>
<th>January</th>
<th>February</th>
<th>March</th>
<th>May</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water temperature (°C)</td>
<td>10.1</td>
<td>12.3</td>
<td>13</td>
<td>16.2</td>
<td>18</td>
<td>18.5</td>
<td>15.6</td>
<td>11.9</td>
</tr>
<tr>
<td>Fish weight (g)</td>
<td>401</td>
<td>784</td>
<td>1089</td>
<td>1062</td>
<td>1357</td>
<td>1614</td>
<td>1831</td>
<td>2803</td>
</tr>
<tr>
<td>Water bacterial count</td>
<td>(4.2 \times 10^7)</td>
<td>(8.7 \times 10^2)</td>
<td>(3 \times 10^1)</td>
<td>(1.5 \times 10^8)</td>
<td>(3 \times 10^8)</td>
<td>(3 \times 10^8)</td>
<td>(5.7 \times 10^8)</td>
<td></td>
</tr>
<tr>
<td>Total bacterial count</td>
<td>(4.5 \times 10^6)</td>
<td>(9.5 \times 10^5)</td>
<td>(1.7 \times 10^7)</td>
<td>(3.8 \times 10^7)</td>
<td>(6.1 \times 10^7)</td>
<td>(4.6 \times 10^7)</td>
<td>(3 \times 10^7)</td>
<td>(1.3 \times 10^7)</td>
</tr>
<tr>
<td>Vibrio sp.</td>
<td>(6.8 \times 10^6)</td>
<td>(4.4 \times 10^6)</td>
<td>(9.9 \times 10^6)</td>
<td>(1.7 \times 10^7)</td>
<td>(2.4 \times 10^7)</td>
<td>(2.3 \times 10^7)</td>
<td>(3 \times 10^7)</td>
<td>(1.7 \times 10^7)</td>
</tr>
<tr>
<td>Aeromonas sp.</td>
<td>(5.2 \times 10^6)</td>
<td>(1.5 \times 10^6)</td>
<td>(5 \times 10^4)</td>
<td>(3.5 \times 10^5)</td>
<td>(4 \times 10^7)</td>
<td>(3 \times 10^6)</td>
<td>(3 \times 10^7)</td>
<td>(1.1 \times 10^7)</td>
</tr>
<tr>
<td>Pseudomonas sp.</td>
<td>(2.1 \times 10^5)</td>
<td>(5 \times 10^3)</td>
<td>0</td>
<td>(1 \times 10^3)</td>
<td>0</td>
<td>2.5 (\times 10^3)</td>
<td>(2.5 \times 10^2)</td>
<td>(3 \times 10^2)</td>
</tr>
<tr>
<td>Plesiomonas sp.</td>
<td>(7.4 \times 10^5)</td>
<td>(1 \times 10^4)</td>
<td>(1.5 \times 10^4)</td>
<td>(3 \times 10^3)</td>
<td>(3 \times 10^3)</td>
<td>(3 \times 10^6)</td>
<td>(3 \times 10^6)</td>
<td>(3.4 \times 10^5)</td>
</tr>
<tr>
<td>Acinetobacter sp.</td>
<td>(2.8 \times 10^6)</td>
<td>(1.5 \times 10^6)</td>
<td>(2.8 \times 10^5)</td>
<td>(3 \times 10^3)</td>
<td>0</td>
<td>0</td>
<td>(3 \times 10^6)</td>
<td>(1.7 \times 10^6)</td>
</tr>
<tr>
<td>Lactic acid bacteria</td>
<td>(3.2 \times 10^5)</td>
<td>(1 \times 10^4)</td>
<td>0</td>
<td>(5 \times 10^3)</td>
<td>0</td>
<td>(1 \times 10^2)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
microbiota correlated with the decrease in bacterial diversity (Figure 2.2 and Table 2.5). Change in the water temperature was also associated with changes in the MC-value of the gut microbiota in fish receiving either diet. In general the MC-values of the gut microbiota decreased throughout the sampling period and as the fish gained weight. This however, was more pronounced during the warmer months of the year as well as immediately after changes from winter diet to summer and vice versa (Figure 2.1). The MC-value of fish receiving diet A decreased significantly (p<0.001) compared to fish fed diet B as water temperatures increased during January and February 2012 (Figure 2.1).

![Figure 2.1](image)

**Figure 2.1:** The MC values of the gut microbiota of salmon fed two different diets over 10 months sampling. * Changes in diet from winter to summer diet and vice versa, ** p= 0.001 differences in the MC-values (as determined using a student’s t-test) of gut microbiota during January and February samplings.

### 2.4.3 Virulence gene profiles

Of the 32 VGs associated with Gram-negative bacteria, only two were detected in the total genomic DNA extracts. Cytolethal distending toxin gene (cdt) was present in all faecal samples, regardless of water temperature or diet (Figure 2.2). Heat-stable enterotoxin gene (east1) was shown only in samples from diet A in July 2011 as well in both diets in November 2011 (Figure 2.2).
**Figure 2.2**: Similarity between the patterns of substrate utilisation by the gut microbiota of Tasmanian Atlantic salmon fed two different diets over 10 months in 2011 and 2012. *cdt*: cytolethal distending toxin; *east1*: a thermostable toxin found among enteroaggregative *E. coli*.

### 2.5 Discussion

In this study it was observed that the microbiota of the hindgut of Atlantic salmon fluctuated as a result of seasonal temperature changes, with diet having little, if no impact on the faecal microbiota. The increase in water temperature from 10.1 to 18.5°C was associated with an increase in numbers of bacteria belonging to different species in both diets. This however, was associated with a disappearance of LAB and *Acinetobacter* species in our samples. Generally, fish receiving diet B, showed the presence of these bacteria during the warmer months of sampling. It has to be noted...
however that the water temperature between our samples that were collected in February (18.5°C) and March (15.6°C) reached 21°C and therefore, we would expect to have observed much higher numbers of bacteria if a sample had been collected during the peak temperature. Previous studies by Ringø et al. (2, 3) have shown that diet to some extent does influence the microbial communities in the fish gut, which is different from what has been reported by others (7). These contrasting results may be due to differences in the number of samples collected and different ranges of seasonal variation and environmental conditions experienced in these studies. In our study we did not find a major difference in bacterial populations of fish receiving two different commercial diets. However, we found that temperature had a greater influence on the number of bacteria, which is contrary to what was observed in Atlantic salmon in Norway (7). This could be due to the fact that fish sampled in that study did not experience as dramatic an increase in temperatures throughout the year as experienced in our study, or other environmental factors such as the number and type of bacteria present in the water played an important role. Another contributing factor could be that although the fish were receiving similar diets in regards to the major components, these may contain other minor ingredients which effect the fish microbiota during high water temperatures.

In our study, none of the fish showed clinical signs of GI disease as evaluated by the veterinarian, although the number of Vibrio were high and the number of LAB were either low or not present in samples collected during warmer months. Therefore, we postulate that either disappearance of LAB from the hind gut may have allowed other bacteria such as Vibrio sp. to overgrow, or the Vibrio species isolated in our study were non-virulent strains or an improved diet formulation would have protected salmon from experiencing intestinal disease. Lack of any VGs associated with pathogenic Gram-negative bacteria in the total DNA extract from faecal biota of these fish supports the idea that Vibrio species and other bacteria which were detected in higher number in samples collected during the warmer months were not pathogenic and that the higher number of these bacteria in the gut of salmon could be due to an increase in their numbers in the surrounding sea water. We did not test for the different bacterial species in the water however the high numbers of total bacteria sampled from the seawater in this study particularly during warm temperatures supports this theory.
Cytolethal distending toxin (cdt) gene, one of the E. coli specific toxin genes, was detected in all of our samples implying that it might be involved in the pathogenesis of disease under favourable conditions such as high temperature. This toxin, with some sequence variation has also been documented in several other bacteria including Haemophilus ducreyi (44), Actinobacillus actinomycetemcomitans (45), Shigella dysenteriae (46, 47) and some human pathogens such as Helicobacter spp. (48). Another E. coli VG (east1) was also present in several of the early samples but many commensal bacteria have previously been shown to carry genes which have been associated with disease and found in isolation do not imply underlying virulence of these bacteria (49). This as well as the lack of appearance in samples collected during warmer periods of the year, rules out its role in the development of GI diseases of salmon.

Whilst the composition of bacterial species in the gut of Atlantic salmon has been studied and established in the past, there has been a lack of research to date on the potential role of these bacteria on the gut health of the salmon, especially during warm seasons, where water temperature favours growth of potentially pathogenic sea bacteria such as Vibrio species. In our study, the salmon farm did not experience any diseases but the high level of Vibrios observed during the warm periods of sampling was associated with a decrease in the number of LAB especially in fish receiving diet A. The LAB have been attributed various protection mechanisms in the host against pathogens, which include the production of bacteriocin (50, 51), the displacement of pathogens from the gut epithelium (52) as well helping achieve optimal nutritional status (53-55). A decline in the number of these bacteria can impede host immunity favouring growth of opportunistic pathogens, which may result in development of diseases. Therefore, mechanisms that improve the stability of LAB, such as the use of probiotics, during warm periods of the year to competitively exclude pathogens from the gut of Tasmanian Atlantic salmon could be highly beneficial for maintaining the health of salmon throughout their growth cycle and during the warm sea water temperature (56).

This study also included the measurement of the functional status of the gut microbiota. Out of the 48 substrates tested, a maximum of 32 substrates were utilised by gut microbiota of salmon receiving both diets. As the water temperature increased, there
was a visible change in the functional status of the gut biota, with a decrease in substrate utilisation regardless of the diet fish received. This change in the pattern of substrate utilisation of microbiota could be due to either partial utilisation of substrates by the remaining bacteria or replacement of the resident bacterial with new groups which had less metabolic capability, or were incapable of fermenting the substrates provided or a combination of all. There was also a direct relationship between the weight of the fish and the decrease in MC-value of gut microbiota over time for both diets. This decrease in MC value may be due to the reduced bacterial diversity in the gut and establishment of certain transient biota into a more stable community as has been shown to occur in other animal species (21). Periods of high stress on the fish, as temperatures increased and peaked between December 2011 and February 2012, were also marked by an abrupt decline in MC-value. The diet fed to fish during these times of stress however did not seem to determine the extent to which the stressor effected the MC-value of the gut biota as there was a significant difference between diet A and B during this time. Interestingly there were three substrates which were utilised by the microbiota of fish fed diet B and not fish receiving diet A during the warmer sea water temperatures namely galactose, maltose and citrate. In respect to bacterial species and substrate utilisation differences, the lack of LAB in the microbiota of fish fed diet A could indicate that these substrates could serve as potential energy sources for LAB.

In conclusion, our data indicate that whilst diets have an important role on growth of Tasmanian Atlantic salmon, development and the stability of gut microbiota in these fish are more affected by temperature. Although the microbiota of the hindgut does not necessarily reflect all the bacteria interacting with the gut epithelium, it is an indicator of the bacteria present and can still provide valuable insight into the changes in bacterial population structure. The high water temperature experienced in this study influenced the function and diversity of the gut microbiota resulting in high populations of *Vibrio* species and fewer LAB. Whether this bacterial population structure is deleterious for Tasmanian Atlantic salmon farm productivity remains to be determined.
2.6 References


CHAPTER 3

3.0 Population dynamics of Vibrio and Pseudomonas species isolated from farmed Tasmanian Atlantic salmon (Salmo salar L.): a seasonal study

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¹ Genecology Research Centre, Faculty of Science, Health, Education and Engineering, University of the Sunshine Coast, Maroochydore DC, Queensland, ² Tasmanian Institute of Agriculture, University of Tasmania, Hobart, Tasmania,


Author contribution:
As second author in this manuscript my contributions involved the initial experimental design of this study, as well as participating in all of sampling trips to Tasmania, sample processing, some data analysis (enumeration) and lastly critical analysis of the manuscript.
3.1 Introduction
Fish are frequently exposed to bacteria through contact with their skin, gills or gastrointestinal (GI) tract (1). A healthy microbiota may help protect the fish against overgrowth of pathogens (2) and even aid in digestion of substrates through the production of dietary enzymes (3). The GI tract microbiota is thought to stabilise after the first feeding (4), and be dependent on factors such as diet (5, 6), surrounding environments (7), geographical location (8) and antibiotic use (9). Moreover, it has been observed that adverse changes occur to the structure and integrity of the gut epithelium in response to some dietary factors and stress (10-12), which may increase susceptibility of the fish to diseases. *Vibrio* and *Pseudomonas* spp. have previously been shown to be part of the GI microbiota of fish (13, 14) including Atlantic salmon (*Salmo salar* L.) (7, 15, 16). These bacteria have also been identified as causative agents of bacterial infection in many marine animals (17-19). *Pseudomonas* are thought to originate from surrounding environmental waters (7), have a role in fish disease (20) and cause food spoilage (21).
We have recently found that *Vibrio* species dominate the cultureable GI microbiota of farmed Tasmanian Atlantic salmon and that their numbers increase as the surrounding water temperature reaches above 13°C (22). This is normally coupled with a decreased diversity in other bacteria. The seasonal changes of the GI microbiota of farmed Atlantic salmon have only sparsely been studied (15), and we wanted to document the prevalence and persistence of individual strains in these fish over seasons.

3.2 Aims of this study
In this study we aimed to characterise *Vibrio* and *Pseudomonas* strains isolated from Atlantic salmon over a seasonal change and investigate their prevalence and persistence in these fish receiving two commercially available diets.

3.3 Materials and Methods
3.3.1 Sampling and isolation of bacterial strains
*Vibrio* and *Pseudomonas* strains were isolated from faeces of commercially farmed Tasmanian Atlantic salmon (*S. salar* L.) on eight occasions between July 2011 and May 2012 as described in Chapter 2.3.1. Atlantic salmon were fed with two very similar
commercially available dietary regimes (designated A and B) which contained very similar protein, lipid and digestible energy content and neither diet was supplemented with probiotics (see Chapter 2.3.1).

Single colonies from TCBS agar were purified on tryptone soy agar (TSA; Oxoid) supplemented with 1% NaCl. Pure isolates were maintained on TSA supplemented with 1% NaCl and frozen at -80°C for long-term storage in 20% glycerol in tryptone soy broth (TSB; Oxoid) supplemented with 1% NaCl. Initial identification was performed with pure colonies that were tested for cytochrome oxidase, catalase activity, motility and Gram staining.

3.3.2 Identification of bacterial strains

3.3.2.1 DNA extraction

Bacterial DNA was extracted using a Bioline ISOLATE II genomic DNA kit (Bioline Australia) following the manufacturer’s guidelines for cell culture extractions with the following adaptation. Two to three colonies from fresh culture were suspended in sterile water as the starting material. Extracted DNA was visualised using gel electrophoresis on a 1% agarose gel pre-stained with ethidium bromide, run at 100 V for 40-60 mins and viewed under UV. DNA was stored at -20°C for further use.

3.3.2.2 Bacterial identification

*Vibrio* and *Pseudomonas* species were initially identified using PCR with genus-specific primers. *Vibrio* species were tested for the presence of the *rpoA* gene using primers which specifically target *Vibrio* species as described before (23). The PCR reaction mixture contained 1 × reaction buffer, 2 mM MgCl₂, 0.2 mM of each dNTP, 0.5 μM of each primer, 1 unit of *Taq* (Bioline) and 1μL of template DNA. The reaction volume was adjusted to 25 μL with sterile water. The PCR cycle was as previously described (22). *Pseudomonas* species were confirmed by screening for previously published genus-specific primers using the previously described reaction mix and thermocycling protocol using a BioRad thermocycler (24).

Confirmation of the isolates was achieved using 16S rRNA gene sequencing PCR using the universal bacteria/archaea specific P11 and bacteria specific P12 primers (25). PCR reaction mixture was made to a final volume of 25 μL and consisted of 12.5 μL Mango
mix (Bioline), 0.2 µM of each primer and 1 µL of template DNA. PCR cycling conditions were as follows: 95°C for 3 min, 30 cycles of 94°C for 30 s, 50°C for 1 min and 72°C for 3 mins with a final extension at 72°C for 6 min on a BioRad Thermocycler. PCR products were sent to Macrogen, Korea for purification and sequencing. The Sequencher v4.8 software (Gene Codes Cooperation, USA) was used to clean up and align sequence reads and searched for matching sequences using the blastn function on the NCBI website (www.blast.ncbi.nlm.nih.gov).

Based on the sequencing results, *Pseudomonas* strains were identified as *P. fragi*. This was confirmed using previously published *P. fragi* species-specific PCR targeting the *carA* gene (21).

### 3.3.3 Typing of isolates

#### 3.3.3.1 PhP typing

*Vibrio* strains were typed using the PhP-RV plates (PhPlate AB, Stockholm, Sweden), which have been specifically developed for typing of *Vibrio* strains, according to manufacturer’s instructions. Briefly, colonies were suspended in the first well of each row containing 325 µL of growth medium comprised of 0.011% bromothymol blue, 0.05% proteose peptone (Difco, Australia), 2% NaCl and 0.8% phosphate buffer (pH 7.5) adjusted to final pH of 7.8 – 8 and aliquots of 25 µL of the suspension were dispensed into the remaining 11 substrate wells of each row containing 150 µL of the growth medium. Plates were then incubated at 28°C and images of the plates were scanned at 16, 40 and 64 hours using a HP desktop scanner. Images were imported into the PhPWIN v6.1 software and were converted into numerical values. Data were then analysed using pairwise comparison and clustered using the unweighted pair group method with arithmetic mean (UPGMA) method. Strains, showing similarity above the established identity of the system (97.5%), were regarded as identical and assigned to a common type (CT), and those below the identity level were regarded as single types (STs) (26).

#### 3.3.3.2 Random amplified polymorphic DNA (RAPD) typing

Bacterial DNA was extracted from pure colonies of a fresh culture by boiling 2-3 colonies grown on TSA supplemented with 1% NaCl in 100 µL of sterile Tris-EDTA (TE) buffer at 95°C for 5 min. DNA was stored at -20°C. Strains were typed using the
PB1 (5-GCGCTGGCTCAG-3) primer as previously described (27). PCR products were visualised on a 1.5% agarose gel run at 90 V for 5 hours, viewed and photographed under UV. Images were imported to the GelCompare II software version 5.1 (AppliedMath, Belgium) for analysis. Dendrograms were created using the UPGMA method with Dice’s coefficient. CT of *Pseudomonas* strains were determined based on their RAPD profiles whereas *Vibrio* CT were determined based on a combination of RAPD profiles and their BPTs.

### 3.4 Results

Identification of the isolates using a combination of genus-specific primers and 16S rRNA gene sequencing showed that *Vibrio* isolates belonged mainly to *V. ichthyoenteri/V. scophthalmi* (n=240) (indistinguishable from one another by 16S rRNA gene sequencing), *Aliivibrio finisterrensis* (n=38) and two other less prevalent strains of *Vibrio* or *Photobacterium* (Table 3.1). Typing of *Vibrio* isolates showed that the dominant *V. ichthyoenteri/V. scophthalmi* strains belonged to 11 CT (CTs) and *Aliivibrio*, the second predominant genera belonged to seven CTs (Figure 1a). These CTs consisted of two to 84 isolates per CT with several strains belonging to single types (ST) in each group (Table 3.1). Amongst *V. ichthyoenteri/V. scophthalmi* strains seven clones were found in fish receiving either diet and were also found in two or more sampling occasions (Table 3.2). Furthermore, the majority of these strains were only found during the summer months when the water temperature exceeded 13°C and the number of *Vibrio* spp., compared to cooler months of the year, was higher (Table 3.2). Despite this increase, the overall proportion of *Vibrio* isolates to the total number of bacteria in the Atlantic salmon faeces was lower than during the cooler months of the year (Table 3.2). The *Aliivibrio* spp., although found in fish receiving either diet, were not persistent and were found sporadically at different sampling occasions (Table 3.2).

Of the 150 *Pseudomonas* isolates, 142 (95%) were identified as *P. fragi*. Typing of these isolates showed the presence of 18 CTs consisting of two to 25 isolates per CT and 53 ST (Figure 4.1b, and Table 3.1). Almost all of these CTs were isolated in the early sampling occasion in spring (September 2011), where the water temperature was 12.3°C. These strains however, disappeared as the temperature increased and re-appeared in samples collected in early autumn (March 2012) when the water
temperature had started to substantially decline (Table 3.3). Although the proportion of these strains over the total number of bacteria was negligible they were more prevalent in fish receiving diet A rather than diet B (Table 3.3).

**Table 3.1:** List of *Vibrio* and *Pseudomonas* species isolated from farmed Tasmanian Atlantic salmon via 16S rRNA gene sequencing. Clonality of the strains was determined by RAPD typing and for *Vibrio* spp. it was a combination of RAPD typing and biochemical fingerprinting

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of isolates</th>
<th>No. CTs (no. of isolates in all CT)</th>
<th>No. of STs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vibrio</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Vibrio ichthyoenteri/V. scophthalmi</em></td>
<td>240 (71 %)</td>
<td>11 (202)</td>
<td>38 (11 %)</td>
</tr>
<tr>
<td><em>Vibrio crassostreae/V. splendidus</em></td>
<td>1 (0.3 %)</td>
<td>0</td>
<td>1 (0.3 %)</td>
</tr>
<tr>
<td><em>Aliivibrio finisterrensis</em></td>
<td>38 (11 %)</td>
<td>7 (28)</td>
<td>10 (3 %)</td>
</tr>
<tr>
<td><em>Photobacterium phosphoreum</em></td>
<td>7 (2 %)</td>
<td>1 (2)</td>
<td>5 (1.5 %)</td>
</tr>
<tr>
<td>Unidentified <em>Vibrio</em> spp.</td>
<td>54 (16 %)</td>
<td>2 (6)</td>
<td>48 (14 %)</td>
</tr>
<tr>
<td><strong>Pseudomonas</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas fragi</em></td>
<td>142 (95 %)</td>
<td>17 (95)</td>
<td>47 (32 %)</td>
</tr>
<tr>
<td>Other <em>Pseudomonas</em> spp.</td>
<td>8 (5 %)</td>
<td>1 (2)</td>
<td>6 (4 %)</td>
</tr>
</tbody>
</table>

* Indistinguishable from another by 16S rRNA gene sequencing
Table 3.2: Prevalence and persistence of Vibrio CTs and STs isolated from farmed Tasmanian Atlantic salmon fed either diet A or B between July 2011 and May 2012. The shaded area indicates the Australian summer months. The Proportion of Vibrio spp. (black shading) to the total number of hindgut bacteria (white shading) is shown as pie charts. Boxed areas show the presence of the same CT in fish receiving either diet.

<table>
<thead>
<tr>
<th>Sampling month</th>
<th>Diet A</th>
<th>Diet B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2011</td>
<td>2012</td>
</tr>
<tr>
<td>Water temp. (°C)</td>
<td>10.1°C</td>
<td>12.3°C</td>
</tr>
<tr>
<td></td>
<td>July</td>
<td>Sept</td>
</tr>
<tr>
<td>Proportion of Vibrio spp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTs (no. of isolates)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V. ichthyoenteri/V. scophthalmi</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT1 (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT2 (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT3 (53)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>CT4 (17)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>CT5 (4)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>CT6 (4)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>CT7 (84)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>CT8 (9)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>CT9 (12)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>CT10 (10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT11 (5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. finisterrensis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT1 (3)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>CT2 (4)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>CT3 (2)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>CT4 (5)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>CT5 (6)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>CT6 (2)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>CT7 (6)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>P. phosphoreum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT19 (2)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Other Vibrios</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT20 (4)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>CT21 (2)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>STs 1-102</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>
Table 3.3: Prevalence and persistence of *Pseudomonas* CTs and ST isolated from farmed Tasmanian Atlantic salmon fed either diet A or B between July 2011 and May 2012. The shaded area indicates the Australian summer months. The proportion of *Pseudomonas* (black shading) isolates over the total number of hindgut bacteria (white shading) have been shown as pie charts.

<table>
<thead>
<tr>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>July</td>
<td>Sept</td>
<td>Nov</td>
<td>Dec</td>
</tr>
<tr>
<td></td>
<td>10.1°C</td>
<td>12.3°C</td>
<td>13°C</td>
<td>16.2°C</td>
</tr>
<tr>
<td>Proportion of <em>Pseudomonas</em></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>CTs (no. of isolates)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. fragi</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT1 (2)</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT2 (9)</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT3 (3)</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT4 (25)</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT5 (3)</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT6 (2)</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT7 (4)</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT8 (2)</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT9 (4)</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT10 (4)</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT11 (2)</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT12 (5)</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT13 (11)</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>CT14 (2)</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT15 (3)</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT16 (12)</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT17 (2)</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other <em>Pseudomonas</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT18 (2)</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>STs 1-53</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>
**Figure 3.1:** UPGMA clustering of *Vibrio* (a) and *Pseudomonas* (b) strains showing genetic relatedness of the strains isolated over one year from Tasmanian Atlantic salmon between July 2011 and May 2012. CTs for *Vibrio* spp. (a) were established by a combination of RAPD typing and a biochemical fingerprinting method. CT5-7, CT9-10 and CT13-18 indicate that the strains belonging to these clusters had identical RAPD pattern but different biochemical phenotypes.
3.5 Discussion

*Vibrio* species have been found in the GI tract of many marine animals including salmon (17-19). Most studies, investigating the prevalence of these bacteria in salmon have focused on one time sampling occasions and have not studied changes in the population structure of *Vibrio* spp. over time (5, 7, 16). Most recently, Hovda and colleagues studied seasonal variation in the gut microbiota of Norwegian Atlantic salmon and identified that *Vibrio* spp. were only found in certain samples (15). In our study, we observed an increase in the number of *Vibrio* as the temperature increased and that the *V. ichthyoenteri*/*V. scophthalmi* group were the major species dominating the *Vibrio* population found during the warmer months. This could be mainly due to the fact that the Norwegian Atlantic salmon do not normally experience the persistent high temperatures (>15°C) during the warmer months of the year, as experienced by the farmed Tasmanian Atlantic salmon in Australia. Interestingly, we found that these bacteria belonged to several CTs, most of which appeared at water temperatures above 13°C. Nonetheless, these data suggest that *V. ichthyoenteri*/*V. scophthalmi* could have better adapted to colonisation of the Atlantic salmon GI tract and increased in number under favourable conditions such as increased water temperature. A similar finding has also been reported by Hovda et al. (15), although these researchers also found the presence of *V. ichthyoenteri* during the cooler months of the year. In the absence of any typing of isolates in their study it is not possible to determine if the isolates found during the cooler months of the year were in fact members of the same clone found in the warmer months. This is further supported by our findings that some CTs of *V. ichthyoenteri*/*V. scophthalmi* were also found in samples collected when the water temperature was below 12°C.

In this study we used 16S rRNA gene sequencing for identification of our isolates however it was not possible to distinguish between *V. ichthyoenteri* and *V. scophthalmi* species, as they are genetically very closely related species. Whilst both species have been found as part of the dominant microbiota in a variety of aquatic animals (15, 28), the host specificity of *V. scophthalmi* has been demonstrated (29), thus suggesting that our *Vibrio* strains are more likely *V. ichthyoenteri* as previously reported in Atlantic salmon (15). *V. ichthyoenteri* has also been found in higher percentages in Senegalese sole (*Solea senegalensis*) fed with a commercial fishmeal based diet (30). In our study,
these bacteria were present in the fish throughout the warmer months and this observation suggests *V. ichthyoenteri* may prosper in situation involving commercial fishmeal based diets. The almost exclusive presence of the *V. ichthyoenteri/V. scophthalmi* group as a constituent of the *Vibrio* population could also suggest a loss of microbial heterogenicity making the fish more vulnerable to opportunistic pathogens (31). This is not unexpected, as it has been previously shown that fish fed commercial diets have a microbiota of comparative lower diversity than their wild counterparts (31). Similarly, captured wild fish have also shown a reduction in the microbiota diversity upon feeding with manufactured diets (32).

The previously non-attended *Allivibrio finisterrensis* was the second most numerous *Vibrio*-like species detected and is a newly described member of the genus *Aliivibrio*, originally isolated from Manila clams, *Ruditapes philippinarum* (33). These bacteria were mainly found during the warmer months although they were not as persistent in the Atlantic salmon as *V. ichthyoenteri*. The fact that these bacteria were also found in multiple samples suggests that they may have been present in the Atlantic salmon gut throughout the warmer months, but in low numbers that escaped our detection. Little is known about the role of these bacteria in the pathogenesis of fish diseases but it has been shown that they are siderophore producers (34). Involved in acquisition of iron from the host, the ability to produce siderophores is regarded as a virulence factor for bacteria (34). However, whether this plays a role in the virulence of this organism towards Atlantic salmon remains to be elucidated.

*Pseudomonas fragi* was also isolated in high numbers in this study. This bacterium however, was only found in the hind gut of salmon either during the cold months of the year or in samples collected during March 2012 when the water temperature was still high i.e. 15.9°C. Typing of these isolates showed a considerable heterogeneity among their population with 17 CTs identified in only two sampling occasions. Whilst this observation was rather surprising, we postulate that these bacteria were outnumbered by the overgrowth of *Vibrio* spp. that occurred during the warmer months of the year. This is supported by the finding that six of the CTs of these bacteria were found in samples collected during September 2011 and March 2012 where the number of *Vibrio* decreased allowing detection of the same clones of *P. fragi* in the gut of the salmon. The presence and/or prevalence of these bacteria however was higher in some fish
however whether this is due to a higher prevalence of these bacteria within the fish gut remains to be elucidated. Alternatively, this could be explained by the fact that in other fish the *Vibrio* strains appeared in the Atlantic salmon hindgut in high number during September 2011 whilst others experienced a high number of *Vibrio* in November. This consequently, could have resulted in less ability to detect *P. fragi* in samples collected from the fish during September 2011.

Other bacteria such as *P. phosphoreum* were also isolated in low numbers (mainly single types) and on three occasions during the study. These bacteria as well as *P. fragi* have both been associated with spoilage of European fish products (21, 35) and their presence in the GI tract of European farmed fish has been noted previously (16). However, the infrequent isolation of these bacteria may indicate that they do not play an important role in the health and stability of the microbiota of farmed Tasmanian Atlantic salmon.

In conclusion, our findings suggest that water temperature has a great effect on the composition of the hindgut microbiota of farmed Tasmanian Atlantic salmon. The higher temperature normally experienced by these fish during summer was shown to promote the growth of *V. ichthyoenteri/V. scophthalmi*. Whilst several CTs of these bacteria were isolated during the summer, certain clones were found to be better adapted to the hindgut of these fish. However, there is also a possibility that their persistence in our samples could have been due to their constant reintroduction from the surrounding water. We also found that such high dominance of *Vibrio* during warmer seasons may outnumber bacteria such *P. fragi*, which in our study was demonstrated by the lack of detection of the same CTs of these bacteria during the warmer months.
3.6 References


CHAPTER 4

4.0 The effect of fish meal composition on the faecal microbiota of farmed Tasmanian Atlantic salmon (*Salmo salar* L.)

Neuman C\(^1\), Hatje E\(^1\), Smullen R\(^3\), Bowman JP\(^2\) and Katouli M\(^1\).

\(^1\)Genecology Research Centre, Faculty of Science, Health, Education and Engineering, University of the Sunshine Coast, Maroochydore DC, Queensland, \(^2\)Tasmanian Institute of Agriculture, University of Tasmania, Hobart, Tasmania, \(^3\)Ridley AquaFeed Pty, Narangba, Queensland, Australia

4.1 Introduction

The gut microbiota of Atlantic salmon has been extensively studied using both culture based and molecular techniques. These studies have identified genera which predominate within the gut including *Vibrio, Pseudomonas, Acinetobacter, Plesiomonas, Aeromonas* (1) and LAB (1, 2). LAB, namely *Lactobacillus* and *Lactococcus*, as well as *Photobacterium phosphoreum*, are dominant groups of bacteria within the gut microflora (3, 4). Many factors can influence the composition of the gut microbiota of adult fish including geographical location (4) and the resulting bacteria present in the surrounding water (1) and the initial microbial composition of the gut after hatching and during the juvenile stages (5). Recently seasonal temperature fluctuations have also been identified as a factor of influence on the gut microbiota (6).

Diet components have been shown to not only affect the health and growth of the fish but are also an important factor for both the development of and shifts in the gut microbiota (7-9). Due to the continual growth of the aquaculture industry increasing cost of fishmeal (FM) and the ecological impact of fishing to feed farmed fish (10), there is a need to find suitable alternative protein sources, such as pea protein (11) or soybean meal (SBM) (12), for use in fish feed formulations (13). The substitution of SBM has been shown to adversely affect Atlantic salmon growth and gut morphology (14-16). The use of prebiotics has been shown to alleviate symptoms by acting in a protective manner on the gut epithelium (17), which could be attributed to modulation of the gut microbiota (18).

Several studies have assessed the impact of variations in the FM inclusion levels on the microbiota of Atlantic salmon (19, 20). Only one study however has assessed such impact during the high summer temperatures (21) but the functional status of the gut microbiota in respond to variation in FM is yet to be evaluated.

4.2 Aims of the study

In view of the above, this study was undertaken to examine changes in the population structure of the gut microbiota of farmed Tasmanian Atlantic salmon in response to varying fishmeal levels and prebiotic additives in the diet.
4.3 Methods

4.3.1 Sampling
Between December 2011 and March 2012 a total of 260 faecal samples were collected over four sampling occasions, roughly every 4 weeks, from Atlantic salmon, reared in sea cages in a commercial fish farm in south of Hobart, Tasmania. Fish were farmed according to standard commercial practices, which included feeding to satiation, examination for Amoebic Gill Disease and subsequent fresh water bathes upon clinical signs of disease. The initial pen from which fish were divided into the feed trial pens was stocked with 61,000 female smolts receiving a standard commercial diet. In December 2011, these fish (pre-trial) were divided into four trial pens, each stocked with 420 fish. From the pre-trial pen and from each trial pen, randomly receiving one of the four different trial diets, 20 fish were caught at random via netting, at each sampling round and anaesthetised using 17ppm isoeugenol (Aqui-S®) according to the commercial fish farms’ procedure, before being gently squeezed to collect the faecal material by the fish farm veterinarian in sterile 50 mL centrifuge tubes. The composition of each of the four trial feed is provided (Table 4.1). Due to the sensitive nature of the feed formulations the composition of the agri-pro prebiotic supplement cannot be provided, however no lactic acid bacteria were used in any of the diets.

<table>
<thead>
<tr>
<th>Components in the fish feed</th>
<th>Trial diet (% Composition of components)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diet 1</td>
</tr>
<tr>
<td>Fat</td>
<td>25</td>
</tr>
<tr>
<td>Vegetable protein</td>
<td>30</td>
</tr>
<tr>
<td>Fishmeal</td>
<td>30</td>
</tr>
<tr>
<td>Land animal protein</td>
<td>14</td>
</tr>
<tr>
<td>Prebiotic supplement</td>
<td>yes</td>
</tr>
<tr>
<td>Digestible energy (Mj/kg)</td>
<td>20.8</td>
</tr>
</tbody>
</table>

Faecal samples were collected from healthy fish, which showed no signs of clinical infection and had not been recently (within the previous 14 days) subjected to fresh water bathing (22). Average fish weights per pen ranged from 1.5 kg (in the first sampling round in December 2011) to 2.44 kg (in the last samples obtained March 2012). At each sampling occasion the water temperature was recorded and ranged from 16°C in December 2011, peaking to 19°C in February and falling back to 15.6°C in
May 2012. Oxygen concentrations were monitored as per standard farming practices and dissolved oxygen concentrations ranged from 5-8.4 mg/L and salinity of the water remained between 34-35 ppt throughout the duration of this study. The sampled pens were located within close proximity to each other (2 m) and the same population of fish was sampled throughout this study. Over the course of the study, fish were switched from the commercial dietary regime to the respective trial diets in December 2011. The faecal samples (n=20) from each pen were pooled into two groups (n=10) per diet to determine intra assay variations and also to eliminate individual differences between fish so that fluctuations in the overall bacterial populations could be assessed. The pooled faecal samples were placed on ice, transferred to the laboratory and processed within 2 h.

4.3.2 Bacterial enumeration, functional status and VG profiles

Samples were processed and bacteria were enumerated as described in section 2.3.2. Identification of bacterial species was performed after growth on selective agar (section 2.3.2) and identified to the species/ genus level using 16S rRNA gene sequencing as described before (section 2.3.2).

All faecal samples were also assessed for the functional status and metabolic capacity of the faecal microbiota as described in section 2.3.3. Total DNA of faecal microbiota was extracted as described before (section 2.3.4) and used to test for the presence of virulence genes using species-specific primers as described in section 2.3.4 (Table 2.4).

4.3.3 Statistics methods

Student’s t-test was used to compare the number of bacteria belonging to Vibrio spp. and LAB to the other bacterial populations. Significance was established as P < 0.05. Student’s t-test was used to compare the MC-value obtained at each sampling occasion between two diets. Significance is established as P < 0.05.
4.4 Results

4.4.1 Bacterial enumeration
Cultivatable bacterial species did not vary greatly between the trial diets or throughout the sampling period. An increase in total bacterial numbers was observed as the water temperature increased with the highest bacterial counts detected during March for most bacterial species, except LAB (Table 4.2). *Acinetobacter* spp. declined in numbers during higher water temperature, i.e. January and February, whilst *Pseudomonas* spp. were never detectable using cultivation methods in any samples collected throughout the trial.

In the samples collected during higher water temperature, LAB were undetectable in both of the low fishmeal diets (diet 3 and 4) and the high fishmeal containing the prebiotic diet (diet 1). The high fishmeal diet (diet 2) however sustained LAB for longer and in high numbers until February (Table 4.2) when temperatures peaked. Generally, the diets containing either high fishmeal or the prebiotic supplement contained cultivatable LAB in higher numbers.
Table 4.2: Number of bacterial species (CFU/g of faecal material) found in the faecal samples of Atlantic salmon over 4 months of sampling from 2011 to 2012. Fish were fed one of four trial diets, diet 1 (high fishmeal (FM) with prebiotic), diet 2 (high FM), diet 3 (low FM) and diet 4 (low FM with prebiotic).

<table>
<thead>
<tr>
<th>Diet</th>
<th>December</th>
<th>January</th>
<th>February</th>
<th>March</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water temperature (°C)</td>
<td>Total bacterial count</td>
<td>Vibrio spp.</td>
<td>Aeromonas spp.</td>
</tr>
<tr>
<td>Diet 1</td>
<td>16</td>
<td>1.4 x 10^6</td>
<td>4.8 x 10^4</td>
<td>4.2 x 10^5</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>1.7 x 10^7</td>
<td>1.9 x 10^5</td>
<td>2.9 x 10^5</td>
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<tr>
<td></td>
<td>19</td>
<td>1.7 x 10^7</td>
<td>1.6 x 10^5</td>
<td>1.3 x 10^6</td>
</tr>
<tr>
<td></td>
<td>16.6</td>
<td>3 x 10^9</td>
<td>3 x 10^8</td>
<td>3 x 10^8</td>
</tr>
<tr>
<td>Diet 2</td>
<td>14</td>
<td>1.4 x 10^6</td>
<td>4.8 x 10^4</td>
<td>4.2 x 10^5</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>2.4 x 10^7</td>
<td>3 x 10^5</td>
<td>4.5 x 10^6</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>4.9 x 10^6</td>
<td>3 x 10^5</td>
<td>2.1 x 10^6</td>
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<td>19</td>
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<td>16</td>
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<td>1.8 x 10^5</td>
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<tr>
<td></td>
<td>17</td>
<td>3 x 10^6</td>
<td>2.5 x 10^5</td>
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<td></td>
<td>19</td>
<td>3 x 10^9</td>
<td>3 x 10^8</td>
<td>3 x 10^8</td>
</tr>
<tr>
<td>Diet 4</td>
<td>14</td>
<td>1.4 x 10^6</td>
<td>4.8 x 10^4</td>
<td>4.2 x 10^5</td>
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<td>17</td>
<td>1.8 x 10^7</td>
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<td>19</td>
<td>3 x 10^9</td>
<td>3 x 10^8</td>
<td>3 x 10^8</td>
</tr>
</tbody>
</table>

4.4.2 Functional status and metabolic capacity

The metabolic functions of the faecal microbiota of fish were compared pairwise and a similarity between the over functional status of the microbiota was established as described earlier (section 2.3.3). Results showed that gut microbiota of fish receiving the high FM with prebiotics and the low FM, i.e. diet 1 and 3, and the high FM diet and low FM diet with prebiotic, i.e. diet 2 and 4, followed a similar trend. There was also no
significant difference between any of the diets in relation to their MC at any of the sampling points (p < 0.05). There was less than 50% similarity between the December and February samples with any of the other faecal samples from the gut microbiota of fish fed diets 2 and 4. However a high similarity (>80%) was seen between the January and March samples from fish receiving the high FM diet (diet 2) (Figure 4.1). On the other hand, the functional status of the microbiota of fish receiving diets 1 and 3 showed higher than 50% at each sampling point. Interestingly the low FM diet (diet 3) showed the highest similarity to initial sample in December (Figure 4.1).

Testing for the presence of virulence genes associated with Gram-negative pathogenic bacteria showed the presence of two genes i.e. cytolethal distending toxin (cdt) in most DNA extracts of faecal microbiota was present in all faecal samples regardless of the diet received by the salmon. The heat stable enterotoxin (east1) gene was also detected in the initial samples collected in fish receiving the high FM with prebiotic diet (diet 1) during January and February (Figure 4.1).

![Figure 4.1:](image)

**Figure 4.1:** Similarity between the patterns of substrate utilization by the gut microbiota of Tasmanian Atlantic salmon fed one of four trial diets, diet 1 (high FM with prebiotic) (1), diet 2 (high FM) (2), diet 3 (low FM) (3) and diet 4 (low FM with prebiotic) (4), over 4 months of sampling from 2011 to 2012. *cdt:* cytolethal distending toxin; *east1:* a thermostable toxin found among enteroaggregative *E. coli.*
Figure 4.2: The MC value of the gut microbiota of salmon fed one of four trial diets, diet 1 (high FM with prebiotic), diet 2 (high FM), diet 3 (low FM) and diet 4 (low FM with prebiotic), over 4 months of sampling from 2011 to 2012.

4.5 Discussion

Small changes in the cultureable microbiota of Tasmanian Atlantic salmon as well as their functional status and MC-value were observed in response to the supplementation of different FM and prebiotics to the fish feed. The increase in water temperature was associated with an increase in the total number as well as some of the bacterial species from the pre-trial commercial diet to the trial diet despite the increase in temperature. Previous reports indicate that the number of bacteria in the gut will normally decrease in response to dietary changes (23). Interestingly, this increase in the number of gut microbiota was associated with an increase in water temperature. We have previously shown a decrease in the number and diversity of the gut microbiota of Tasmanian Atlantic salmon during the period of high water temperature (Chapter 2) (6). There are several reasons for this, including the fact that the water temperature was already high at the time of the initial sampling and therefore, we might have missed shifts in the number of bacteria in the fish samples. Alternatively, it could be due to the fact that the trial feed contained FM that may support the microbiota of the gut. Other factors such as stocking density (24) might have a role by reducing the stress level on caged fish. Supplementation with prebiotics did not show any effect on the number of cultivatable
LAB in this study. This is not entirely surprising as the role of prebiotics is to stimulate the growth of LAB in the gut and is usually inferred through the presence of increased bacterial numbers in the faecal material (25), however the site of colonization may not coincide with the sampling site which may cause difficulties in the verification of colonisation in vivo (26). Other studies have also shown that synbiotics, which is the combined use of pre- and probiotics, improves the colonisation of probiotic strains (27) and this could be an attractive avenue for future feed trials.

Ringsø et al. (2006) have shown that fish fed the high FM diets have higher total viable bacterial counts, as well as a different microbiota composition compared to fish receiving a SBM diet (28). Increased bacterial diversity has also been observed in individual fish fed diets with increasing SBM levels (21). This was not observed in this study where the number of cultivatable bacterial species present in the gut microbiota of fish fed either the high or low FM diet remained very similar with only one log difference at any one time. We postulate that this could be partly due to the presence and to a certain extent the concentration of FM in the diets. This was not unexpected, as other studies have also shown that diet components have an effect on the gut microbiota composition (29, 30). In this respect, the supplementation of the prebiotic was only affecting the concentration of LAB and that was only seen in the high FM diet trial.

This may also be a direct effect of the use of culture based techniques in this study instead of using a molecular based method such as 16S rRNA gene sequencing which would make it possible to detect smaller changes in the microbiota including species which are non-cultureable (23). In this study however, the aim was to detect major shifts rather than small variations that may occur in the gut microbiota upon feed composition. Nonetheless, the measurement of the overall metabolic function of the gut microbiota in combination with the traditional culture-based technique provided additional information on the impact of feed composition on gut microbiota of salmon.

The number of LAB varied between the four diets. The high FM in combination with the prebiotic supplement in diet 1, as well as the low FM diets (diets 3 and 4), did not sustain LAB populations as the water temperature increased, however the high FM diet alone (diet 2) promoted the growth of these species. This is an interesting observation as our previous study suggests that the inclusion of prebiotics into feed during the summer months may help sustain LAB. In fact, supplementation of fish feed with prebiotics has...
previously been shown to support beneficial species in the fish gut (13). A decrease in LAB in response to the low FM supplement was contraindicative to results observed previously in Rainbow trout where an increase in LAB was observed in fish fed plant-based food (23, 31, 32). The use of molecular techniques may improve the detection sensitivity of shifts in the microbiota in response to varying FM inclusion levels.

The gene encoding for cytolethal distending toxin was present in all samples obtained from the fish, regardless of the sampling time or diet received. This toxin has been found in *E. coli* as well as several other bacteria including *H. ducreyi* (33), *A. actinomycetemcomitans* (34), *S. dysenteriae* (35, 36) and some *Helicobacter* spp. (37). The presence of this VG in all faecal samples tested could indicate the presence of one or more of the above bacteria in the faecal samples of fish. In the present study we did not focus on detecting these opportunistic pathogens and therefore cannot comment on the presence and role of this gene in the pathogenesis of fish disease especially as all fish from which faecal samples were collected were quite healthy. Interestingly, we identified the presence of this virulence gene in faecal samples of salmon in our previous study (Chapter 2). Heat stable enterotoxin (*east1*) was also detected in the initial samples as well as the high FM diet containing the prebiotic supplement (diet 1). Although no major differences between cultivatable bacterial species were observed, this VG in certain samples could allude to the presence of bacterial species which cultivation based methods cannot detect or were not tested for in our study.

In general there was no similarity of the functional status of the microbiota in relation to the FM or prebiotic supplement. Furthermore, there was not a great difference in the bacterial population diversity between the diets, however the ability of the faecal microbiota to utilise substrates was lower in the diets containing the low FM with or without the prebiotic supplement. This may be due to the negative impact that the substitution of plant-based proteins can have on the GI health (38) and possibly by extension the GI microbiota of salmon. The decreased MC value observed in diet 2 compared to diet 1 may be due to the presence of a higher number of LAB in the fish receiving diet 2, which tend to have a slower metabolic capability than some Gram-negative species.
The MC value of the faecal microbiota varied greatly between the high and low FM diets, as well as between the prebiotic and non-prebiotic diets high FM diets, despite the fact that there was no difference between the number of cultivatable bacteria in samples from all diets. This was interesting as the MC value is an indication of the microbiota as a community to utilise substrates and may indicate either the presence of different bacterial species in samples from the high FM /prebiotic supplement diets, which were not assessed in this study, or could be due to the presence of several highly metabolically active clones of the same bacterial species found in the fish receiving the other diets. It has been shown that there is a direct relationship between the weight of the fish and the decrease in MC-value of gut microbiota, normally due to the reduced bacterial diversity and the establishment of a stable community (39). This was only observed in the low FM diets (diet 3 and 4). The abrupt decline in MC value in fish receiving the low FM diets, between December and February, as was previously noted (Chapter 2) and it is speculated that the diets used in the previous study may also have been low in FM composition.

In conclusion, the supplementation of varying levels of FM in the fish feed was not able induce shifts in the microbiota of Atlantic salmon studied. However, the MC value of the microbiota of fish fed the high FM diet was higher than other groups. Despite this observation, the inclusion of prebiotics did not effect the number of cultivatable LAB. Further studies, however, using molecular techniques may help allude to the other changes occurring in the microbiota in response to differing FM levels.
4.6 References


CHAPTER 5

5.0 Characterisation of lactic acid bacteria isolated from the hindgut of farmed Tasmanian Atlantic salmon (Salmo salar L.)

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Image source: http://www.nutraingredients-usa.com/Research/Probiotics-may-counter-obesity-and-diabetes-NIH-study
5.1 Introduction
The aquaculture industry has undergone rapid growth within the last 20 years as a result of both market demand and ocean fish stock depletion (1, 2). This rapid growth has led to challenges such as the control of disease and ensuring the health and growth of fish stocks, which is managed through the use of both antibiotic and antifouling agents (3). The large-scale use of antibiotics over the past decade has led to the growing problem of antibiotic resistance in both fish pathogens and commensals (4-6). Antibiotic use has also been shown to disrupt the normal microbiota, and in fish suffering from gastrointestinal (GI) tract infections, can greatly increase disease susceptibility and the likelihood of further infection (7, 8). Unregulated use can also have public health consequences due to the consumption of food contaminated with antibiotic resistant bacteria or exposure to animals fed antibiotics causing increased risk of colonisation or infection of humans with resistant bacteria or inducing antimicrobial resistance in the normal microbiota (9). As the industry is seeking more sustainable and environmentally friendly methods, the use of probiotics offers a suitable alternative, although it is yet to be routinely applied to large-scale operations. Since the first application of a probiotic in aquaculture in 1986 (10) many proposed probiotic candidates have been identified, most of which are either lactic acid bacteria (LAB) or belong to genera *Vibrio*, *Bacillus* or *Pseudomonas* (11, 12). In aquaculture the desired health benefits conferred by probiotics is through improvement to the gut microbiota (11) by pathogen inhibition (13, 14), increased nutrient digestibility (15, 16) and increased stress tolerance of the fish (15, 17). This improvement to fish health in turn leads to a decrease in mortality rate, and subsequently a decrease in financial losses (18, 19). Before probiotic strains can be approved for commercial use, they need to be identified and characterised for their *in vitro* and *in vivo* function to assess their safety in animals produced for food (20).

5.2 Aims of this study
The aim of this study was to characterise a collection of LAB, isolated from the faecal microbiota of Atlantic salmon maricultured in southeastern Tasmania, for their potential use as a probiotic in the industry.
5.3 Methods

5.3.1 Sampling and LAB isolation
Faecal samples were collected from two farms as previously described (section 2.3.1 and 4.3.1) Faecal samples were diluted (1:1 w/v) in sterile PBS (pH 7.4). One hundred microliters was spread on MRS agar supplemented with 1% nystatin, dissolved in DMSO, to inhibit the growth of fungus and yeast. Plates were incubated at 20°C for 24-48 hours. Isolates were purified on MRS agar, Gram-stained, tested for oxidase reaction and maintained in MRS broth (Oxoid) with 20% glycerol at -80°C.

5.3.2 Biochemical fingerprinting of LAB
Putative LAB isolates were typed using a rapid biochemical fingerprinting method PhP-LB (PhPlate AB, Stockholm, Sweden) specifically developed for typing LAB. The bacteria were each mixed with 8 mL of PhP-LB medium and 150 µL of this suspension was inoculated into designated rows (24 substrates) in the 96-well microplates. Plates were incubated at 30°C and images of plates were scanned at 24, 48 and 72 h using a HP Scanjet 4890 desktop scanner. After the final reading of plate images, all images were imported into the PhPlate software (PhPWin version 4.24, PhPlate AB, Stockholm, Sweden) where the absorbance of each reagent was converted to numerical values ranging from 0-30 and the mean absorbance value from all individual readings was calculated for each reagent giving a fingerprint made of 24 numerical values.

Similarity among substrate utilisation of each isolate was calculated after pair-wise comparison of fingerprints and expressed as the correlation coefficient using the PhP-Win 4 software as previously described (21) and clustered into a dendrogram according to the unweighted pair group method with arithmetic averages (UPGMA) (21). Strains that belonged to CTs i.e. having the same biochemical fingerprints, were selected for further investigation.

5.3.3 Identification of LAB
Strains belonging to CTs that were present either over several sampling occasions or found in the faeces of salmon receiving different diets or from different farms were selected for further characterisation and were identified to the species level using 16S rRNA gene sequencing. The genomic DNA of each CT representative was extracted using a QIAGEN DNeasy Blood & Tissue kit (Qiagen, Australia) according to
manufacturer’s instructions and stored until further use at -20°C. PCR for 16S rRNA gene sequencing was performed on each of the CT representatives using the universal bacteria/archaea specific P11 and bacteria specific P12 primers (23). The final PCR reaction mixture was made to a volume of 25 µL, which consisted of 12.5 µL Mango mix (Bioline, Australia), 0.2 µM of each primer and 1 µL of template DNA. PCR cycling conditions were as follows: 95°C for 3 min, 30 cycles of 94°C for 30 s, 50°C for 1 min and 72°C for 3 min with a final extension at 72°C for 6 min on a BioRad Thermocycler. PCR products were sent to Macrogen, Korea for purification and sequencing. The Sequencher software version 4.8 (Gene Codes Cooperation, USA) was used to trim and align sequence reads. Searches for sequence matches were performed using the blastn function on the NCBI website (www.blast.ncbi.nlm.nih.gov).

5.3.4 Cytotoxicity of LAB strains

The Atlantic salmon kidney (ASK) cell line (CRL-2747) has been derived from healthy primary epithelial kidney cells of Atlantic salmon and has been immortalised. The cells were maintained at 20°C in L-15 medium (Lonza, Australia) supplemented with 20% foetal bovine serum (FBS; Lonza) and 1% Penicillin/streptomycin solution (Lonza) in 100% air. LAB strains (n=73) were tested for their cytotoxicity towards ASK cells as previously described with adaptation (24). In brief, LAB strains were grown in double strength MRS broth for 18 to 48 h (depending on the isolate requirement) at 30°C with agitation (150 rpm). Cell free supernatant was obtained by centrifugation of the broth at 2500 rpm for 10 min followed by filtration of the supernatant through a 0.22 µm pore-size filter. Filtrate was stored at -20°C until use (24). ASK cells were seeded into a 96-well tissue culture plates (Nunc, Australia) and grown at 20°C in L-15 medium (Lonza) supplemented with 20% FBS without antibiotics for ~7 days until 100% confluence. Filtrates from LAB were diluted (1:10 and 1:100) in sterile PBS and 100 µL of diluted supernatant was inoculated in each well. All tests were performed in triplicate. The strains were deemed cytotoxin-producers if cytotoxic effects (cell rounding and death) were displayed in greater than 50% of cells as previously determined (24). Isolates showing cytotoxicity against the ASK cells were excluded from further characterisation.
5.3.5 Adhesion capability

Strains that did not show cytotoxicity were further tested for their ability to adhere to ASK cells. ASK cells were seeded onto 12 mm diameter × 1 mm thickness glass coverslips within a 24-well tissue culture plate and grown to ~75% confluence in culture medium at 20°C and 100% air. Cells were rinsed three times with 1 mL of L-15 to remove any residual antibiotics and replaced with culture medium without antibiotics. Bacterial strains were grown overnight in MRS broth at 30°C with agitation (100 rpm), centrifuged at 2500 rpm for 10 min and bacterial pellets were resuspended in sterile PBS (pH 7.4). Bacterial concentrations were adjusted to ca. 1 × 10^9 cells/mL and 100 µL of this suspension was inoculated into wells in duplicate. Cells were incubated at 20°C for 1.5 h before rinsing each well three times with 1 mL of sterile PBS to remove any non-adherent bacteria. Coverslips were fixed in 95% ethanol for five min, Gram-stained and viewed using light microscopy (1000× magnification). Bacterial adhesion was assessed according to Grey and Kirov (1993), where the percentage of adherent bacteria was determined by the presence of bacteria on 100 randomly selected cells and the degree of bacterial adhesion was assessed by counting the number of attached bacteria on 25 randomly selected ASK cells (25). Adhesion rates were expressed as low (1-10 bacteria/cell), medium (11-19 bacteria/cell) and high (>20 bacteria/cell). Strains that adhered to <10% of ASK cells were deemed non-adherent (25).

5.3.6 Antibiotic resistance

LAB strains were tested for their antibiotic resistance according to the methods recommended by the Clinical and Laboratory Standards Institute (CLSI) guidelines (26). Minimum inhibitory concentration tests were performed in 5 mL of Mueller-Hinton (MH, Oxoid) broth for the Enterococcus spp. and 5 mL of MH broth supplemented with 5% lysed horse blood for the Pediococcus spp. and the Weissella spp. All LAB strains were tested against ampicillin (8 µg/mL), penicillin (8 µg/mL) and chloramphenicol (8 µg/mL, 16 µg/mL, 32 µg/mL) and the Pediococcus and the Weissella strains were additionally tested against gentamicin (4 µg/mL, 8 µg/mL and 16 µg/mL) (25). The Pediococcus strains were also tested against imipenem monohydrate (0.5 µg/mL) (26). This involved inoculation of a bacterial colony into MH broth and incubation at 30°C and 100 rpm for 18-24 h. Growth was determined as positive upon
visual inspection of turbid or non-turbid broth (26). All strains were additionally tested in 5 mL of MH broth containing kanamycin (16 µg/mL), tetracycline (4 µg/mL, 8 µg/mL and 16 µg/mL), vancomycin (4 µg/mL, 16 µg/mL and 32 µg/mL), streptomycin (32 µg/mL, 64 µg/mL) and for high resistance *Enterococcus* strains against streptomycin (1000 µg/mL) (26), or erythromycin (0.5 µg/mL, 4 µg/mL and 8 µg/mL) as previously described (27).

**5.3.7 Bacteriocin production by the LAB isolates**

The ability of the LAB strains to produce bacteriocin and/or other compounds that inhibit the growth of pathogenic bacteria was tested according to the previously described method with some modification (27). A number of type culture strains, as well as several highly adherent strains isolated in another study using the same samples (29) were selected and used as indicator strains. These included: *Acinetobacter baumannii* (ATCC 19606), *A. calcoaceticus* (ACM 686), *Aeromonas eucrenophila*, *A. hydrophila* (RBH1), *A. jandaei* (ATCC 49568), *A. media* (ATCC 33907), *A. schubertii* (ATCC 43700), *A. veronii* biovar sobria (5Ag), *A. veronii* biovar sobria (7Bg), *Klebsiella oxytoca* (ATCC 8724), *K. pneumoniae* (ATCC 13883), *Pseudomonas aeruginosa* (ATTC27853), *Vibrio parahaemolyticus* (ATCC 33844), *V. harveyi* (ATCC 142126) and *V. alginolyticus* (ATCC 17749). From the collection of bacteria previously isolated from the Atlantic salmon (Neuman *et al.* submitted), the following bacterial strains were included; *Shewanella baltica*, *Providencia heimbachae* and unidentified strains belonging to the genera *Shewanella* and *Lysinibacillus* (one each).

LAB strains were grown in MRS broth at 30°C with agitation (100 rpm) for 16 h and the cell free supernatant was collected after centrifugation followed by filtration through a 0.22 µm pore size filter. Filtrate was stored at -20°C until used later. Indicator strains (i.e. *V. harveyi* and *V. alginolyticus*) were grown in TSB with 1% NaCl at 28°C or in TSB at 30°C (*S. baltica*, unidentified strains belonging to *Shewanella* and *Lysinibacillus* spp. and *P. heimbachae*) and at 37°C for all other strains listed above. Two microliter aliquots of the filtered supernatant were spotted onto MRS agar plates and depending on the requirement of the indicator bacterium, the MRS agar was overlaid with the agar medium that supported the growth of the indicator strain. The indicator strain was inoculated at a concentration of 10^5 CFU/mL on the top agar layer and incubated.
overnight at the appropriate temperature. Growth inhibition of the indicator strains was scored positive if the clear zones were larger than 2 mm diameter as recommended (27).

5.3.8 Survival through the simulated GI tract
Each of the LAB strains was assessed for their ability to survive an in vitro digestion model as previously described by Fernández et al. (2003) with some modification (31). Isolates were grown in 5 mL of MRS broth overnight, washed and resuspended in sterile PBS. Strains were adjusted to a concentration of $10^9$ CFU/ml and 200 µL of this mixture was inoculated into 2 mL of GI medium, at a final pH 2, containing the following; 125 mmol$^{-1}$ NaCl, 7 mmol$^{-1}$ KCl, 45 mmol$^{-1}$ NaHCO$_3$ and 3g/l$^{-1}$ pepsin and incubated at 30ºC on a rotary shaker at 200 rpm for 3 h to simulate peristalsis. Samples were serially diluted and plated onto MRS agar at 0, 90 and 180 min and cultivated on appropriate medium to assess bacterial viability (31).

5.3.9 Competitive adhesion capability
The ability of the LAB strains to exclude pathogenic bacteria from adhering to ASK cells was assessed as previously described (30) with some modification. Pathogenic strains used for this assay have been described above. Only ATCC strains that showed moderate to high level of adhesion to ASK cells (25) were used to challenge the LAB strains. ASK cells were grown as described for the adhesion assay above and 100 µL of the bacterial suspension was inoculated into duplicate wells at a concentration of ca. $10^9$ CFU/mL and allowed to adhere for 1.5 h to the ASK cells. One hundred microliters of each pathogenic strain at a concentration of ca. $10^9$ CFU/mL was used for the challenge study. Displacement was calculated as the percentage change in the adhesion capability of each pathogenic strain alone or in the presence of the LAB strains (30).

5.4 Results

5.4.1 Identification of Lactic acid bacteria (LAB) from Atlantic salmon
Initial typing of the LAB isolates with the PhPlate system showed the presence of 17 CTs and 68 single types. Strains that were either present in several samples or isolated from fish receiving different diets (n=73) were chosen for further tests. These strains belonged to 10 CTs (i.e. CT 2- 7, 9, 15- 17). Identification of these isolates using 16S rRNA gene sequencing showed that they belonged to six different species, namely
Enterococcus casseliflavus, E. faecalis, E. faecium, Pediococcus acidilactici and Weissella hellenica.

5.4.2 Cytotoxicity assay of LAB strains
Of the 73 LAB isolates, 24 (32.9%) displayed cytotoxic effects towards ASK cells. Cytotoxicity was not limited to any one CT or bacterial species however the percentage of cytotoxic strains belonging to each CT varied. W. hellenica isolates belonging to CT 15 and 16 showed the lowest overall cytotoxicity with only 25% (4/16) of all isolates lysing ASK cells (Table 5.1), followed by E. faecium where 25% of the isolates showed cytotoxicity (Table 5.1). The number of E. casseliflavus and E. faecalis strains showing cytotoxicity was 27% and 29% respectively. Overall, the bacterial species displaying the highest cytotoxicity i.e. >50% were P. acidilactici and Pediococcus sp. Strains belonging to CT 6 and 16 were the only strains that did not show any cytotoxicity (Table 5.1). The lack of cytotoxicity did not correlate with any particular sampling time, salmon farm, diet or bacterial species. Isolates which showed cytotoxicity towards ASK cells were excluded from further characterisation.

5.4.3 Adhesion capability of LAB to Atlantic salmon cells (ASK)
The LAB strains that showed no toxicity toward ASK cells (n=49) were tested for their adhesion to this cell line. The majority of LAB strains (41/49) showed a low adherence (2-10 bacteria/cell) to ASK cells. Five strains displayed medium level of adherence, three of which were E. casseliflavus strains (one from CT 2 and two from CT 4) while the other two strains were W. hellenica (CT 16) and E. faecalis (CT 17) respectively (Table 5.1). Three strains showed the ability to adhere at a high level to the ASK cells with the highest adhering strains belonging to the species P. acidilactici (54 bacteria/cell; CT 7) (Table 5.1). Two other strains also showed adhesion level of 21 and 23 bacteria/cell and belonged to W. hellenica (CT 15).
Table 5.1: Number of strains belonging to different species and common types (CT) showing cytotoxic effect on and their adhesion capability to Atlantic salmon kidney (ASK) cells

<table>
<thead>
<tr>
<th>Species and CT</th>
<th>Cytotoxic</th>
<th>Adhesion capacity of non cytotoxigenic CTs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Yes</td>
</tr>
<tr>
<td>E. casseliflavus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT 2</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(14%)</td>
</tr>
<tr>
<td>CT 4</td>
<td>24</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(33%)</td>
</tr>
<tr>
<td>CT 6</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. faecium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT 5</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(25%)</td>
</tr>
<tr>
<td>E. faecalis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT 17</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(29%)</td>
</tr>
<tr>
<td>P. acidilactici</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT 3</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(33%)</td>
</tr>
<tr>
<td>CT 7</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(75%)</td>
</tr>
<tr>
<td>Pediococcus sp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT 9</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(50%)</td>
</tr>
<tr>
<td>W. hellenica</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT 15</td>
<td>14</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(29%)</td>
</tr>
<tr>
<td>CT 16</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOTAL (ALL)</td>
<td>73</td>
<td>24</td>
</tr>
</tbody>
</table>
5.4.4 Antibiotic resistance of LAB strains

The LAB strains that showed no toxicity toward ASK cells (n=49) were tested for their antibiotic resistance. Antibiotic resistance was not limited to any one CT or LAB species, with observable differences in their patterns of resistance, however an overall high resistance to both chloramphenicol and vancomycin was found. All of the *Pediococcus* strains showed resistance to imipenem and ampicillin whilst all of the *Enterococcus* strains were susceptible to ampicillin (Table 5.2). Most of the LAB strains showed susceptibility to streptomycin however five *E. faecalis* strains (CT17) showed the ability to resist a concentration of 1000 µg/mL of this antibiotic. Four of these strains also showed complete resistance to kanamycin and chloramphenicol (Table 5.2).
Table 5.2: The antibiotic resistance patterns of lactic acid bacterial strains belonging to ten different common types (CTs). Imi: imipenem (0.5 µg/mL); amp: ampicillin (8 µg/mL); gen: gentamycin (4 µg/mL, 8 µg/mL and 16 µg/mL); chl: chloramphenicol (8 µg/mL, 16 µg/mL, 32 µg/mL); pen: penicillin (8 µg/mL); kan: kanamycin (16 µg/mL); tet: tetracycline (4 µg/mL, 8 µg/mL and 16 µg/mL); van: vancomycin (4 µg/mL, 16 µg/mL and 32 µg/mL); str: streptomycin (32 µg/mL, 64 µg/mL); ery: erythromycin (0.5 µg/mL, 4 µg/mL and 8 µg/mL).

<table>
<thead>
<tr>
<th>Species (CTs)</th>
<th>Percentage of strains showing resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
</tr>
<tr>
<td>E. casseliflavus (2)</td>
<td>6</td>
</tr>
<tr>
<td>E. casseliflavus (4)</td>
<td>16</td>
</tr>
<tr>
<td>E. faecium (5)</td>
<td>2</td>
</tr>
<tr>
<td>E. casseliflavus (6)</td>
<td>3</td>
</tr>
<tr>
<td>E. faecalis (17)</td>
<td>5</td>
</tr>
<tr>
<td>P. acidilactici (3)</td>
<td>2</td>
</tr>
<tr>
<td>P. acidilactici (7)</td>
<td>2</td>
</tr>
<tr>
<td>Pediococcus sp. (9)</td>
<td>1</td>
</tr>
<tr>
<td>W. hellenica (15)</td>
<td>10</td>
</tr>
<tr>
<td>W. hellenica (16)</td>
<td>2</td>
</tr>
</tbody>
</table>
5.4.5 Bacteriocin production by the LAB isolates

All 49 LAB isolates produced bacteriocin-like compounds that inhibited the growth of up to six of the ATCC strains they were tested against. The highest level of inhibition was seen against the *Shewanella* spp. with 41 out of 49 LAB strains inhibiting bacterial growth (Table 5.3). The production of bacteriocin-like compounds was not limited to any particular CT or bacterial species however the inhibition of growth was species specific. None of the LAB were able to inhibit *A. sobria*, *A. jandaei*, *A. hydrophila*, *A. schubertii*, *V. harveyi*, *P. aeruginosa*, *K. oxytoca*, *K. pneumonia*, *P. heimbach*, *S. enterica* or the two *Acinetobacter* spp. *A. veronii* biovar sobria was only inhibited by two strains of *E. casseliflavus* and the *Lysinibacillus* strain was only inhibited by one *E. casseliflavus* and one *E. faecium* strain (Table 5.3)
Table 5.3: Percentage of LAB strains belonging to 10 common types (CTs) producing bacteriocin-like activity and inhibited the growth of 10 selected ATCC strains. No activity was observed against A. sobria, A. jandaei, A. hydrophila, A. schubertii, V. harveyi, P. aeruginosa, K. oxytoca, K. pneumonia, P. heimbach, S. enterica or the two Acinetobacter spp. strains.

<table>
<thead>
<tr>
<th>Species (CT)</th>
<th>Percentage of strains showing bacteriocin activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A. veroni by sobria</td>
</tr>
<tr>
<td>E. casseliflavus (2)</td>
<td>17</td>
</tr>
<tr>
<td>E. casseliflavus (4)</td>
<td>6</td>
</tr>
<tr>
<td>E. faecium (5)</td>
<td>0</td>
</tr>
<tr>
<td>E. casseliflavus (6)</td>
<td>0</td>
</tr>
<tr>
<td>E. faecalis (17)</td>
<td>0</td>
</tr>
<tr>
<td>P. acidilactici (3)</td>
<td>0</td>
</tr>
<tr>
<td>P. acidilactici (7)</td>
<td>0</td>
</tr>
<tr>
<td>Pediococcus sp. (9)</td>
<td>0</td>
</tr>
<tr>
<td>W. hellenica (15)</td>
<td>0</td>
</tr>
<tr>
<td>W. hellenica (16)</td>
<td>0</td>
</tr>
</tbody>
</table>
5.4.6 Survival through simulated environment of the stomach

Survival of the 49 LAB strains was low and at 90 min only seven isolates were capable of surviving, as was observed through enumeration. These included 3 strains of *E. casseliflavus* (i.e. strains 1-242 belonging to CT1; 13-89 belonging to CT4 and 4-236 belonging to CT12) showing adhesion capabilities of 5, 10 and 2 CFU/cell respectively, as well as 4 *W. hellenica* strains (i.e. strains H117, 19-291 and 19-298 belonging to CT15 and 19-302 belonging to CT16) displaying adhesion capabilities of 8, 4, 4, 17 CFU/cell respectively. The highest survival rates were observed by strains 1-242 and 13-89 (6.2 × 10^3 and 4.9 × 10^3 CFU/mL respectively) whilst *E. casseliflavus* strains 4-236 showed the lowest cell survival (2 × 10^2 CFU/mL). The three strains from CT 15 showed low survival rates of 1.1 and 1.2 × 10^3 as well as 4 × 10^2 CFU/mL at 90 min. Strain 19-302 not only survived 180 min of peristalsis but also increased in numbers from 2.1 × 10^3 CFU/mL at 90 min to 2.8 × 10^4 CFU/mL at 180 min.

5.4.7 Competitive adhesion assay of the LAB bacteria

Based on the results obtained from the adhesion assay, antibiotic resistance testing and the ability of the LAB strains to produce bacteriocin-like compounds, four strains were selected for competitive adhesion assay against 16 ATCC strains. The selected strains included one strain of *P. acidilactici* belonging to CT 7 (strain 4-251), three strains of *W. hellenica* belonging to CT 15 (strains 19-449 and H123) and CT 16 (strain 19-302). Of these, the *P. acidilactici* strain 4-251 showed the highest competitive level causing a decrease in the adhesion capability of 15 of the 16 adherent ATCC strains (Table 5.4). The *W. hellenica* strain (19-302) on the other hand, showed the least competitive inhibition, decreasing the adhesion level of only 10 of the 16 ATCC strains and increasing the adhesion of three (Table 5.4). The percentage decrease in pathogen adhesion ranged from 14 % to 100 % and this was independent of the type of pathogen or LAB strains tested. The *W. hellenica* strains (H123) and (19-449) were able to decrease the adhesion level of 13 and 12 ATCC strains respectively (Table 5.4).
Table 5.4: Percentage reduction in the level of adhesion of 16 selected pathogenic strains to the ASK cells in the presence of selected LAB strains *P. acidilactici* (strain 4-251) and three strains of *W. hellenica* (19-449, H123 and 19-302). The level of adhesion of the 16 pathogens to ASK cells have also been given.

<table>
<thead>
<tr>
<th>Pathogenic strain tested (strain number)</th>
<th>CFU/cell</th>
<th>+ <em>W. hellenica</em> (19-449)</th>
<th>+ <em>P. acidilactici</em> (4-251)</th>
<th>+ <em>W. hellenica</em> (H123)</th>
<th>+ <em>W. hellenica</em> (19-302)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. veroni bv sobria</em> (strain 568)</td>
<td>20</td>
<td>-85</td>
<td>-90</td>
<td>-90</td>
<td>-75</td>
</tr>
<tr>
<td><em>A. jandaei</em> (567) (ATCC49568)</td>
<td>15</td>
<td>+20</td>
<td>-60</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>A. sobria</em> (7Bg)</td>
<td>17</td>
<td>+47</td>
<td>-59</td>
<td>-29</td>
<td>0</td>
</tr>
<tr>
<td><em>A. media</em> (565)</td>
<td>14</td>
<td>-14</td>
<td>-29</td>
<td>-57</td>
<td>-7</td>
</tr>
<tr>
<td><em>A. hydrophila</em> (R1)</td>
<td>12</td>
<td>-50</td>
<td>+25</td>
<td>-50</td>
<td>-50</td>
</tr>
<tr>
<td><em>A. eucrenophila</em> (533)</td>
<td>19</td>
<td>-79</td>
<td>-84</td>
<td>-89</td>
<td>-32</td>
</tr>
<tr>
<td><em>A. shibertii</em> (534) (ATCC 43700)</td>
<td>11</td>
<td>-36</td>
<td>-27</td>
<td>-100</td>
<td>-36</td>
</tr>
<tr>
<td><em>A. calcoaluicus</em> (ACM 686)</td>
<td>5</td>
<td>-60</td>
<td>-80</td>
<td>-60</td>
<td>-80</td>
</tr>
<tr>
<td><em>K. oxytoca</em> (ATCC 8724)</td>
<td>9</td>
<td>-100</td>
<td>-78</td>
<td>-41</td>
<td>-56</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> (ATCC 13883)</td>
<td>5</td>
<td>-100</td>
<td>-100</td>
<td>-60</td>
<td>+40</td>
</tr>
<tr>
<td><em>Lysinibacillus</em> (H89)</td>
<td>13</td>
<td>-77</td>
<td>-69</td>
<td>-62</td>
<td>-54</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> (ATCC27853)</td>
<td>9</td>
<td>-44</td>
<td>-78</td>
<td>-41</td>
<td>-67</td>
</tr>
<tr>
<td><em>P. heimbach</em> (16-84)</td>
<td>3</td>
<td>+34</td>
<td>-33</td>
<td>0</td>
<td>+67</td>
</tr>
<tr>
<td><em>S. baltica</em> (2-250)</td>
<td>17</td>
<td>+65</td>
<td>-53</td>
<td>+29</td>
<td>-53</td>
</tr>
<tr>
<td><em>Shewanella</em> sp. (2-244)</td>
<td>14</td>
<td>-93</td>
<td>-57</td>
<td>-57</td>
<td>0</td>
</tr>
<tr>
<td><em>S. enterica</em> subsp. enterica* (ATCC 13311)</td>
<td>27</td>
<td>-56</td>
<td>-59</td>
<td>-56</td>
<td>-63</td>
</tr>
</tbody>
</table>
5.5 Discussion

In this study 160 LAB, previously isolated from the faecal hindgut of Atlantic salmon, were characterised for their potential use as probiotics in the aquaculture industry. PhP typing revealed 10 CT that were present over several samples or several diets and sequencing of representative strains showed these isolates belonged to several species; *P. acidilactici*, *W. hellenica*, *E. faecalis*, *E. faecium* and *E. casseliflavus* all of which have been previously isolated from aquaculture sources (27, 32-34).

The production of cytotoxic compounds in a stressed environment, ie. lack of nutrients and interbacterial competition for space, has the potential to be harmful to the host (35). Although each bacterial species was cytotoxic to ASK cells, *Pediococcus* spp. were more likely to be capable of toxin production. This is an interesting observation, as *P. acidilactici* has to date not been shown to be pathogenic to fish. Rather, strains have demonstrated potentially beneficial to the gut microbiota of Tilapia (*Oreochromis niloticus*) (35), improve both microvilli morphology (37, 38) and reduce spinal deformities in Rainbow trout (*Oncorhynchus mykiss*) (39) with one strain currently approved for inclusion into feed (40). Whilst *W. hellenica* has been isolated in the past from the intestinal system of Flounder it has also been shown to be an opportunistic pathogen in Rainbow trout (41) which could explain why cytotoxic compounds were produced by some strains.

All of the 49 non-cytotoxic LAB were capable of producing bacteriocin-like compounds that inhibited the growth of at least two of the pathogens against which they were tested. Other studies have shown *P. acidilactici* capable of bacteriocin production that was particularly effective against *Vibrio* spp. as well as other Gram-negative bacteria (42). In this study, although we observed some growth inhibition towards *V. alginolyticus* and *V. parahaemolyticus* by some of the *P. acidilactici* strains, the majority of the LAB were unable to inhibit the growth of the tested *Vibrio* spp. They were however effective against other Gram-negative species, which mirrors observations made in other studies (42), which tested isolates from non-human sources (43). *W. hellenica* isolates tested also did not display inhibition of *Pseudomonas* spp. as previously reported, however they showed minimal inhibition towards some *Aeromonas* spp. and *V. alginolyticus*, contradicting previous findings (44). The *Enterococcus* spp. also showed varying ability to inhibit the growth of the pathogens through bacteriocin production with no pattern
evident. Although previous research indicated only *E. faecium* capable of Gram-negative bacterial inhibition (45-47), in our study we observed one strain capable of inhibition of a *Lysinibacillus* sp.

The overall observed adhesion by the 49 non-cytotoxic LAB strains was low with only several strains displaying medium to high adherence, regardless of the species or CT these strains belonged to. This could be explained by the fact that the isolates originated from the hindgut faecal microbiota and although this contains bacteria that interact directly with the gut epithelium it also contains some transient species. Of the strains showing medium to high adhesion the *P. acidilactici* and *W. hellenica* isolates displayed higher adherence to fish cells than the *Enterococcus* spp., indicating their increased potential to exclude pathogens.

All LAB tested were capable of competitively excluding a number of pathogens, although to varying degrees. Whilst other studies have shown that *P. acidilactici* and *Enterococcus* spp. have the potential to exclude enteric pathogens (48, 49), results from our study showed that *W. hellenica* also shared the same ability. The ability to exclude pathogens however has been shown to be strain specific, depending both on the LAB and pathogen used (30) and was also shown in this study. Interestingly, the same strain of LAB capable of completely excluding adhesion by some pathogen also caused other pathogens to increase their adhesion capability. This increased pathogen adhesion has been shown to occur before with different strains (50) and could be due to co-aggregation between the bacteria (51), exopolysaccharides secreted by the LAB (52) or another form of interaction between the bacteria which is currently unknown (53).

Like adhesion, survival through a simulated GI tract environment is strain specific with the majority of tested strains unable to survive acidic conditions and therefore making these potentially unsuitable for use as probiotics. Low or no survival of probiotic strains under simulated GI conditions has however been shown before (54) and as encapsulation of probiotic strains in food particulates allows for greater survival rates (55), strains should be retested under encapsulated conditions.

Overall it is apparent that two isolates belonging to the same species or even the same CT can display varied abilities in their adhesion capability, pathogen displacement or even survival through the gastrointestinal tract. This can make the selection of potential
probiotic strains difficult due to the variance between isolates and highlights the importance to characterise the individual isolates in depth prior to application.

In conclusion, LAB bacteria in this study were isolated from the hindgut of Atlantic salmon and showed the ability to inhibit a variety of known pathogens either through the secretion of bacteriocin-like compounds or through competitive adhesion. This information indicates the possible application of some these strains (n=4) for use as probiotics in the salmonid industry. As it is unlikely any one probiotic would fulfil all required criteria, further investigation into the combined use of several strains is necessary. Whether these strains would be of benefit in vivo and how these would be administered to their full potential (i.e. through the inclusion into the fish feed or by application into the surrounding water) would need further investigation.
5.6 References


40. EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP). 2012. Scientific Opinion on the safety and efficacy of Pediococcus acidilactici (CNCM I-3237, CNCM MA 18/5M—DSM 11673) and Pediococcus pentosaceus (DSM 23376, NCIMB 12455, NCIMB 30237 and NCIMB 30168) as silage additives for all species. EFSA J. 10(6): 2733.


emulsions formulated with sweet whey as emulsifier and survival in simulated gastrointestinal conditions, Food Res. Int. 42(2): 292-297.
CHAPTER 6

6.0 Assessing the impact of fish feeds on the gut microbiota of Tasmanian Atlantic salmon (*Salmo salar* L.) using a semi-continuous culture

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6.1 Introduction

The initial transient gut microbiota of fish originates after hatching from the surrounding environment and depending on the fish species develops into a stable community usually during or after the juvenile stages (1). There are many factors that influence the gut microbial community in adult fish such as the bacteria present in the surrounding water during initial colonisation of the gut, diet and the nutritional status, as well as the age of the fish (2-5). It has been shown that other environmental conditions such as geographical location also effect microbial gut composition (3, 6-10). There are several different fermentation models to study the gut microbiota, however, the use of either a semi-continuous or a continuous culture is shown to better simulate the gastrointestinal system of humans and other mammals (11). The functional status of the whole gut microbiota to break down and ferment substrates can also be used to measure the effect of various diet components (12). Moreover, semi-continuous and continuous fermenter models have been used in the past to assess the effect of lactic acid bacteria (LAB) (13), diet components (14) or the effect of both pre- and probiotics on the gut microbiota of humans (15). Other studies have focused on other mammals such as rabbits (16) and cows (17), however, no studies have investigated the impact of feed on the gut microbiota of fish, and in particular Atlantic salmon, in a semi-continuous fermenter model.

6.2 Aims of the study

Farmed Tasmanian Atlantic salmon often experience high water temperatures during summer, peaking at or above 20°C. As feed components are known to be an important factor for the development and/or shift in the gut microbiota in fish, this study aimed to investigate the possibility of using a semi-continuous fermentation model to study the impact of commercially available fish feeds, varying in protein and lipid composition, on the gut microbiota of Atlantic salmon during periods of high water temperatures i.e. 20°C.
6.3 Methods

6.3.1 Fermenter media and gut microbiota inocula

A total of twenty litres of seawater was collected in sterile containers (1 L on each sampling occasion), transported on ice to the laboratory and sterilised via autoclaving after the addition of fish feed. Two commercially available fish feeds (A and B) with different protein: fat ratios (Table 1), suitable for the different growing seasons, were used to prepare a sterile 1 % (w/v) solution of which a 2 L volume served as the working medium for the fermenter. Three Atlantic salmon (Salmo salar L.), with an average pen weight of 2.8 kg per fish, reared in sea cages in a commercial fish farm south of Hobart, Tasmania, were caught at random via netting, from a pen stocked with 70,000 female smolt. The fish were sacrificed using an overdose of the anaesthetic isoeugenol (Aqui-S®) according to the commercial fish farm procedure by the staff veterinarian (ethics number UTAS A12001). Experiments comparing the impact of feed A and B on the gut microbiota of Atlantic salmon were performed one month apart, due to the logistical problem associated with simultaneous testing. However, the faecal material used as inocula for both experiments was collected from the same cage and the same group of fish. The water temperature at the time of the hindgut collections for testing feed A was 11°C and 13°C when collecting samples to test feed B, respectively. At each occasion, the hindgut of each fish was removed onsite and transported on ice to the laboratory where the faecal contents was inoculated into the fermenter within 8 h from collection. Anaerobic semi-continuous culture fermentation, conducted in a 5 L culture vessel of a laboratory fermenter (BioFlo/CelliGen 115 Fermenter/Bioreactor), was achieved by continuous flushing of the headspace of the culture vessel using CO₂ gas as previously described (18). The hindgut contents of the fish were pooled to minimise individual differences between the fish (19) and were directly inoculated into the vessel through the inoculation port. The fermenter was set at 100 rpm agitation throughout the experiment to simulate peristalsis. The anaerobic environment was monitored and maintained throughout the experiment using gas flow controllers. The experiment was repeated at 20 °C for each fish feed.

As a negative control, conical flasks containing a 1 % (w/v) solution of fish feed in sterile seawater were used. The conical flasks were shaken on a rotary shaker at 100 rpm at 20°C and routinely sampled and spread on Tryptone Soya Agar (TSA) (Oxoid,
Australia) without dilution. At the time of both hindgut collections, the water temperature ranged between 11-13 °C.

Table 6.1: Composition (expressed as percentage) of the fish feeds A (summer feed) and B (winter feed) used in this study. Ratios of vegetable protein to land animal protein as well as the ratio of fish oil to poultry oil is also given.

<table>
<thead>
<tr>
<th>Composition</th>
<th>Diet A (% composition)</th>
<th>Diet B (% composition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vegetable protein : land animal protein</td>
<td>1 : 1.2</td>
<td>1 : 3.9</td>
</tr>
<tr>
<td>Fat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fish oil : poultry oil</td>
<td>50 : 50</td>
<td>25 : 75</td>
</tr>
<tr>
<td>Fishmeal</td>
<td>25</td>
<td>20</td>
</tr>
<tr>
<td>Other</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Digestible energy (MJ)</td>
<td>20.1</td>
<td>20.05</td>
</tr>
</tbody>
</table>

6.3.2 Feeding regime and sampling

Once inoculated, the culture was grown under batch conditions for 24 h after which it was switched to semi-continuous culture (20). Based on the feeding routine and subsequent faecal excretion of the farmed Atlantic salmon, as shown previously (21), it was calculated that the volume of feed and sample to be replaced each day was 31.8 mL per fish. As our working volume in the culture vessel was 2 L, which roughly equated to the hindgut contents of 20 Atlantic salmon (on average 100 mL per sampled fish), the total volume of working medium to be replaced each day was calculated to be 636 mL (21). Samples were collected on days 0, 1, 6 and 12 by use of the sterile collection port, immediately prior to feeding with the same amount of working medium. Bacteriological analysis, functional status of the fermenter microbiota and analysis of their metabolic capacity (MC) was performed on the initial inocula and all subsequent samples collected from the fermenter.

6.3.3 Bacterial enumeration

The initial inocula was diluted (1:1 w/v) followed by serial dilution in sterile PBS (pH 7.4) and 100 µL of each dilution was spread on a number of selective and non-selective agar to target the main bacterial groups commonly found in the gut of Atlantic salmon (3, 6, 7). TSA was used to obtain a total bacterial count, Herellea agar (22) for isolation of Acinetobacter spp., Pseudomonas agar (Difco, Australia) for isolation of
*Pseudomonas* spp., TCBS for isolation of *Vibrio* spp., MRS for isolation of LAB, and IBBS for isolation of *P. shigelloides*. Plates were incubated for 24-48 h at 20°C. This served as the baseline counts of all bacterial species before fermenter inoculation (Day 0). A serial dilution was made of the sample collected from the fermenter at each time point and bacteria were enumerated on the agar as described for the initial inocula.

6.3.4 Functional status and metabolic capacity of the faecal biota

The fermenter microbiota were tested for their functional status and MC-value as described previously in Chapter 2. The only modification was the PhP-48 plate inocula where 5 mL of each sample collected from the fermenter was centrifuged at 2500 rpm for 10 minutes and the bacterial pellet was resuspended in 15 mL of PhP medium and 150 µL of the suspension was used to inoculated each well.

6.4 Results

6.4.1 Bacteriological analysis

An increase in the diversity of cultureable bacterial species was found in samples subjected to diet A over the 12 days of the experiment. This was associated with a decrease in the total number of cultureable bacteria from $2.5 \times 10^{11}$ to $3 \times 10^5$ CFU/mL after day one thereafter stabilising at $7.5 \times 10^8$ CFU/mL for the rest of the experiment (Figure 6.1a). Similarity among the functional status of the bacterial biota, which was very low during the first 24 h, increased and samples collected on days 6 and 12 had a highly similar functional status, which was an indication of a similar bacterial biota. There was an increase in the number of certain bacterial species such as *Vibrio*, *Pseudomonas*, *Acinetobacter* and LAB in samples collected after day 6 (Figure 6.1a). The total number of bacteria in the faecal sample used for feed B was much lower than in samples collected for the first fermenter study assessing feed A (Figure 6.1b). Some differences were observed in the number and presence of smaller bacterial groups between the two feeds after 12 days. This included an increase in the number of *P. shigelloides* and the absence of LAB in fermenter with feed B (Figure 6.1b). However, despite these differences in bacterial numbers, the functional status of the faecal biota in fermenters receiving either feed A or B was highly similar within the 12 days of sampling (Figure 6.1c).
**Figure 6.1:** Number of bacterial species in the gut microbiota of Atlantic salmon and per mL of the fermenter culture over 12 days of the study using feed A (a) or B (b). (c) shows similarity between the functional status of the gut microbiota receiving feeds A or B (boxes) during the 12 days of sampling. Total number of bacteria was measured on pooled faecal samples of salmon before inoculation and is based on CFU/g of faecal material. LAB: Lactic acid bacteria.

### 6.4.2 Substrate utilisation and MC value

The pattern of substrate utilisation of faecal microbiota subjected to both feeds showed an increase in the overall similarity of the microbiota between samples collected on days 6 and 12. During this period the metabolic profile of bacterial microbiota subjected to both feeds showed a high similarity throughout the study with the maximum similarity obtained between day 6 and 12 (Figure 6.1a and b).
Table 6.2: Degree of substrate utilisation, displayed as weak (+), moderate (++) or complete (+++), by the gut microbiota subjected to either feed A or feed B at each sample point over the course of 12 days of fermentation.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Feed A</th>
<th>Feed B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 1</td>
</tr>
<tr>
<td>Mannonic acid lactone</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>L-Arabinose</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>D- Xylose</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Galactose</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Maltose</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Trehalose</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Palatinose</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Sukrose</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lactose</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Melibiose</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lactulose</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Gentobiose</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Melezitose</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Raffinose</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Insine</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Adonitol</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Inositol</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D-Arabitol</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glycerol</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Maltitol</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dulcitol</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sorbose</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Deoxy-glucose</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Deoxy-ribose</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D-Fucose</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L-Fucose</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Tagatose</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Amygdalin</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Arbutin</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>β-Methyl-glucoside</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5-Keto-gluconate</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Gluconate</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Melbionate</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Galacturonic acid</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Salicin</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Citrate</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Fumarate</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Malinate</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>Malonate</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>L-Tartarate</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Urea</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Ornithine</td>
<td>+</td>
<td>+++</td>
</tr>
</tbody>
</table>
Comparison of metabolic profiles of microbiota subjected to differing feeds showed that there were similarities between the faecal microbiota of each sample collected over 12 days (Figure 6.1c). This was associated with an increase in the MC values of both faecal microbiota at the temperature tested (Figure 6.2).

![Figure 6.2: Changes in the metabolic capacity (MC) value of the gut microbiota of Tasmanian Atlantic salmon fed feed A (high protein/low fat) or B (low protein/high fat) over 12 days of fermentation measured using PhPlate-48 to show the potential of gut microbiota to utilise different substrates.](image)

### 6.5 Discussion

Feed components have been shown to be an important factor for the development and/or shift in the gut microbiota in fish (23-25). In this study we assessed whether a semi-continuous fermenter model could be used to study the impact of two commercially available feeds, i.e. summer and winter feeds, on the gut microbiota of Tasmanian Atlantic salmon. The semi-continuous fermenter model employed in this study was designed to mimic the feeding and gastric emptying of the salmon, as shown in previous studies mimicking the human gut (20). To further strengthen this model, the fermenter working medium used was sterilised seawater sourced from the same farm that the hindgut samples were collected from. Although the feeds tested in this study were designed for low (winter) and high (summer) temperatures, the aim was to assess the impact of feeds on the gut microbiota during times of higher water temperatures (20°C) experienced by the farmed salmon in Tasmania.
Different feed components appear to select for different bacterial species. Feed A (summer feed) contained a digestible protein (DP): digestible energy (DE) of 19.6, with an equivalent fish: poultry oil ratio of 50:50 (see Table 1) which appeared to sustain LAB populations. Although we did not test the impact of the individual feed components this has been shown previously (26). This feed was also associated with the growth of Vibrio spp. in the fermenter vessel. In a separate study it was shown that there was an increase in the number of Vibrio spp. in the hindgut of Atlantic salmon during the warm seasons when the water temperature increased to 20°C (Chapter 2) and therefore it was speculated that the higher water temperature is the main driver for this shift. This was observed in the fermenter model for feed A. The faecal samples collected from salmon and used for testing initially contained a small population of Vibrio spp., which was observed in the previous study also when the temperature was low. Based on this observation, it is suggested that the high water temperature, rather than the summer feed, impacts the growth of Vibrio spp. in the gut of farmed Atlantic salmon.

Feed B (winter feed) was comprised of a DP:DE ratio of 16.3, with a mix of fish oil to high poultry oil ratio of 25:75 respectively. Changes in the gut microbiota of Atlantic salmon exposed to this feed, unlike with feed A, were subtle. It has to be noted that the initial faecal sample used for testing the impact of this feed contained a high number of Vibrio spp. and therefore this population did not increase dramatically at the temperature set in the fermenter (20°C). Another possible explanation could be that the environmental temperature during the collection of the faecal sample for this test was higher i.e. 13°C than the one collected for testing feed A. As a result this had already favoured the growth of Vibrio spp.

A large decline in the total number of bacteria was observed in the microbiota subjected to feed A. As previously reported, this is not uncommon as the fermenter model itself has been shown to lead to the rapid loss of less competitive bacteria due to the presence of unprotected free cells in the microbiota rather than their natural state within the faecal matrix of the gut (27-30). Interestingly this phenomenon was not observed for the microbiota subjected to feed B where the number of bacteria in the inoculum was low, i.e. 10^6, from the beginning. An alternate explanation could be that faecal samples collected for feed B had already experienced a natural bacterial washout of less
competitive species due to the higher temperatures experienced by the sampled fish. As the aim of this study was to determine the effectiveness of using a fermenter to study the impact of feed and not temperature on the microbiota of salmon, further research and temperature trials are required to draw conclusions based on how initial starting water temperatures affect the microbiota.

In this study it was shown that the fermenter model appears to be suitable to assess impact of different feeds on hindgut bacterial populations of Atlantic salmon, regardless, of the fact that different starting inocula were used to test both feeds. Variability among the number of bacterial populations belonging to smaller groups was observed with a decrease in the number of *P. shigelloides* subjected to feed A and an increase in the microbiota subjected to feed B. The lack of growth of LAB in response to feed B (winter feed) indicates that either these bacteria require a specific feed component similar to what was found in feed A (summer feed) or that these bacteria were not present in the initial faecal sample. Alternatively, it is possible that LAB were present at undetectable levels over the course of fermentation due to the overgrowth of other bacterial species such as *Vibrio* spp.

The MC value used in this study is an indication of the ability of gut microbiota to utilise different substrates as a community. The MC value is high when one or few bacterial species present in the community have a high metabolic function. Alternatively, it can be due to the high diversity of bacterial species in the community each contributing to the utilisation of different substrates. Therefore, the results of MC values should be evaluated in conjunction with the functional status of the microbiota. In this study, the low MC value of the early gut microbiota sample could be attributed to the initial shift of the hindgut microbiota from the gut environment to the fermenter where the only source of food is the fish feed. This may require a period of adaptation for the microbiota and probably the production of required enzymes. However, within a day the community started utilising the feed components provided and this was associated with an increase in MC values, which was also shown in changes in the composition of the gut microbiota. The correlation between bacterial diversity and MC-value has been shown previously (31). Over 12 days of fermentation the MC values of the microbiota increased as bacterial diversity and their substrate utilisation pattern increased. There were marked differences between the MC values of the gut microbiota...
receiving either of the two feeds after 12 days, which could possibly be attributed to the higher number of metabolically active bacteria present in feed B. Furthermore the high number of LAB present in samples collected from the microbiota receiving feed A could explain the lower MC value as these bacteria are not as metabolically active as some of the Gram-negative species found in the microbiota receiving feed B.

An interesting observation of this study was the fact that despite the differences in the composition of the feeds, the response of the gut microbiota of Atlantic salmon to either feed showed a high similarity at every sampling round. This indicates that despite small differences in the number and species of bacteria, the pattern of substrate utilisation was highly similar between the two sets of microbiota. We postulated that this could in part be due to the fact that the fermenter model and the temperature used in this study did not select for the growth of specific bacteria, rather the community as a whole, making the semi-continuous fermenter model used in this study a valuable tool to assess the impact of feeds on the composition of gut microbiota. Zampa et al. (2004) used a semi-continuous fermenter model to assess the effects of digestible carbohydrates metabolism by the gut microbiota of healthy people. The results showed that their fermenter model was a suitable system to assess the changes in metabolic function of the gut microbiota (20), indicating a usefulness of the model also in other species, including fish, as shown in this study. This model could also be further developed to assess the compatibility of candidate probiotics with normal gut microbiota.

The feed A, used in this study has been referred to as the “summer feed” by the feed industry supplying this feed and is designed to promote the growth of salmon during the warmer months of the year. In this study we maintained the fermenter temperature at 20 °C which is normally experienced by the Tasmanian Atlantic salmon. Comparison of this with feed B (winter feed) showed that the diversity of the bacterial species (except LAB) was unlikely to be dramatically affected by the feed regime at the temperature used. In view of the fact that warmer water temperatures normally support the growth of pathogenic bacteria such as Vibrio spp. as shown in chapter 2, the high level of LAB in the gut might be beneficial to the fish (32, 33).

It has been suggested that pooled faecal samples may suffice the need for replication, however this may also lead to inter-microbiota interactions favouring the growth of certain bacterial species and therefore leading to an unrepresented microbiota (34).
Future studies could counteract this issue by using the same inoculum from several fish (10), which would provide enough faecal material to avoid consecutive sampling, as this has been shown to temporarily alter the microbiota (35). In our study due to logistical constraint we were not able to use the same inocula to test the two feeds simultaneously. However, the consistency of the data obtained from the MC value and the level of substrate utilisation as well as the overall increase in the number of certain species i.e. *Vibrio* spp., which dominate the gut of Atlantic salmon, implies that the inocula collected at different time intervals had little effect on the dominant bacterial species.

Whilst the use of culture based techniques to investigate the impact of feed on the gut microbiota of fish can provide a valuable insight into bacterial population dynamics, the use of molecular techniques may provide additional information on small changes in the cultivatable and non-cultivatable bacteria occurring in a fermenter study such as this one. On the other hand, the present study provided information on the functional status and MC of the gut microbiota of fish over time, an approach which has not been utilised to its full extent in other studies. We would also like to emphasis that in our fermenter model the impact of the gut physiology of fish has not been taken into account and therefore data obtained from this fermenter study should be interpreted with care.

In conclusion, this study showed that the semi-continuous fermenter model used could offer a valuable tool for assessing the impact of feed as well as factors such as environmental temperature fluctuations on the hindgut microbial community of fish. Further refinement, by including other environmental factors present in the fish gut and varying environmental temperature, is however needed, to capture the true potential of this system for future studies.
6.6 References


CHAPTER 7

7.0 General Discussion

The aquaculture industry has undergone a large expansion due to the continual increase in demand for fish and fish products (1, 2). Due to the nature of intensive farming systems, the need to manage disease outbreaks is of great importance. Australia’s geographical location provides some natural protection from diseases affecting both wild and farmed Atlantic salmon however, aquatic environments are excellent disseminators of disease causing bacteria and Gram-negative bacteria, such as *Vibrio*, *Pseudomonas* and *Aeromonas* spp., are still a major problem to the industry (3-5).

Farmed Atlantic salmon in Tasmania are cultivated towards their upper thermal limit (6) and therefore experience higher temperatures for a longer period than other parts of the world, such as Norway or Chile (7, 8). This study was initiated based on the outbreak of SGS that occurred in one of the salmon farms sampled in previous years (9). The fish farm has since implemented a number of procedures to improve the husbandry practices including an improvement to the fish diet by changing the feed formulation. The outcome of such improvement resulted in the maintenance of fish health throughout the production period. During the sampling periods of this study, although the water temperatures were high, these did not reach those recorded in the previous years during which fish were afflicted with SGS.

This study identified water temperature as a key factor for the presence of high numbers of bacteria and in particular *Vibrio* spp. in the gut of Atlantic salmon. The faecal samples did not carry any VGs commonly found among pathogenic *Vibrio* spp. and other Gram-negative bacteria and therefore these bacteria were regarded as non-pathogenic. Their high number in the gut of Atlantic salmon was first reported during the outbreak of SGS in this farm during 2009 but that report did not investigate the pathogenesis of these bacteria or their involvement in SGS. The fact that these bacteria were also found in the gut of healthy salmon in this study indicates that the high number of these bacteria was merely the result of their overgrowth in the surrounding water of the cages as the temperature increased and therefore fish received them through regular consumption of water during feeding. The presence of a high number of bacteria in
water samples collected from this farm during the summer months of the year supports this conclusion, however a study to type the bacteria found in the water column compared to the faecal samples is required to confirm this. An alternative explanation would be that this increase in bacterial numbers occurs within the gut of salmon, where the environment for growth is ideal due to free access to nutrients. Therefore, having been encased in a protective milieu rather than present in a planktonic form would help these bacteria to proliferate. Whether these bacteria also interacted with the gut epithelium of the salmon is unknown as this study only tested the faecal bacteria most of which could be transient strains. For a GI illness such as SGS to occur, several factors may be involved which are not limited to but include, high water temperatures which would lead to overgrowth of pathogens (10), a weakened immune system of the fish due to the environmental stress (11, 12) and/or total disruption of normal microbiota, as observed during antibiotic therapy (13) and finally substandard husbandry practices, such as over stocking (12) and poor diets (14-16). Previous outbreaks of SGS on one of the farms sampled in this study led to the development of an improved feed composition and improved farm management. It can therefore be postulated that the impact of a high number of Vibrio spp., during periods of high water temperatures, was minimal. It is also possible that the outbreaks of SGS in the same farm in previous years may be due to a different bacterial species or different strains of Vibrio that were not present in the water during the course of this study. Identification and typing of the Vibrio spp. in this study indicated the presence of two major species throughout the warmer months of the year. Interestingly, within each species there were several strains, all of which persisted throughout the high water temperature periods. Characterisation of these strains and the presence of specific VGs associated with those strains may help to identify if any particular clone had a more dominant role in the colonisation of the gut of the salmon and could provide insight into their potential involvement in disease development in fish. Therefore it is possible that one or a few types of these bacteria were more dominant than the others and potential pathogens may constitute a subset of these types. Similarly, typing of the P. fragi strains that dominated the microbiota during the cooler months of the year showed the presence of different types. The presence of non-pathogenic bacteria, such as LAB and Pseudomonas, during the cooler periods of the year may suggest that one or more of these species, if they indeed persist over the warmer months could have a protective role in the gut of Atlantic salmon.
The lack of known VGs in faecal samples from salmon in this study does not necessarily imply the absence of a putative VG among some of the bacterial strains found in the gut. This may rather be a result of sequence variation in the VGs of the bacteria or due to different virulence mechanisms possessed by these bacteria. It has to be noted however that the presence of one VG alone also may not be enough for full expression of pathogenicity of a bacterium as non-pathogenic bacteria have been found to carry “VGs” (17, 18). In this regard, the presence of *cdt* gene in all faecal samples from healthy salmon may indicate that either this gene was not able to cause disease by itself or was not expressed or if it was, it might have been in low quantity not enough to initiate the disease.

The impact of diet on the health of fish has been well defined (14-16), however the role of diet on the composition of microbiota and their functional status in salmon has not been investigated. The novel approach used in this study, provided information on the changes that occur in the capacity of the gut microbiota to act as a metabolically active system, which in this study was collectively referred to as MC-value. The MC-value demonstrated an association between a decrease in bacterial diversity and the metabolic potential of the microbiota as temperatures increased. This suggests that either the overgrown species were unable to ferment the available substrates or that they were metabolically less active. To a certain extent, this theory may explain why the bacteria that dominated the gut microbiota did not cause any visible disruption to the health of the salmon. Analysis of the metabolic potential of *Vibrio* spp. present at the gut epithelial level would provide a better understanding as to the interactions between these bacteria and the host. Assessment of the immune function of the fish during periods of high water temperatures and high numbers of *Vibrio* spp. would provide additional information regarding the lack of SGS seen in the present study and subsequent years.

Since the last outbreak of SGS, there has been an improvement in the feed formulations supplied to the fish farms. This study had the opportunity to investigate the impact of feed components, in particular FM and prebiotics, on the composition of the microbiota in Atlantic salmon during higher water temperatures. Whilst the prebiotics alone supported the growth of LAB, the level of FM inclusion did not affect the composition of the microbiota. However, a combination of a high FM and a prebiotic supplement
supported the persistence of LAB during higher temperatures, even though these bacteria disappeared due to the overgrowth of other species. Therefore, from a feed industry point of view, the substitution of FM, with other more cost effective components such as soybean meal, should be considered in conjunction with a prebiotic supplement. It has to be noted however that the use of a prebiotic supplement can only improve the function of the existing gut microbiota and therefore the impact of low FM diets on the growth performance of the fish has to be assessed first before any formulation changes are made.

From an industry perspective the performance of the feed in terms of fish growth is important and for this reason it is necessary to perform feed trials to assess the impact of new diet formulations. This however is not only time consuming but is also costly to both the feed companies and the fish farms. In this project, an in vitro model was developed to allow the initial assessment of the influence of feed components on the microbiota of fish. This in vitro model may help improve the feed formulation before a feed trial takes place. Fermentations models have been used in other studies to effectively model the response of the gut microbiota of mammals to feed components (19, 20) or the inclusion of probiotics into the feed (21). Although the composition of the initial microbiota used to test the two fish feeds was somewhat different, it was possible to effectively model shifts in the population structure and assess the impact of different feed components on the structure and function of the gut microbiota. Further studies are needed to address some of the larger issues still concerning the use of fermentation systems to model the gut microbiota such as the physiology of the gut, including the different compartments and dietary enzymes, something that was not possible to assess in this study.

The exact role of LAB within the hindgut of Atlantic salmon in Tasmania remains unknown however this study was able to characterise a number of persistent LAB strains isolated from the salmon gut. These strains showed the ability to exclude pathogenic bacterial species from adhering to ASK cells in vitro and/or inhibit their growth via production of bacteriocin-like compounds. Further study is also required to determine whether any of these potential candidate strains would be able to survive through the GI tract and colonise the salmon gut in vivo. One such hurdle is the route of strain administration to fish, as it is necessary for the fish to receive the strain in
numbers high enough to enable them to colonise the gut. Moreover it is important to establish whether these bacteria, would confer protective effects. Some environmental factors such as increased water temperatures (22) have been shown to negatively affect the ability of LAB to provide protection from pathogens. It has also been shown that interactions between the pathogens and LAB can lead to co-aggregation (23). This phenomenon, which was also observed in our study can promote adhesion of pathogens to epithelial cells and increase the likelihood of disease development. The selection of a suitable LAB strain as a probiotic candidate must therefore be undertaken with care to ensure the health of the fish. This study did not investigate the cumulative effect which has been observed from the use of two or more LAB strains in combination (24) and therefore future studies should also include testing the possible beneficial effect of these four candidate strains in combination before in vivo trials are initiated.

Molecular studies assessing the impact of diets on the gut microbiota of Atlantic salmon have shown identification of different bacterial species in the gut. These methods, although highly useful, may not be applied to studies that aim to evaluate the health and function of this complex microbial milieu. The use of alternative characterisation methods of the microbiota, such as assessing the functional status and their MC-value showed shifts in the composition and number of bacterial species. It also provided an estimation of the ability of this community to break down the feed components to be fully utilised by the gut microbiota. This is important, as the gut microbiota is responsible for the production of several digestive enzymes (25). Further studies, however, may help allude to the other changes occurring in the gut microbiota in response to feed components.

7. 1 Future directions

In this study although temperature was identified as a key factor in shifts in the gut microbiota, the exact cause of SGS still remains unknown. Factors such as the high water temperature and the overgrowth of bacteria such as Vibrio spp. together with subpar diets and poor husbandry practices may collectively provide a suitable ground for the development of fish diseases such as SGS. Typing and full characterisation of dominant clonal groups of bacteria such as the Vibrio spp. during increased water temperatures, as well as assessment of the immune response of the fish may provide a
better insight on the mechanism of disease development. Furthermore, future studies should aim towards the identification of non-pathogenic bacteria that can competitively grow in the gut of salmon during the warm seasons together with feed supplements, such as FM and/or prebiotics that might be advantageous for salmon to maintain a healthy and diverse microbiota during the production cycle. Characterisation of LAB strains identified several potential probiotic candidates capable of both reducing bacterial adherence, as well as producing bacteriocin-like compounds. However, further evaluation of these strains is required to determine their potential use in the industry, including successful colonisation of the salmon gut in vivo, as well as the optimal route of administration. The semi-continuous culture fermenter developed in this study provides a suitable model to assess the effect of different feeds on the gut microbiota and how it affects their metabolic function. This model provides a future cost effective tool for feed companies to initially assess the impact of the newly formulated fish feed on the composition of the gut microbiota before the need for feed trials.
7.1 References

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