The diversity of the gastrointestinal bacterial community and its relationship to Atlantic salmon health and productivity

By

Kamarul Zaman Bin Zarkasi

B.Sc. (Hons) Microbiology, Universiti Sains Malaysia

M.App.Sc. Microbiology, University of Tasmania

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Kamarul Zaman Bin Zarkasi

B.Sc. (Hons), M.App.Sc. (Microbiology)

Supervisor: _____

Associate Professor John P. Bowman

Head of School: _____

Professor Holger Meinke

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Declaration of Originality and Authority of Access

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Kamarul Zaman Bin Zarkasi

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Statement of Co-Authorship

The following people and institutions contributed to the publication of work undertaken as part of this thesis:

Kamarul Z. Zarkasi, School of Land and Food, University of Tasmania (Candidate)

John P. Bowman, School of Land and Food, University of Tasmania (Supervisor)

Mark L. Tamplin, School of Land and Food, University of Tasmania (Co-supervisor)

Richard S. Taylor, CSIRO Agriculture Flagship (Co-supervisor)

Guy C.J Abell, CSIRO Agriculture Flagship (Co-supervisor)

Brett D. Glencross, CSIRO Agriculture Flagship

Mohammad Katouli, University of the Sunshine Coast

Christina Neuman, University of the Sunshine Coast

Eva Hatje, University of the Sunshine Coast

Author details and their roles:

Paper 1: Pyrosequencing-based characterization of gastrointestinal bacteria of Atlantic salmon (*Salmo salar* L.) within a commercial mariculture system

Located in Chapter 2

Kamarul Z. Zarkasi (68%) designed and conducted the experiment, analysed the data and wrote the manuscript. John P. Bowman (10%), Richard S. Taylor (5%), Guy C.J Abell (5%), Mark L. Tamplin (3%), and Mohamad Katouli (3%) contributed to experimental design, analysed the data, and edited the manuscript. Christina Neuman (3%) and Eva Hatje (3%) offered sampling and laboratory works.

Paper 2: Atlantic salmon (*Salmo salar* L.) gastrointestinal microbial community dynamics in relation to digesta properties and diet

Located in Chapter 3

Kamarul Z. Zarkasi (60%) designed and conducted the experiment, analysed the data and wrote the manuscript. John P. Bowman (15%), Richard S. Taylor (15%) Guy C.J Abell (4%), Mark L. Tamplin (3%) and Brett Glencross (3%) contributed to experimental design, analysed the data, and edited the manuscript.

Paper 3: *In vitro* growth characteristics of dynamic Atlantic salmon (*Salmo salar* L.) gastrointestinal microbial community in relation to different diet formulations

Located in Chapter 4

Kamarul Z. Zarkasi (69%) designed and conducted the experiment, analysed the data and wrote the manuscript. John P. Bowman (15%), Guy C.J Abell (5%) Richard S. Taylor (5%), Mark L. Tamplin (3%) and Brett Glencross (3%) contributed to experimental design, analysed the data, and edited the manuscript.

We the undersigned agree with the above stated "proportion of work undertaken" for each of the above published or submitted peer-reviewed manuscripts contributing to this thesis

(Assoc. Prof. John P. Bowman)   (Prof. Holger Meinke)

Supervisor   Head of School

School of Land and Food   School of Land and Food

University of Tasmania   University of Tasmania

12 February 2015   12 February 2015

Statement on Ethical Conduct

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University. The research included in this thesis was conducted under Animal Ethics permit A0012001.

Kamarul Zaman Bin Zarkasi

University of Tasmania

Hobart

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Thesis Summary

The Atlantic salmon (*Salmo salar* L.) gastrointestinal tract has a dynamic microbial community and its structure has been suggested to be a factor influencing fish health and productivity. Less productive salmon growth performance during times of temperature stress has been suggested to be linked to various factors including diet quality and the incumbent gastrointestinal tract microbial community. As salmon farming is one of Tasmania's largest agrisector industries, there is a strong motivation to better understand, protect and sustain salmon health and productivity. The aim of this thesis was to providing strategic information in relation to how gastrointestinal microbiology relates to farm environmental conditions and diet composition. Overall, this study provides improved understanding of farmed salmon gastrointestinal bacterial communities, what drives their dynamism and describes the relative impact of diet, environment and farm management factors.

The relationship of Atlantic salmon gastrointestinal tract bacteria to environmental factors, in particular water temperature within a commercial mariculture system, was investigated. Salmon gastrointestinal tract bacterial communities of commercially farmed in south-eastern Tasmania was analysed, over a 13 month period across a standard commercial production farm cycle, using 454 16S rRNA-based pyrosequencing. Faecal bacterial communities were highly dynamic but largely similar between randomly selected fish. In post-smolt, the faecal bacteria population was dominated by Gram-positive fermentative bacteria, however by mid-summer members of the family *Vibrionaceae* predominated. As fish progressed towards harvest, a range of different bacterial genera became more prominent corresponding to a decline in *Vibrionaceae*. The sampled fish were fed two different commercial diet series with slightly different protein, lipid and digestible energy levels; however the effect of these

differences was minimal. The overall data demonstrated dynamic hind gut communities in salmon that were related to season and fish growth phases but were less influence by differences in commercial diets used routinely within the farm system studied. This study provides understanding of farmed salmon gastrointestinal bacterial communities and describes the relative impact of diet, environmental and farm factors.

Less productive Atlantic salmon growth performances have been linked to temperature stress, diet and indirectly linked to the incumbent GI tract microbial community. To obtain knowledge that may aid management of salmon production during abnormally warm summer periods as well as to better understand salmon GI tract microbial community dynamics a feeding trial was performed over a summer period. The diets were tested over a 5 month period in relation to a commercial standard diet that has intermediate protein levels (45:25 protein:lipid, 35% fishmeal, IntPro). Modified diets tested included a low (10% w/v) fish meal content diet (LFM diet); a diet with a high protein and energy content (50:20 ratio, 21.4 MJ/kg digestible energy, HiPro) and a low protein, low energy diet (40:30, 19.7 MJ/kg, LoPro). A six point categorical scoring system was developed to describe expressed digesta consistency, where a low score describes 'normal' faeces and a high score denotes casts (pseudofaeces), or empty hind gut. The "faecal score" was used as a proxy for gut function. Faster growing fish generally had lower faecal scores and this was due to accelerated growth of sexually immature subpopulations while the diet cohorts showed comparatively little difference in terms of faecal score though the overall lowest scores were observed after 5 months with the LoPro diet. The GI tract bacterial communities assessed with 16S rRNA amplicon pyrosequencing were dynamic over time with the LoPro diets most strongly shifting the community structure in relation to the commercial standard diet used. During the

summer period the LoPro diet cohort and to a lesser extent all other cohorts with standard fish meal content had transient increases in GI tract community diversity mainly represented by an increased abundance of anaerobic (*Bacteroidia* and *Clostridia*) and facultatively anaerobic (lactic acid bacteria) taxa. The digesta had enriched populations of these groups in relation to faecal casts. The majority of samples (60-86%) across all diet cohort faecal communities were eventually dominated by the marine-derived, bile-tolerant marine facultatively anaerobic genus *Aliivibrio*. The results suggest that time (incorporating seasonal changes in temperature) and diet is potentially related to faecal microbial community structure. Categorization of the digesta via the faecal scoring system revealed strictly anaerobic taxa were comparatively more abundant in firmer, normal faecal samples that are also rich in plant chloroplast material suggesting significant diet digestion had occurred therein. Anaerobes were comparatively much less populous in pseudofaeces, which is generally associated with poor feeding. These community shifts possibly through formation of different levels of metabolites and/or immune system stimulation could influence salmon physiology and farm-level performance outcomes.

In order to better understand microbial changes within the salmon GI tract at the dietary level, the microbial community dynamics were assessed within a simple *in vitro* growth model system. In this system the growth and composition of bacteria were monitored within diet slurries held under anaerobic conditions inoculated with salmon faecal samples. This system was assessed using total viable bacteria counts (TVC), automated ribosomal intergenic spacer analysis (ARISA), and 16S rRNA pair-end Illumina-based sequence analysis. A total of 5 complete diets were tested including low fish meal (LM), low protein (LP), high protein (HP), a commercial standard diet with intermediate protein and lipid content (CS) and a CS diet

version where fish oil was completely replaced with poultry oil (PO). In addition plant meals (lupin kernel meal and pea extract, referred to as the LK and PE diets) were tested in isolation to determine if plant-derived material promotes the growth of specific bacteria. The *in vitro* model cultures were incubated at 20ºC to simulate warm summer temperatures. The microbial growth in the diet slurries after a lag phase of ~3 h grew over a 24 h period with a progressive decline in pH. TVC counts indicated growth on MA and TCBS plates were equivalent indicating most bacteria that grew were bile salts tolerant. ARISA and Illumina sequencing data revealed there was very clear separation between the complete diets and the LK and PE plant meal diets suggesting bacteria that grew were very distinct. The sequencing analysis showed in the case of the complete diets those members of the genera *Aliivibrio*, *Vibrio* and *Photobacterium* became greatly predominant. However based on replicated experiments there was evident stochasticity of what exact species became dominant. *Vibrionaceae* may have become predominant due to their rapid growth capacity, relatively high abundance within the starting faecal material and salt tolerance though several other bacterial taxa were also present in great abundance initially. The LK and PE diets only allowed the growth of the aerobic genus *Sphingomonas* no other faecal-associated bacterial grew including *Vibrionaceae* suggesting a combination of protein and lipid diet components structure the salmon GI tract community.

Taken together the data suggests acyclic dynamism in farmed Atlantic salmon GI tract populations is the norm with largely *Vibrionaceae* predominant beyond the post-smolt phase. *Vibrionaceae* are successful owing to their bile tolerance and rapid growth on diet nutrients. In future studies, to achieve demonstrable farm-level performance outcomes via diet manipulation focus should be placed on dietary additives and experimental strategies

instigated to more stably manipulate and influence GI tract communities, especially if probiotic supplementation is intended. Such experiments will need to be performed in tandem with deeper investigations of salmon physiological responses (cell biology, gene expression, protein profiles) during both optimal feeding periods and during periods in which feeding is suppressed or halted due to thermal stress and/or behavioural changes and correlate these responses to GI tract microbial communities. Furthermore, the functional role of GI tract bacteria needs to be more deeply examined to determine if metabolites and/or cellular interactions influence salmon immune or hormonal system responses. Integrated, these approaches can potentially lead to deeper understanding of how salmon GI tract bacteria interact within salmon in relation to environmental drivers and also give clues of how management strategies can be altered to maximise production during change for the long term.

Publications and presentations from this thesis

The following publications and presentations have resulted from work associated with the thesis.

Publications

Zarkasi KZ, Abell GCJ, Taylor RS, Neuman C, Hatje E, Tamplin ML, Katouli M and Bowman JP (2014) Pyrosequencing-based characterization of gastrointestinal bacteria of Atlantic salmon (*Salmo salar* L.) within a commercial mariculture system. *Journal of Applied Microbiology* **117:** 18-27. doi: 10.1111/jam.12514. (Manuscript submitted: 30th January 2014. Manuscript accepted: 28th March 2014).

Presentations

Zarkasi KZ, Goossen K, Abell GCJ, Taylor RS, Tamplin ML and Bowman JP (2012) Molecular and culture based characterisation of gastrointestinal tract bacteria of Atlantic salmon (*Salmo salar* L.) within a commercial mariculture system in south-eastern Tasmania, Australian Society for Microbiology Annual Scientific Meeting 2012, 1-5 July 2012, Brisbane, Queensland, Australia. Abstract number 555.

Zarkasi KZ, Abell GCJ, Taylor RS, Tamplin ML and Bowman JP (2012) Molecular and culture based characterisation of gastrointestinal tract bacteria of Atlantic salmon (*Salmo salar* L.) within a commercial mariculture system in south-eastern Tasmania, 6th Annual Graduate Research-Sharing Excellence in Research Conference 2012, 6-7 September 2012, University of Tasmania, Hobart, Australia. Abstract number 134.

Zarkasi KZ, Taylor RS, Abell GCJ, Tamplin ML and Bowman JP (2014) The effect of dietary formulations and environmental factors on Atlantic salmon gastrointestinal microbial community, World Aquaculture Conference and Trade Show 2014, 7-11 June 2014, Adelaide, South Australia, Australia. [Conference Extract]

Zarkasi KZ, Abell GCJ, Taylor RS, Neuman C, Hatje E, Tamplin ML, Katouli M and Bowman JP (2014) Pyrosequencing-based characterisation of gastrointestinal microbiota of commercially farmed Atlantic salmon (*Salmo salar* L.) in south-eastern Tasmania, Australian Society for Microbiology Annual Scientific Meeting 2014, 6-9 July 2014, Melbourne, Victoria, Australia. Abstract number 79. [ASM BD Student Travel Award]

Zarkasi KZ, Abell GCJ, Taylor RS, Glencross B, Tamplin ML and Bowman JP (2014) Effect of different diet composition and environmental factors on Atlantic salmon (*Salmo salar* L.) gastrointestinal tract microbial community, 15th International Symposium on Microbial Ecology, 24-29 August 2014, Seoul, South Korea. Abstract number 266. [UTAS Travel Award]

Zarkasi KZ, Taylor RS, Abell GCJ, Glencross B, Tamplin ML and Bowman JP (2015) The relation of dietary formulation and digesta properties on the Atlantic salmon (*Salmo salar* L.) gastrointestinal microbial community, World Aquaculture 2015, 26-30 May 2015, Jeju, South Korea. Abstract number 169.

List of Abbreviations

The following abbreviations and acronyms have been used in this thesis:

16S rRNA	16S ribosomal ribonucleic acid
AGD	amoebic gill disease
ANOVA	analysis of variance
ARISA	Automated ribosomal intergenic spacer analysis
BLAST	Basic Local Alignment Search Tool
BW	body weight
bp	base pair
BSA	Bovine serum albumin
CAP	canonical analysis of principal coordinates
CF	Condition factor
CFU	colony-forming unit
CO_2	carbon dioxide
df	degree of freedom
DE	digestible energy
DGGE	Denaturing Gradient Gel Electrophoresis
DHA	docosahexaenoic acid
DNA	deoxyribonucleic acid
EPA	eicosapentaenoic acid
FOS	fructooligosachharides
FPH	fish protein hydrolysates
GDP	gross domestic product
GI	gastrointestinal
GOS	galactooligosaccharides
IMD	the index of multivariate dispersion
LAB	lactic acid bacteria
MA	marine agar
MDS	multidimensional scaling
MOS	mannanoligisaccharide
MRS	De Man-Rogosa-Sharpe

MS	mean squared
NCBI	National Centre for Biotechnology Information
NGS	next generation sequencing
NMDS	Nonmetric multidimensional scaling
OTU	Operational taxonomic unit
PCR	polymerase chain reaction
ppm	parts per million
PERMANOVA	permutation multivariate analysis of variance
RGI	Relative growth index
RNA	ribonucleic acid
SGR	specific growth rate
SGS	summer gut syndrome
spp.	species
TCBS	thiosulfate-citrate-bile salts-sucrose
TRFLP	terminal restriction fragment length polymorphism
UV	ultra violet

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Chapter 1:

Atlantic salmon gastrointestinal microbiology: a review

1.1 Introduction

1.1.1 The Tasmanian salmon farming industry

The growth of the global salmon farming industry is one of the best examples of commercially successful aquaculture. Since the industry became established in early 1980s, it became a profitable aquaculture industry worldwide in most temperate countries including Scotland, Norway, Canada, Chile, Australia, Ireland, New Zealand and the United States (Knapp *et al.* 2007). Salmonid culture began in the 19th century in Europe when spawning and freshwater rearing techniques were developed for restocking of rivers and lakes. By late 1960s, the modern techniques of salmon culture in floating sea cages were initiated in Norway, and in the 1980s commercial salmon farming was well established (Asche *et al.* 2003). Since then, the salmon farming industry has become the main supplier (60%) of salmon products worldwide replacing the wild salmon fishing industry (Fig. 1.1). Farming of Atlantic and Pacific salmonids has huge economic potential in temperate marine countries such as Norway, Ireland, Scotland, Chile, Canada, Australia, New Zealand and the United States due to ideal environmental conditions, well managed supply chains, a favourable domestic business climate since salmon has a relatively high commercial value (Knapp *et al.,* 2007). Due to its requirement for access to clean oceanic waters, the salmon farming industry has become a key economic driver in developing remote coastal communities over the past 30 years.

a)
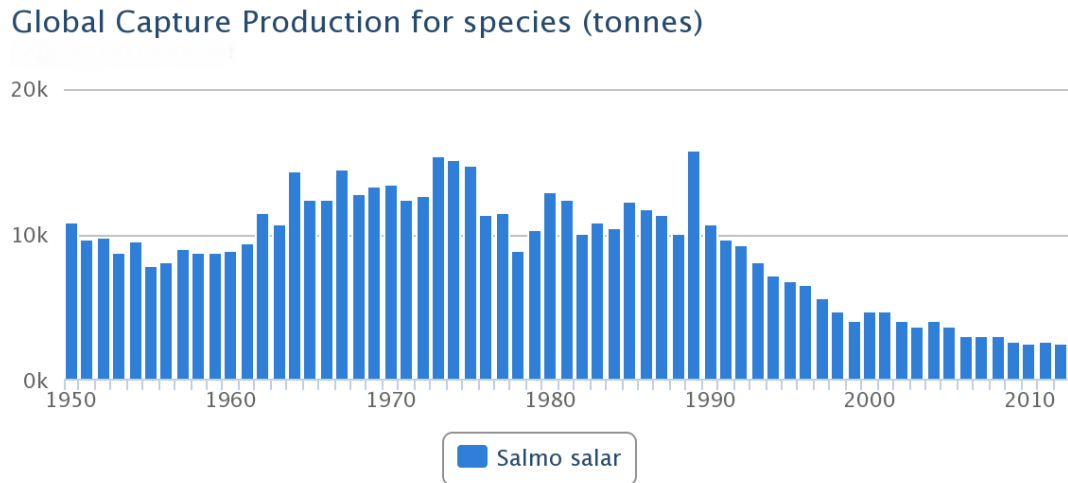
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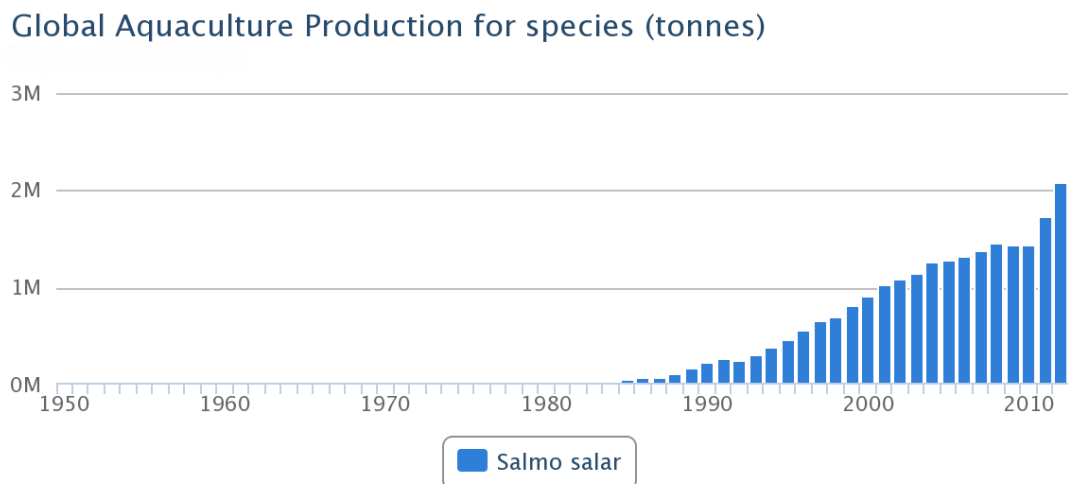
Figure 1.1: Global wild captured vs farmed aquaculture production for species, a) captured production, b) aquaculture production. Source data: FAO Fisheries and Aquaculture Department, FAO (2013).

Atlantic salmon originate from the temperate coats of the North Atlantic Ocean, being endemic to North America and Europe. A single strain was imported to the Gaden hatchery in New South Wales from the River Philip (Nova Scotia) in the 1960's with the initial aim of stocking freshwater lakes for sport fishery. Descendants of these fish were transferred to the Salmon Enterprises of Tasmania (SALTAS) hatchery in Tasmania in the mid-1980's as the foundation to the Tasmanian salmon farming industry. Tasmanian production has since expanded rapidly. Over the last decade, Australian salmonid production (mainly Atlantic salmon) increased by 171%, from 16,220 tonnes in 2001-2002 to 42,978 tonnes in 2012-2013. This was largely driven by the strong production growth in Tasmania (Table 1.1). In 2011-2012, salmonid production in Australia increased by 19% and surpassed sardine production to be Australia's highest species group produced in volume terms (ABARES, 2013). Tasmania's salmon industry is worth $506 million as Australia's highest value fishery and makes up around 1.5% of the world's overall salmon production and possesses a very good international reputation for its superior quality due to its clean farming waters, lack of major diseases and excellent all year round farming conditions (Tasmanian Department of Primary Industries, 2012).

Table 1.1: Salmonids production for 2011-2012 by state in Australia

	New South Wales	Victoria	Tasmania	Other states
Value AU$'000	2,200	3,870	506,446	61
Quantity (tonnes)	200	536	43,249	4
Production (%)	0.45%	1.22%	98.32%	0.01%

Source of data: ABARES (2013)

Atlantic salmon is the largest food trade item for Tasmania (Tasmanian Department of Primary Industries, 2012), contributing $160 million GDP into the Tasmanian economy. Almost 93% of Tasmanian salmon productions (2011-2012) are sold to the Australian domestic market while only 7% is exported. The industry is still expanding rapidly, mostly due to increase demand from consumers for a healthy source of omega 3 polyunsatureated fatty acids, as well as assisted by Tasmania's environmental conditions, which is ideal for salmon farming. Furthermore, strict quarantine controls on importation of salmonids products has played a significantly role in protecting the salmon industry from many of the serious diseases that affect salmon production in other countries. This quarantine control has prevented the importation of new genetic stocks, so the industry is reliant upon the initial River Philip strain. This strain has been managed to improve commercial traits using modern genetic selection techniques since 2005. The main traits in the selection goal are resistance to amoebic gill disease (AGD), growth, sexual maturation and harvest quality (Kube *et al.,* 2012). Most of Tasmanian's salmon farms are located at D'Entrecasteaux Channel and Huon River in the state's southeast and Macquarie Harbour on the west coast, and employ around 1,280 people across Tasmania.

According to the report by Australian Bureau of Agricultural and Resource Economics, ABARES (2013), seafood consumption increased at an average annual rate of 3% between 2000 and 2013. Apparent consumption of seafood per person increased at an average annual rate of 1 per cent, from 13 kilograms per person in 2000–01 to 15 kilograms per person in 2012–13 exceeds the consumption of sheep meat and lamb. At the same time, per person consumption of beef and veal has declined since 2006. Danenberg & Mueller (2011) reported Australians were consuming on average 3.1 meals a week that included a seafood component, with fresh salmon became top five of fish consumed. The key reasons provided

by survey respondents for consuming seafood included for better health, taste, ease of preparation, diversification from meat consumption and reasonable prices.

Salmon farming rapid increase and its inherent competitive market advantages makes the salmon industry a critical agribusiness within the Tasmanian economy. Considerable research and development (R&D) investment has been made in the areas of environmental management, nutrition, stock management, disease control, physiology, and selective breeding (Kube *et al.,* 2012). Tasmania in being the major supplier of salmonids to the Australian domestic market has also been driven by the success in effectively marketing salmon to the Australian consumer (Fisheries Research and Development Corporation 2007) with resultant increased consumption (50% increase between 2006 to 2013 fro, 1.1 to 1., Tassal Group Inc. Annual report 2013). Atlantic salmon products are exported throughout the Asian region including Japan, Hong Kong, Indonesia, Thailand, China, Taiwan, Singapore, Malaysia, Vietnam, Guam, India and the Philippines (Tasmanian Department of Primary Industries 2012). The Tasmanian Atlantic salmon industry has a relatively disease free status, the most significant disease problem is Amoebic gill disease (AGD) (Munday *et al.* 2001; Powell *et al.* 2001; Vincent *et al.* 2006; Taylor *et al.* 2007) that requires regular fish handling and freshwater bathing.

1.1.2. Atlantic salmon (*Salmo salar*) lifecycle

In the wild, Atlantic salmon spawn their eggs in highly oxygenated fast-flowing freshwater streams in autumn. The length of incubation of the eggs within the river bed gravel depends upon water temperature, after approximately 440 degree days (sum of daily temperatures) the fish hatch as alevins, the mouthparts of the alevin are undeveloped and they derive nourishment from the yolk-sac which protrudes the ventral surface. The yolk sac

gradually absorbs over three to four weeks (dependant upon temperature). The final absorption of the yolk sac coincides with the development of the mouth, digestive tract and excretory organs, so that the fry are able to first feed. They work their way up through the gravel and begin to live on small zooplankton. As the fry develop they make longer forays for larger food items. Depending upon temperature, fish take a year or more to develop as fingerling and parr before they can migrate out to sea.

The process of smoltification consists of a number of independent but coordinated changes in the biochemistry, endocrinology, morphology, and behaviour of the juvenile salmon, including alterations in lipid metabolism, osmoregulation, oxygen transport, buoyancy, growth, colour, shape, rheotaxis and schooling behaviour which are preparatory to maximise the success of downstream migration and ocean life (Stefansson *et al.,* 2008). The transformation from freshwater parr to seawater adapted smolt is decided by body weight and synchronised by natural day length cues from winter to spring. Fish may spend 1 to 5 years at sea before returning to their natal river as mature adults to spawn (Hansen & Quinn, 1998). Atlantic salmon are adapted to the prevailing environmental conditions that they encounter both in freshwater and at sea (Hansen & Quinn, 1998). The most common life history pattern for Atlantic salmon is thus anadromy, but resident individuals and populations of the species are also well known (Jonsson & Jonsson, 1993). As a result some populations of salmon do not go to sea, and these populations may be landlocked, with physical barriers preventing anadromy (Verspoor & Cole, 1989).

Commercially farmed Atlantic salmon go through a production cycle that mirrors nature (ISFA, 2015) but freshwater growth is closely controlled by temperature and nutrition to advance or delay production timing (Figure 1.2). The Tasmanian industry is unique in utilising all-female stock, which naturally mature later than male fish, thus ensuring harvest

quality and year-round harvest production. The process of all-female production involves androgen hormonal treatment of potential broodstock to produce XX neomales as potential broodstock. When crossed with normal females this results in 100% female offspring. Spawning of genetically selected females and neomales occurs in autumn (May), with up to 40% of eggs subjected to hydrostatic pressure shock to induce triploidy to prevent maturation. The development of the embryo begins (Pelletier *et al.,* 2009) leading to alevins that begin to hatch after approximately four weeks of the eyed egg stage. The yolk sac is absorbed enabling the fish to move and swim, this process may take 3 weeks and then active feeding will begin using artificial feeds (ISFA, 2015).

During the freshwater stage, growth rates dramatically increase and requirements for feed, oxygen and water flow significantly increase. The process of smoltification is controlled by growth rate, grading, temperature control and lighting before trucking the fish to sea. The lead groups of fish are available to stock to sea as 'out of season smolt' by late summer to mid-autumn and are followed by 'marine pre-smolt' groups between autumn and spring. Finally, the spring smolts are stocked in September-October (Figure 1.2). Fish spend 15-18 months in floating net-pens at sea prior to reaching harvest size of around 5 kg (ISFA, 2015; Pelletier *et al.,* 2009). Utilising different smolt types, farm sites and production plans the industry is able to guarantee year-round harvest to maintain the flow of product to customers. Fish are seined and pumped into harvest vessels, stunned, bled and then transfer for processing as premium gutted, portioned or smoked product. This combination of supply predictability and consistent high quality allows Tasmanian farmed salmon to command a high price and stable customer base.
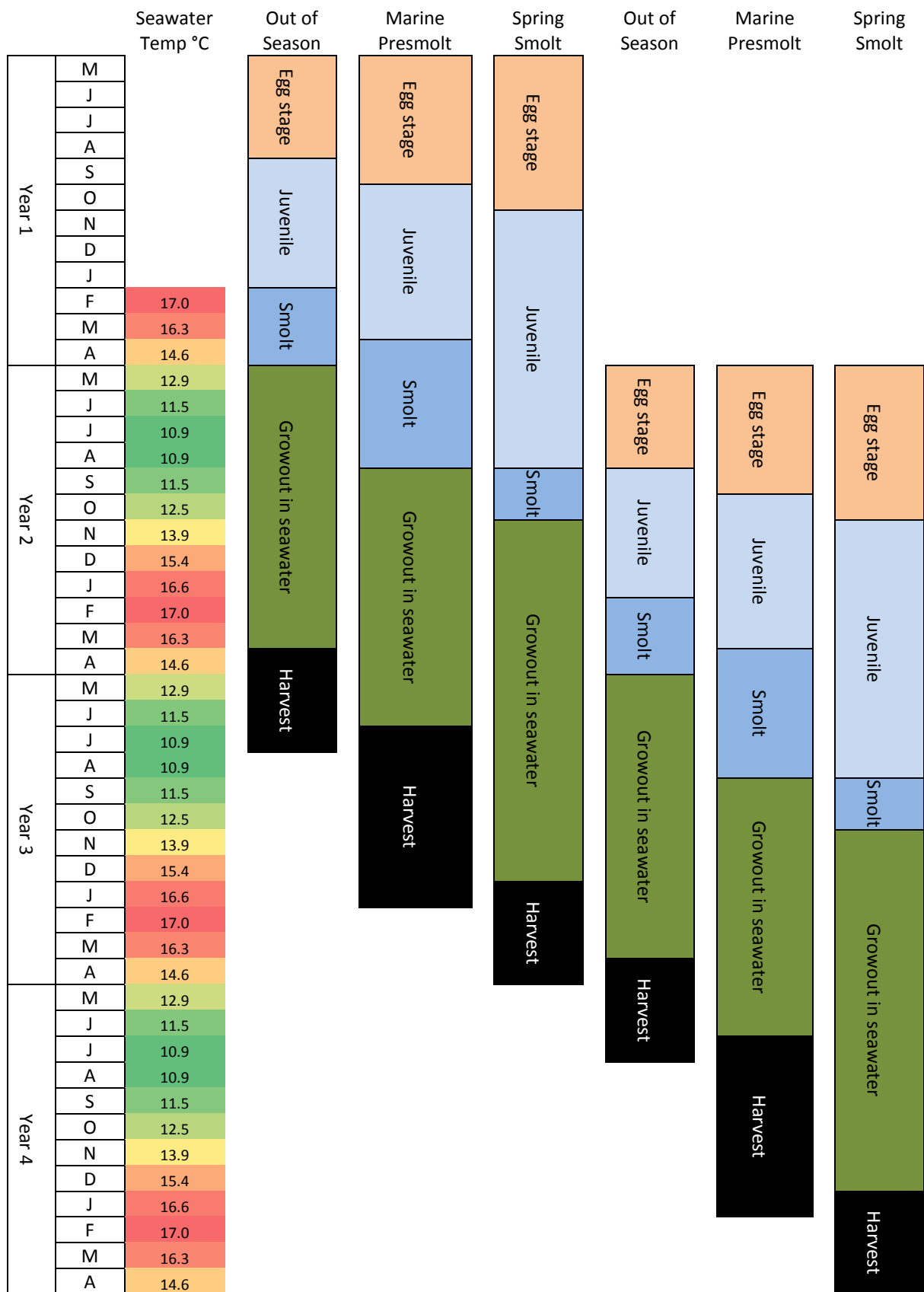
Figure 1.2: Production cycle of farmed Atlantic salmon in Tasmania over two generations.

1.1.3 "Summer Gut Syndrome" in Tasmanian Atlantic salmon

The optimum temperature for growth of Atlantic salmon in sea water occurs at 13-15°C (Handeland *et al.* 2003), with upper critical temperatures around 22°C (Jonsson and Jonsson 2009). Salmon respond to suboptimal thermal fluctuations by behavioural, biochemical and physiological modifications in order to maintain cellular homeostasis and physiological performance (Claireaux *et al.* 2000; Farrell and Richards 2009). Average sea water temperatures (at 5 m depth) in south eastern Tasmania are close to this optimum (average 14°C). However, during the summer season, water temperatures average is around 17-18°C and peaks at 20°C with surface temperatures reaching 23°C. Coupled with lowered oxygen availability this imposes limitations on stock performance for the industry (Clark and Nowak 1999). It is predicted that thermal tolerances will be exceeded more frequently due to climate change and ocean warming, which is likely to increase production cost, degraded fish health and increased disease issues and impact fish welfare (Battaglene *et al.* 2008).

Tasmanian salmon farmers have recorded levels of inappetance in stock in some summer periods (especially 2007-2009) which lead to a high proportion of underweight fish with poor condition. This seasonal production issue has raised concerns within the Tasmanian Atlantic salmon industry due to impacts it has on production efficiency, potentially leading to approximately 20 per cent increase in production costs and increased time to market weight. This summer production issue is characterised by fish with yellow/white casts in the hindgut or no digesta, and frequent observation of reddened hind gut epithelial layer tissue. This suggestions involvement of GI processes in the overall problem which is referred to as "Summer Gut Syndrome" (SGS) or Irritable Gut Syndrome (IGS). To date, no specific sustainable management strategy to mitigate SGS has been developed. However husbandry practices such as lowered stocking density, improved net hygiene and water flow

management by aeration in times of slack tide and high temperature have achieved some level of control of the problem.

The seasonal SGS issue remains largely undefined but commercial experience has demonstrated that antibiotic treatment appears capable of resolving the problem temporarily (Green *et al.* 2013). This suggests the possible involvement of bacterial agents in SGS. However, no evidence of standard disease like signs such as, lesions typical of bacteraemia or parasitic intestinal disease manifests. A change from nil antibiotic usage on Tasmanian marine farms in the mid 1990's to over 9 tonnes in 2007 was almost exclusively a result of the need to control SGS, and was a major concern for the industry due to negative public perception of product quality and environmental impact (Australian Broadcasting Corporation, 2009). Beside general husbandry practices the two major factors implicated in the onset of SGS are elevated water temperatures and diet quality. At above 16°C Atlantic salmon become stressed and exhibit fatigue (Oppedal *et al.* 2011) and thus thermal stress is likely the arena in which the SGS problem is set.

The development of manufactured pellet feeds lead to the rapid growth of global salmon aquaculture. The cooking processes required to produce pellets ensures that they are free of pathogens and are robust for transportation, silo storage and suitable for high volume delivery by blower or water cannon into sea cages. Tasmanian salmon feeds available from the mid-1980's were made largely from fish meal and fish oils with carbohydrate binders and produced by the steam method, whereby raw materials were blended and steam cooked to 70°C before being pressed through a ring dye. This process produced a high protein (45-50%) product with limited capacity to incorporate lipids (typically 15-18%).

By the late 1990's extruded feed technology became available, whereby ingredients are steam super-heated to 120°C and pressure extruded, the sudden change in pressure causing the pellet to expand. Protein is the most expensive portion of fish feed, extrusion allows proteins to be replaced with oils, which are flooded into the pellet matrix by vacuum coating. Extruded feeds are typically 'high energy' having dietary oil levels up to 30% and digestible energy above 20 MJ/Kg. Extrusion produces a more durable pellet, increased carbohydrate and nutrient digestibility and lowered feed conversion ratio (FCR). The ability to alter protein:energy ratios allows feeds to be tailored to life stage requirements of the fish (Hillestad and Johnsen 1994; Einen and Roem 1997). The extrusion cooking process (which breaks down heat labile anti-nutrients) and increased global research on salmon nutrition paved the way for fish meals and oils to be replaced by alternative animal and plant sources, thus lowering dependence upon marine raw materials and promoting 'least cost' blending from alternative sources depending upon price and availability.

Around the year 2002 as feed energy increased and least cost diet strategies were introduced, the seasonal production issue was first noticed. Bacteria in Atlantic salmon gastrointestinal (GI) tract appear to be highly dynamic environment over its lifetime. In wild salmon the community likely stabilises while in maricultured salmon, the bacterial community may be more undeveloped or simpler and thus more heterogeneous (Ringø *et al.* 1995). Researchers in Norway suggested the bacterial community in Atlantic salmon GI tract is highly influenced by the local environment, especially temperature, and salinity, as well as dietary regimes (Ringø *et al.* 2010). As found in other animals GI tract microorganisms are likely to be highly important for fish development, promotion of metabolism, and maturation of innate immune responses (Olafsen 2001). Therefore, increased knowledge of the diversity

of bacteria that inhabit the GI tract of commercial fish species may lead to benefits in the management and productivity of fish in aquaculture systems.

1.2 Interaction between fish and bacteria

Fish and most marine organisms inhabit environments that are relatively rich in bacteria and other microorganisms (Hansen and Olafsen 1999). Interaction between fish and bacteria can be categorised as parasitism, mutualism and commensalism. Each category is based on the interaction effect on each population (Atlas and Bartha 1986). Parasitism is a bacterial population gaining benefits at the expense of the host fish. Commensalism is a bacterial population gaining benefits, but the host is unaffected. Mutualism is where both bacteria and the host gain benefits from their interaction (Madigan *et al.* 2009). The relationship between fish and bacteria is difficult to generalise because of the diverse range of fish species, the wide variety of metabolic capabilities of bacteria, differences in the environments fish inhabit, and the diverse range of bacteria that can occur within and on fish.

Fish and bacteria interact since mucus layers of the skin, GI tract and gills provide bacteria a surface to colonise (Austin 2006). Mucus layers purposely act as protective layers for the fish. However, they can also provide adhesion sites for bacteria (Cahill 1990). Nevertheless, bacteria have not been found attached to nor colonise healthy internal fish organs such as the liver, kidney and spleen (Austin 2006). The gastrointestinal tract epithelial layer is one of the surfaces which are commonly colonised by bacteria. The ability to grow and multiply on the mucus layer surface can be placed into two major categories, indigenous bacteria and transient bacteria (Madigan *et al.* 2009). Indigenous bacteria can grow and multiply on the surface of the host fish, while transient bacteria only attach for a short time and generally do not grow significantly. Ringo *et al.* (2003) and Ringo *et al.* (2007) reported

that bacterial pathogen attachment to the GI tract is often the first step of infections. The location of the bacterial attachment and ability to colonise the GI tract determines the importance of the bacterial species in fish (Cahill 1990).

Several studies have described that the salmon gastrointestinal tract bacterial community can be divided into two major groups, either autochthonous bacteria (normal flora of endothermic animals) or allochthonous bacteria (microorganisms which are transient visitors of the GI tract which pass through the gut over time) (Savage, 1989; Ringø *et al.,* 1995). Bacterial groups described as autochthonous bacteria in salmonids potentially include *Enterobacter*, *Pseudomonas* and *Acinetobacter* because they have been isolated from both free-living and cultured fish (Ringø *et al.,* 1995). Previous research indicated variability in the salmon intestinal microflora reflecting the GI tract community is determined by the exposure to changes in surrounding water, such as the transition from freshwater to saltwater (Austin, 2006).

Due to the sudden change from freshwater to seawater environments following stocking to sea cages, it is possible that there is disruption of both autochthonous and allochthonous communities, though some bacteria may survive well in both environments. This is based on the observations that intrusions of waters with different salinity levels affects ecosystem functioning that is conducted by microorganisms (Neubauer *et al.,* 2013) and also direct affects microbial cells (Nelson *et al.,* 2014). It is possible that community perturbations, where some bacteria are promoted due to inherent resilience to change in the environment may follow short term exposure regular freshwater immersion to control AGD. If this tends to occur, the microbes that can tolerate environmental shocks such as changes in salinity or presence of oxygen may become more predominant within salmon GI tracts.

Seawater can function as a medium for growth and the transport of bacteria compared to the air, which has been thought only to function as a transport medium (Hansen and Olafsen 1999). Thus, bacteria native to the local seawater are responsible for many marine fish diseases. Only very few bacteria in seawater are actually obligate pathogens (dependent on a living host for their propagation). The majority of bacteria are opportunistic pathogens that are present as part of the normal seawater microbiota (Hansen and Olafsen 1999).

Environmental factors directly or indirectly contribute to the occurrence of opportunistic and pathogenic bacteria in fish. As an example, *Vibrio anguillarum* counts in seawater can increase up to 10^3 fold due to discharge of carbohydrate-containing waste water (Larsen 1985). Moreover, virulence determinants of bacteria may be regulated by environmental factors or may only be expressed under specific conditions (Griffiths 1991). Thus, alterations in seawater temperature, oxygen concentration, pH, osmotic strength and pollutions may allow bacteria to colonise, invade and penetrate to the host tissues (Hansen and Olafsen 1999). In addition, chemical or abrasive forces may also impair the integrity of the mucus layer and facilitate bacterial access to host epithelial surfaces (Hansen and Olafsen 1999).

The salmon GI tract microbial community likely varies on the basis of differences in diets, environmental factors, seasons, geographic locations and overall ecosystems. Many of these factors have not been investigated in depth so far but theoretically all may collectively combine to hugely impact GI tract communities and thus eventually influence salmon health and production. Diets appear to be one of the most important factors that shape the salmon microbial GI even though the impact is still not clear. The influence of high temperature and summer season might contribute to the increase number of certain bacteria which can respond

strongly to water temperature, such as *Vibrio*, which can grow rapidly between 13-22ºC (Kaspar and Tamplin 1993).

1.2.1 Bacterial concentration in fish and aquaculture environment

Maeda (2002) demonstrated that bacteria show different distribution patterns and population densities in marine environments. As an example, coastal sea sediment may contain more than 10^7 colony forming units/g wet weight of sediment, while in natural seawater and waters within aquaculture farms can reach a level of 10^6 cells/ml. The main bacteria in aquaculture waters were identified as *Vibrio, Pseudomonas, Flavobacterium, Acinetobacter,* and *Pseudoalteromonas* (Maeda 2002). Certain bacteria such as *Vibrio* spp. can grow quickly when the marine environment is enriched by organic matter. Moreover, *Vibrio* spp. has a high growth rate and ability to adapt to oxygen deficient conditions, makes them able to quickly react within and grow in eutrophic aquaculture environments (Maeda 2002).

According to Maeda (2002) and Ringø *et al.* (1995) the main pathogenic bacteria infecting fish are *Vibrio anguillarum*, *Vibrio parahaemolyticus, Photobacterium damselae, Edwardsiella tarda, Tenacibaculum maritimum* (formerly *Flexibacter maritimus*), *Aeromonas hydrophila* and some *Streptococcus* spp., while *Lactobacillus* spp. also formed a high percentage of intestinal tract bacterial colonists. The bacteria in fish may have widely different effects either positive, neutral or negative involving activities ranging from pathogenesis to probiotic activity.

1.2.2 Bacterial survival strategies in the marine environment

Pathogenic bacteria may survive for months or years in water and sediment, requiring them to develop strategies that help them to sustain viability in variable environments and to adapt in nutrient fluctuations availability (Morita 1982; Roszak and Colwell 1987; Hansen and Olafsen 1999). At least some bacteria are able enter a dormant stage to overcome stressful and/or extreme conditions (Watson *et al.* 1998). An important microbe to the salmon industry able to do this is *Aliivibrio salmonicida*, which is able to survive extreme conditions such as starvation for lengthy periods (Hansen and Olafsen 1999). Dormancy characteristics can be identified by increased resistance to heat and UV irradiation, and synthesis of starvation survival proteins (Roszak and Colwell 1987; Hansen and Olafsen 1999).

1.2.3 The establishment of bacteria inside gastrointestinal tract

The range of bacteria isolated from the fish generally is related to the bacterial load of their surrounding habitat and varies with temperature, water salinity and suspended organic matter (Cahill 1990) as well as the timing of fish exposure to bacteria. Fish can be first exposed during the egg stage or during larval development. Diverse groups of bacteria have been found associated with marine fish eggs, both under natural and artificial culture conditions (Olafsen 2001). Moreover, both pathogenic and non-pathogenic bacteria can be found within fish eggs (Hansen and Olafsen 1999). This suggests that the establishment of bacteria inside the intestinal tract of fish can commence as early as the egg stage of life fish. Ringø *et al*., (1995) also reported, bacteria are introduced to the early development of fish larvae when first-feeding commences.

Bacteria often however, occur at heavy loads during the hatching process (Ringø *et al.* 1995; Olafsen 2001). Therefore, bacteria are also introduced during the hatching process

prior to the first feeding process. Bacteria entering the GI tract along with the seawater, also establishes the gut flora in fish (Ringø *et al.* 1995). In addition, the bacterial communities in fish re-continually affected by the fish diet as well as the ambient water (Hansen and Olafsen 1999). Thus, environmental factors and input of nutrients, both contribute to the presence and variation of bacteria inside the intestinal tract. Recent studies have been conducted to understand the potential of bacterial populations in the fish GI tract for their growth and survival. Some of these bacteria were defined as probiotic bacteria, which can benefit the host fish by various means (Verschuere *et al.* 2000; Geovanny D *et al.* 2007).

1.2.4 The role of bacteria in the fish gastrointestinal tract

Bacteria present in animal and human GI tract play an important role for their host in terms of nutrition, absorption, digestion and metabolism. Some roles are specific and not carried out by the host including for example fermentation of non-degradable oligosaccharides, degradation of xenobiotics, synthesis of growth factors, and metabolism of bile compounds (Guarner and Malagelada 2003; Tlaskalová-Hogenová *et al.* 2011). Gastrointestinal bacteria also contributes to the development and maintenance of mucosal and systemic homeostasis of the host as well as host defences and longevity (Rolfe 2000; Neish 2009). Despite numerous positive interactions between microorganisms and hosts, bacteria are also clearly capable of numerous negative interactions such as parasitism, infectious disease, toxigenesis and less defined disturbance of gut function (Tlaskalová-Hogenová *et al.* 2011).

In fish, bacteria play an important role in the GI tract likely much like other animals and fundamentally associated with nutrient gain by the host or on the other hand the through

diseases (Harris 1993). Moreover, fish health is suggests to be dependence on the immediate environment and thus fish are exposed to a potentially wide variety of microorganisms that may colonise either internally or externally (Gómez and Balcázar 2008). These microbes may colonise fish when conditions are favourable; therefore establishment of normal protective microorganisms is important in maintaining fish health as well as excluding potential invaders (Balcázar *et al.* 2006). Recently, potential of normal microorganisms to affect the overall health status of fish has been underestimated.

The fish health status can be improved by the role bacteria play within the fish GI tract, in which the bacteria create a favourable habitat for themselves by modulating expression of host genes by interacting with GI tract tissue (Balcázar *et al.* 2006; Gómez and Balcázar 2008). The normal GI tract microbiota, found to be the suite of species compatible with the host biology likely significantly influences the innate immune system which can enhance disease resistance of fish via stimulation of production of antimicrobial peptides, lectins, lysozyme, anti-proteases and natural antibodies (Gómez and Balcázar 2008).

Bacteria have diversified enzymatic potential within the GI tract and might be beneficial or interfere in numerous ways (Bairagi *et al.* 2002). Bacteria are important owing to their fermentative and nutrient synthesis capacities (Nayak 2010). For example the host microbiota is capable of digesting otherwise ingestible material such as cellulosic material that can be added in high fibre fish diets (Saha *et al.* 2006). Moreover, previous studies suggested that bacteria present in the GI tract can produce digestive enzymes such as amylase, cellulose, lipase and protease apart from endogenous sources. This activity would be beneficial because it would enhance digestion and provide dietary energy (Bairagi *et al.* 2002) as well as potential probiotic effects via accumulation of acidic end-products. Bacteria are

suspected in playing important roles in postnatal maturation of fish gut immune functions, thus shifts in the bacterial community in fish GI could influence fish immune status and gut homeostasis (Gaboriau-Routhiau *et al.* 2009). Thus, beneficial bacteria introduced to farmed fish by incorporation into fish diets (Saha *et al.* 2006) have potential for disease control, and increasing growth performance due to improved nutrient utilization.

1.3 The Atlantic salmon gastrointestinal microbial community

The GI microbial community in fish is considered an important factor for the fish and contributes to fish health, growth and productivity (Ringø *et al.* 1995; Olafsen 2001; van Kessel *et al.* 2011). Moreover, the fish intestine has been described as an important route for invasion of pathogenic bacteria and subject to microbial colonisation (Hovda *et al.* 2007; van Kessel *et al.* 2011). Previous studies described salmon GI microbial communities as being influenced by several factors that included a diet regime (Ringø *et al.* 2006a; Bakke-McKellep *et al.* 2007; Ringø *et al.* 2008; Reveco *et al.* 2014), antibiotics (Burridge *et al.* 2010), temperature (Cahill 1990; Pankhurst and King 2010), salinity (Sullam *et al.* 2012), ecosystems and geographic location (Holben *et al.* 2002). Therefore, understanding the fish GI microbial community is important for identifying its potential in determining fish health and growth capacity and more broadly may lead to improved knowledge that could inform farm management practices and reduce negative impacts to farm fish stocks.

Furthermore, the majority of Atlantic salmon mariculture takes place in marine net cages, where the fish's ability to adjust to fluctuations in the natural environment is limited within the confines of the production enclosure. Therefore, the physical environment changes

may induce a stress response that incurs a physiological energy cost to the fish (Oppedal *et al.* 2011). The GI microbiota in fish has been suggested as highly important for fish development, including promotion of metabolism and maturation of innate immune response (Waagbø 1994; Ringø *et al.* 1995). Improved understanding of bacteria types and other life forms, which inhabit the fish intestinal tract, and understanding of how the surrounding environment and dietary regime influence the intestinal fish microbiota, may eventually provide benefits in the management and productivity of aquaculture systems.

1.3.1 The diversity of Atlantic salmon gastrointestinal microbial community

The salmon GI microbiota and other fish varieties have previously been investigated using culture-dependent methods (Cahill 1990; Ringø and Birkbeck 1999) and more recently using molecular-based methods (Holben *et al.* 2002; Hovda *et al.* 2007; Navarrete *et al.* 2009). The latter approach, avoids an inaccurate reflection of the microbial composition since uncultivable bacterial genera can be detected (Suau *et al.* 1999; Hovda *et al.* 2007). One of the obvious bacteria genus that can be missed in culture-dependent studies are the lactic acid bacteria but with recent molecular-based methods, this bacterial group is easily identified and has been described as one of the major components of the gut microbiota especially in healthy fish (Hovda *et al.* 2007). This Gram-positive fermentative bacterial group seems to make an important contribution to salmon GI tract function and is presumed to provide benefits through immunomodulatory effects and pathogen antagonistic interactions via adherence to the intestinal epithelial cell layer, as well as by providing nutrients by contribution to the digestion process (Ringø and Gatesoupe 1998; Balcázar *et al.* 2006; Hovda *et al.* 2007; Balcázar *et al.* 2008; Ringø *et al.* 2010).

The salmon GI tract is inhabited by many different microorganisms, and the bacterial genera that have been isolated include both Gram-negative and Gram-positive bacteria (Table 1.2). In addition, the species distribution seems to differ from the foregut through to the hind gut (Hovda *et al.* 2007). The vast dominant culturable bacterial genera discovered from the salmon GI tract identified by previous studies include *Vibrio* spp., *Aliivibrio* spp., *Photobacterium* spp., *Enterobacteriaceae*, *Flavobacterium* spp., *Pseudomonas* spp., *Lactobacillus* spp. and *Lactococcus* spp., (Cahill 1990; Ringø *et al.* 1995; Ringø and Birkbeck 1999; Holben *et al.* 2002; Hovda *et al.* 2007). Other abundant bacterial genera present includes *Mycoplasma* spp., *Carnobacterium* spp., *Citrobacter* spp., and *Clostridium* spp. were also identified using molecular-based methods (Holben *et al.* 2002; Hovda *et al.* 2007).

Table 1.2: List of bacterial genera commonly found in the salmon GI tract

Genus	References
Acinetobacter	(Holben *et al.* 2002), (Hovda *et al.* 2007), (Navarrete *et al.* 2009)
Aeromonas	(Holben *et al.* 2002), (Skrodenyte-Arbaciauskiene *et al.* 2008), (Askarian *et al.* 2012)
Bacillus	(Hovda *et al.* 2007), (Askarian *et al.* 2012), (Hovda *et al.* 2012)
Brevundimonas	(Hovda *et al.* 2007)
Burkholderia	(Holben *et al.* 2002)
Campylobacter	(Holben *et al.* 2002)
Carnobacterium	(Burr and Gatlin 2005), (Skrodenyte-Arbaciauskiene *et al.* 2008)
Chryseobacterium	(Burr and Gatlin 2005), (Skrodenyte-Arbaciauskiene *et al.* 2008)
Citrobacter	(Holben *et al.* 2002)
Clostridium	(Holben *et al.* 2002)
Cytophaga	(Holben *et al.* 2002)
Enterobacter	(Holben *et al.* 2002)
Enterobacteriaceae	(Burr and Gatlin 2005), (Skrodenyte-Arbaciauskiene *et al.* 2008)
Enterococcus	(Burr and Gatlin 2005), (Skrodenyte-Arbaciauskiene *et al.* 2008)
Flavobacterium	(Navarrete *et al.* 2009)
Fusobacterium	(Holben *et al.* 2002)
Janthinobacterium	(Hovda *et al.* 2007)
Lactobacillus	(Holben *et al.* 2002), (Hovda *et al.* 2007), (Hovda *et al.* 2012)
Lactococcus	(Hovda *et al.* 2007), (Burr and Gatlin 2005), (Hovda *et al.* 2012)
Microbacterium	(Burr and Gatlin 2005), (Skrodenyte-Arbaciauskiene *et al.* 2008)
Methylobacterium	(Holben *et al.* 2002)
Mycoplasma	(Holben *et al.* 2002)
Peptostreptococcus	(Holben *et al.* 2002)
Photobacterium	(Holben *et al.* 2002), (Hovda *et al.* 2007), (Hovda *et al.* 2012)
Plesiomonas	(Skrodenyte-Arbaciauskiene *et al.* 2008)
Pseudomonas	(Hovda *et al.* 2007), (Navarrete *et al.* 2009), (Askarian *et al.* 2012)
Psychrobacter	(Hovda *et al.* 2007), (Askarian *et al.* 2012)
Serratia	(Burr and Gatlin 2005), (Skrodenyte-Arbaciauskiene *et al.* 2008)
Staphylococcus	(Askarian *et al.* 2012)
Streptococcus	(Holben *et al.* 2002)
Vibrio	(Hovda *et al.* 2007), (Hovda *et al.* 2012)
Weissella	(Hovda *et al.* 2012)

1.3.2 Effect of diet on salmon GI tract microbial communities

Salmon health and the relation to the GI tract microbiota are still poorly defined. However, the GI tract microbial communities are more likely greatly influenced by diets used in the aquaculture industry. This theory has been supported by substantial research (Ringø and Olsen 1999; Korsnes *et al.* 2006; Ringø *et al.* 2006a; Ringø *et al.* 2006b; Bakke-McKellep *et al.* 2007; Kotzamanis *et al.* 2007; Ringø *et al.* 2008; Askarian *et al.* 2012; Landeira-Dabarca *et al.* 2013). Diet has been explored that targets fish GI health (Dimitroglou *et al.* 2011) and switching diet regime seems to shift microbial diversity. The correlation between what is optimal for fish growth performance and the GI tract microbial community is still largely unknown.

Manipulation of diet content to maximize fish growth performance has been performed in many different ways. Primarily, it has involved optimising protein/lipid ratios (Hillestad and Johnsen 1994; Ringø and Olsen 1999; Lee and Kim 2001; Korsnes *et al.* 2006; Kotzamanis *et al.* 2007), digestible energy levels (Korsnes *et al.* 2006; Kotzamanis *et al.* 2007) and digestibility (Landeira-Dabarca *et al.* 2013). Diet supplements, fish meal and oil substitutes such as soybean (Carter and Hauler 2000; Ringø *et al.* 2006b; Bakke-McKellep *et al.* 2007; Ringø *et al.* 2008), lupin kernel (Carter and Hauler 2000; Glencross *et al.* 2008; Salini and Adams 2014), microalgae (Borowitzka 1997; Spolaore *et al.* 2006; Zhao *et al.* 2006), complex carbohydrates (Ringø and Olsen 1999; Korsnes *et al.* 2006), chitin (Askarian *et al.* 2012), probiotics cultures and prebiotics compounds (Austin *et al.* 1995; Verschuere *et al.* 2000; Burr and Gatlin 2005; Balcázar *et al.* 2006; Ringø *et al.* 2006a; Bakke-McKellep *et al.* 2007; Merrifield *et al.* 2010), can potentially modify GI tract communities based on a number of culture-dependent and molecular-based analyses. Overall data from previous researches suggests that GI tract microbial communities of salmon and other fish are sensitive

to dietary changes and local aquatic environments and these changes likely have consequences in aquaculture applications.

In general, farmed salmon fish require fishmeal and fish oil, which contains high-quality protein and rich in essential amino acids (Sargent and Tacon 1999). Other ingredients such as wheat, poultry meal and vitamins can also been added. These components known widely, are used in fish farmed today as well as many research has been conducted to find other potential diet ingredients which can improve fish diet for better health status and productivity.

1.3.2.1 Protein

The protein and energy level were found affected the salmonid fish because improper balance of protein and energy ratio have increased fish production cost (Lee and Kim 2001). Previous studies have found salmon growth improved or decreased if protein diet and energy ratio were manipulated (Einen and Roem 1997) and it also suggested that diet with high energy and less protein, or fish protein hydrolysates (FPH) addition can optimise the growth of salmon (Hillestad and Johnsen 1994; Lee and Kim 2001). Moreover, studies have found that FPH used as substitutes in fishmeal to enhance marine fish growth, or replacing some percentage fish meal with FPH, positively affected the growth of marine fish as well as improved marine fish development (Kotzamanis *et al.* 2007).

Despite it benefiting marine fish growth and development, manipulation of fish meal such as replacement with FPH can also boost bacterial proliferation (Kotzamanis *et al.* 2007). Monitoring microbiota is important to make sure that they are not detrimental to marine fish because opportunistic bacteria such as *Vibrio* species can infect fish larvae for example, and

potentially result in high mortality rates (Gatesoupe 1997). Unfortunately, no intensive study of protein diet manipulation on salmon guts bacteria has been performed to date.

1.3.2.2 Soybean meal

Soybean meal has been studied to investigate its potential for farming fish as a main diet ingredient or dietary supplement for fish. It has shown as having the best potential for replacing fish meal protein and to improve salmon productions in the commercial farm (Carter and Hauler 2000). Moreover, soybean as a supplementary added diet in salmon has confirmed previous observations that the fish gut microbial community are sensitive to dietary manipulation (Bakke-McKellep *et al.* 2007; Ringø *et al.* 2008). *Psychrobacter aquimaris*, *Psychrobacter maritimus*, *Psychrobacter submarinus* and *Psychrobacter okhotskensis* previously were either not reported or rarely found in the GI tract of fish, but research on GI tract microbial community associated with salmon fish feed with soybean products by Ringø (2008) and Bakke-McKellep et al., (2007), found these species become important. The study also indicated dominant bacterial species of *Psychrobacter cibarius*, different from the dominant bacteria (see below) found in salmon fed conventional diets. The dominant bacterial species as previous findings were *Aeromonas* spp. (Skrodenyte-Arbaciauskiene *et al.* 2008), *Mycoplasma* spp. (Holben *et al.* 2002), *Photobacterium* spp. (Hovda *et al.* 2007), *Pseudomonas* spp., *Shewanella* spp. (Navarrete *et al.* 2009) and *Vibrio* spp. (Hovda *et al.* 2007). This finding potentially proves the influence of diet manipulations to the microbial diversity in the fish gut as suggested by prior studies (Ringø and Olsen 1999; Ringø *et al.* 2006a; Ringø *et al.* 2006b). Despite the potential benefit of soybean meal, negative reaction to the soybean meal occurs in salmon unless the soybean has been suitably pre-treated (Romarheim *et al.* 2011).

1.3.2.3 Lupin kernel

Lupin kernel has become a routinely added ingredient into fish diet because it contains a high crude protein content and well-balanced amino acid composition (Glencross *et al.* 2011; Salini and Adams 2014). Lupin kernel meal is extracted from the genus *Lupinus*, simply known as lupin, native to North, Latin and South America. *Lupinus* species that have been used as sources of lupin kernel meals includes *Lupinus angustifolius* (Glencross *et al.* 2008), *Lupinus albus* (Salini and Adams 2014), *Lupinus luteus* (Glencross *et al.* 2011) and *Lupinus mutabilis* (Molina-Poveda *et al.* 2013). As lupin kernel meal contains high crude protein, it can potentially replace the protein content in some of the fishmeal without effecting fish health and productivity (Glencross *et al.* 2011; Salini and Adams 2014).

Previous researches found the growth performance of salmon was significantly affected by the lupin kernel meals due to lupin kernel ingredient improving the nutritional content such as organic matter, nitrogen and energy, making this ingredient highly useful in fish diets (Glencross *et al.* 2008; Glencross *et al.* 2011; Salini and Adams 2014). Lupin cultivars such as *L. luteus* can also be useful to improve lipid metabolism and hence fish performance (Salini and Adams 2014). The effect of lupin kernel on salmon gut bacterial community is limited and still not well studied (Knudsen *et al.* 2008), but because lupin kernel was found influence other GI tract bacteria of other marine species, it potentially could impact on the salmon GI tract microbial communities as well (Silva *et al.* 2011).

1.3.2.4 Microalgae

Microalgae, mostly cyanobacteria have been used as food for many years, and it is popular as an alternative protein source for humans and animals (Jensen *et al.* 2001; Spolaore *et al.* 2006; Zhao *et al.* 2006; Sharma *et al.* 2011). The commercial cultivation of

microalgae started in early 1960's in Japan and since then its production has expanded across the globe (Borowitzka 1999). As in humans, cyanobacteria such as *Spirulina* has been used as added ingredients in animal feed like fish and farmed fish (Zhao *et al.* 2006). *Spirulina* is a good source for proteins, vitamins and polysaccharide. It also alters the pigmentation of the flesh due to a high content of the carotenoids astaxanthin and canthaxanthin, which are usually supplemented in salmon diets (Řehulka 2000) since colouring of the flesh of salmonids is an import aesthetic quality of salmonid products (Certik and Shimizu 1999; Thajuddin and Subramanian 2005; Spolaore *et al.* 2006). Moreover, microalgae supplements, depending on the species used, may contain highly unsaturated essential fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Reitan *et al.* 1997).

Researchers previously found that fish dietary intake of microalgae can potentially increase fish growth rate even though no positive impact on feed conversion efficiency and mortality levels were observed (Zhao *et al.* 2006). In order to be used in the aquaculture industry, the microalgae strains must be both easily cultured, not active, and non-toxic to both humans and fish. More recently, trends show that cyanobacteria have been avoided as feed supplements in the aquaculture industry due to their high cost (Borowitzka 1997; Pulz and Gross 2004). The effect of microalgae supplements on salmon GI tract bacterial community structure has yet to be studied, because the practice of using this type of supplement is still rare in the salmon industry. Since microalgae was found to affect the GI tract of humans and other animals, which are fairly stable, it is also likely this supplement will influence the salmon GI tract microbial community if it was added to salmon diets (Skjermo and Vadstein 1993; Parada *et al.* 1998).

As an aside, some species of cyanobacteria are not suitable for human foods and could impact negatively on fish health due to production of toxins (Sellner 1997).

Cyanobacterial blooms due to either toxin production or their subsequent environmental impacts (e.g. localized hypoxia) also tend to be harmful to farmed fish (Sevrin-Reyssac and Pletikosic 1990).

1.3.2.5 Probiotics

Probiotics are beneficial bacteria that have the potential to control pathogens through a variety of mechanisms, and have been widely used in human and animal nutrition as well as in aquaculture (Gomez-Gil *et al.* 2000; Verschuere *et al.* 2000; Balcázar *et al.* 2006). In aquaculture, probiotics has been added into fish diets as an alternative to antibiotic treatment. Previous research suggests that the addition of probiotics may enhance and improve fish health as well as survival (Korsnes *et al.* 2006). Probiotic bacteria may act to improve fish health by: i) competitive exclusion of pathogenic bacteria by ingesting probiotics bacteria to reduce or eliminate opportunistic pathogens (Balcázar *et al.* 2006); ii) contribution of enzymatic and nutrients sources to fish GI tract digestive processes (Sakata 1990); and/or iii) enhancement of immune responses. Prior studies have reported that oral administration of *Clostridium butyricum* to rainbow trout enhanced vibriosis resistance of the fish (Sakai *et al.* 1995); and also had antiviral effects (Girones *et al.* 1989).

In several recent studies it has been suggested that the GI microbiota of fish play a role as a defensive barrier against enteric pathogens (Gómez and Balcázar 2008; Ringø *et al.* 2010). It can be considered therefore, that probiotic candidates could induce similar or enhanced benefits in salmon. Lactic acid bacteria (LAB) such as *Lactobacillus*, *Carnobacterium*, *Pediococcus* and *Lactococcus* are the most prevalently proposed probiotics in aquaculture for the control certain bacterial genus of *Vibrio*, *Pseudomonas* and *Aeromonas*

(Balcázar *et al.* 2006; Balcázar *et al.* 2008). Moreover, lactic acid bacteria appear to be major components of healthy adult specimens of various farmed fish species, including salmon (Hovda *et al.,* 2007) so it is suspected they have roles in salmon growth, presumably by nutrient conversion and possibly immunomodulation.

Probiotics in salmon industry however, are still considered new and not widely used as supplementary diet ingredients. However research seems to be positive in terms of understanding their efficacy. For example, a *Carnobacterium* strain isolated from Atlantic salmon was effective at controlling infections caused by *Vibrio ordalii*, *Yersinia ruckeri* and *Aeromonas salmonicida* in fry and fingerlings (Robertson *et al.* 2000). In addition, probiotics bacteria has been found potentially reduced mortality rates from *Aeromonas salmonicida* infections (Austin *et al.* 1995).

1.3.2.6 Prebiotics

Probiotic agents face tough challenges in commercial aquaculture because of the difficulty to maintain viability of the bacteria in feed during storage, especially after pelleting. Probiotic bacteria are leached from the feed particles in rearing water, during feed handling and also during feed preparation (Merrifield *et al.* 2010). Probiotic agents also must be able to compete in the GI tract. Therefore, prebiotics has been suggested to be implemented into commercial aquaculture to replace or complement the use of probiotics. Prebiotics mainly consist of oligosaccharides promoting beneficial bacterial growth within the GI tract (Gibson *et al.* 2003). Studies suggested potential prebiotic applications for salmonid such as mannanoligisaccharide, inulin, galactooligosaccharides and fructooligosachharides (Bakke-McKellep *et al.* 2007; Grisdale-Helland *et al.* 2008).

Inulin is a polydisperse carbohydrate consisting mainly of fructose, and it can be found in variety of vegetables and fruits such as asparagus, garlic, onions and bananas (Roberfroid 1993). This ingredient has potential application in aquaculture because it has prebiotic potential even though it is not a natural fiber in fish diets (Ringø *et al.* 2006a). Previous studies showed inulin was associated with changes in the fish GI microbial communities such as decreased bacterial population level (Bakke-McKellep *et al.* 2007), and induced the growth of certain potentially beneficial bacteria such as *Carnobacterium* spp., that could have prebiotics effect on the fish GI tract (Ringø *et al.* 2004; Ringø *et al.* 2006a) as mentioned previously.

1.3.3 Effect of antibiotics

Antibiotics are widely applied in the farmed, fish industry mainly to eliminate pathogenic bacteria and to treat bacterial infections in fish (Armstrong *et al.* 2005; Austin and Austin 2007; Burridge *et al.* 2010; Cabello *et al.* 2013). Landeira-Dabarca *et al.,* (2013) describe that bacterial load in fish was decreased in fish fed diet supplemented with antibiotics which proved the antibiotic benefits to control bacterial in fish. However, despite the positive results of antibiotic use, certain issues have been raised that are concerned about their application in the farming of fish. This includes the presence of residual antibiotics in the farmed fish that could affect the safety of the food (Burridge *et al.* 2010), and the possibility of drug-resistant strains of bacteria transferring resistance to pathogens (Ringø *et al.* 1995). The most popular antibiotics currently used in the farming of fish include oxytetracycline, oxolinic acid, flumequin, furazolidone, tylosin, florfenicol, quinolones,

tribrissen and amoxycilin (Rae 1992; Ringø *et al.* 1995; Burridge *et al.* 2010; Haque *et al.* 2014; Shah *et al.* 2014) with oxytetracycline, tylosin, florfenicol and quinolones mostly used in salmon aquaculture (Cabello *et al.* 2013).

The use of antibiotics also can eradicate beneficial bacteria and could promote the proliferation of antibiotic-resistant opportunistic pathogens due to minimization of competition (Ringø *et al.* 1995). An example of such is antibiotic treatment using oxytetracycline, which has a dual effect on reducing bacterial population densities and diversity. A study showed oxytetracycline eliminated most bacteria but caused *Aeromonas* species to nearly completely dominate the community (Navarrete *et al.* 2009). Thus, it raises a concern, because some species of *Aeromonas*, such as *A. salmonicida*, *A. caviae* and *A. hydrophila* are common pathogens of fish even though *Aeromonas* is a genus widely isolated from the gut (Huber *et al.* 2003; Romero and Navarrete 2006; Navarrete *et al.* 2008). Our knowledge about the impact of antibiotic treatment on the salmon intestinal tract bacterial community is still quite limited, but according to Navarrete *et al.,* (2008) antibiotics as well as other less understood factors can reduce salmonid GI tract bacterial population densities and diversity.

Another concern of antibiotic effects is its influence on the aquacultural environment because it leads to a major alteration of the biodiversity of the aquaculture environment by replacing susceptible bacterial communities with resistant ones (Cabello *et al.* 2013). This problem potentially increases the resistance of bacteria to certain important antibiotics typically used in mariculture such as oxytetracycline, florfenicol, amoxycilin and quinolones (Rae 1992; Ringø *et al.* 1995; Burridge *et al.* 2010; Haque *et al.* 2014; Shah *et al.* 2014), and thus potentially increases the risk of disease in mariculture. Antibiotic resistant bacterial increase may be even a greater risk and most studies on antibiotic resistance focus only on

culturable bacteria, which constitute only a small proportion of the total bacteria present in the aquatic environment (Bissett *et al.* 2006). Therefore, the effect of antibiotic usage in the aquaculture industry needs to be carefully considered and monitored.

1.4 Environmental factors affect salmon GI microbial communities

Environmental factors include temperature, pH, salinity, rainfall and dissolved oxygen concentration, all of which can influence and control the bacterial populations within the water mass, and cause physiological stress on fish (Horsley 1977). Some of these environmental factors also likely have a significant effect on the bacterial flora in GI tract.

1.4.1 Effect of temperature

Temperature has a fundamental effect on bacterial growth and all bacteria have specific minimum, optimum and maximum temperatures determined by inherent thermodynamic constraints (Corkrey *et al.* 2012). The increasing water temperature due to climate change may initially be beneficial in terms of increased growth rates, but there is a small thermal window beyond which temperature increase has a detrimental effect upon growth, disease susceptibility and reproduction (Pankhurst and King 2010). Atlantic salmon are temperature sensitive (Oppedal *et al.* 2011) and show a strong dependence on temperature for their reproduction cycle and reproductive development (Pankhurst and King 2010). Previous studies have defined the temperature between 6ºC and 22ºC as the temperature limits for salmon growth with an optimum temperature of 14ºC (Elliott and Hurley 1997).

Salmon actually has a higher tolerance to warm temperatures compared to many other temperate fish species (McCullough 1999), and this fact makes them suitable for growth in

Tasmania which has warmer temperature during summer, and often experiences a continual 4-month period at above 16ºC. However, Tasmanian mariculture industry is potentially impacted during extreme summers and long-term temperature increases could eventually constrain and then harm productivity and economic sustainability of salmon mariculture in this region. Moreover, certain fish diseases such as amoebic gill disease (AGD) become increasingly prevalent at higher temperatures (Adams and Nowak 2003; Steinum *et al.* 2008). Salmon growth may decline by 20% to 25% if water temperature increases from 16°C to 20°C (Oppedal *et al.* 2011), depending on the associated husbandry–based management of farmed populations. Moreover, temperature also potentially affect the fish digestive physiology as described by Amin *et al.,* (2014), because temperature has substantial effect on the nutrient utilization efficiency, increase energy intake and energy loss, reduce dissolve oxygen (DO) level which is important for fish growth. All this factors could accelerate fish stress that can lead to the ability of fish limits to consume feed. Currently, a few husbandry strategies such as lowering stocking density and optimized seasonal diets, water flow and oxygen availability have been applied to minimize the temperature effects.

1.4.2 Effect of season

As salmon GI microbial communities are sensitive to the changes of temperature, it has been assumed that GI microbiota was also affected by seasonal variation; Hovda *et al*., (2012) reported otherwise, but the study about seasonal variation on salmon was minimal. Hovda *et al.,* (2012) suggested that seawater seasonal variation did not show a direct correlation between water temperature and bacterial density in the case of farmed Atlantic salmon. This finding is in contrast with other researchers who reported that GI bacterial

communities in fish vary seasonally (Macmillan and Santucci 1990; Ahmed *et al.* 2004; Hagi *et al.* 2004; Lau *et al.* 2007). Studies on tilapia (Ahmed *et al.* 2004) and freshwater fish (Hagi *et al.* 2004) found that seasonal variations have fundamental effects on fish GI microbiota. The different conclusions probably are caused by factors such as geographic location and the surrounding environment, since the composition of bacterial communities has been suggested to be strongly determined by the external environment properties in which they are found (Fierer and Jackson 2006; Sullam *et al.* 2012).

1.4.3 Effect of geographic location and different ecosystems

The salmon GI microbial communities have been studied by relatively few researchers so far and interestingly findings are quite divergent with most of the focus on the most abundant bacteria within the salmon in the GI tract. These different observations (described in more detail below) could derive from different geographical locations. Studies have been performed in Scotland (Holben *et al.* 2002), Norway (Holben *et al.* 2002; Hovda *et al.* 2007), Lithuania (Skrodenyte-Arbaciauskiene *et al.* 2008), and Chile (Navarrete *et al.* 2009), as well as in different ecosystems (fresh, estuarine, marine or farm) and populations (juvenile, wild and adult). Difference in the surrounding environments is suggested to be the main factor influencing the differences in GI tract microbial since every location has differ in term of temperature range. For example Norwegian water temperature is generally quite cool year around and this could have a strong effect on seawater taxa that might be capable of colonizing the salmon intestinal tract (Hovda *et al.* 2007).

Moreover, the bacterial genera found within surrounding waters also slightly different between geographic locations, contributed by the environmental conditions such as salinity,

oxygen concentration, and presence of pollutants (Eiler *et al.* 2006). Studies have described the GI microbiota of Norwegian farmed salmon were predominantly by the bacterial genera *Acinetobacter* (Holben *et al.* 2002), *Vibrio*, *Photobacterium* and *Pseudomonas* (Hovda *et al.* 2007; Hovda *et al.* 2012), while the genus *Mycoplasma* was found to dominate in Scottish farmed salmon in one study (Holben *et al.* 2002). *Mycoplasma* was also found in wild Scottish salmon (Holben *et al.* 2002). Nevertheless bacterial genera dominant in Chilean salmon instead include *Shewanella* and *Microbacterium* (Navarrete *et al.* 2009), while in wild Lithuanian salmon, *Aeromonas* and *Carnobacterium* were the most frequently isolated (Skrodenyte-Arbaciauskiene *et al.* 2008). Other bacterial genera detected include *Citrobacter*, *Enterobacter, Clostridium*, *Fusobacterium*, *Lactococcus*, *Lactobacillus*, *Weisella*, *Photobacterium* and *Streptococcus* (Table 1.3) (Holben *et al.* 2002; Hovda *et al.* 2007; Skrodenyte-Arbaciauskiene *et al.* 2008; Navarrete *et al.* 2009).

Table 1.3: The most abundant bacterial genera in the salmon gastrointestinal tract collected from different geographic locations and ecosystem.

Genus-level identification is based on 16S rRNA sequences.

Genus	Relative abundance (%)	Geographic location	Eco-systems	References
Acinetobacter	~27-55	Norway	farmed	(Holben *et al.* 2002; Hovda *et al.* 2007)
Aeromonas	13.5	Lithuania	wild	(Skrodenyte-Arbaciauskiene *et al.* 2008)
Campylobacter	~5	Scotland	farmed	(Holben *et al.* 2002)
Carnobacterium	15.4	Lithuania	wild	(Skrodenyte-Arbaciauskiene *et al.* 2008)
Enterobacter	~4	Norway	farmed	(Holben *et al.* 2002)
Enterobacteriaceae	15.4	Lithuania	wild	(Skrodenyte-Arbaciauskiene *et al.* 2008)
Lactobacillus	~4	Norway	farmed	(Holben *et al.* 2002)
Lactococcus	11.5	Lithuania	wild	(Skrodenyte-Arbaciauskiene *et al.* 2008)
	~6	Norway	farmed	(Hovda *et al.* 2007)
Microbacterium	~25-58	Chile	farmed	(Navarrete *et al.* 2009)
Mycoplasma	25	Norway	farmed	(Holben *et al.* 2002)
	81	Scotland	farmed	(Holben *et al.* 2002)
	96	Norway	wild	(Holben *et al.* 2002)
Photobacterium	~46	Norway	farmed	(Hovda *et al.* 2007)
Plesiomonas	19.2	Lithuania	wild	(Skrodenyte-Arbaciauskiene *et al.* 2008)
Pseudomonas	~16	Norway	farmed	(Hovda *et al.* 2007)
	~20	Chile	farmed	(Navarrete *et al.* 2009)
Shewanella	~75-92	Chile	farmed	(Navarrete *et al.* 2009)
Vibrio	~50	Norway	farmed	(Hovda *et al.* 2007)

1.5 Objectives of the thesis research

This thesis has been instigated to investigate Tasmanian farmed Atlantic salmon GI tract microbiology. The work will test possible causal links of fish growth, feed conversion efficiency, water temperature, and general farm management to GI tract microbial populations. The project will study the effect of different diets and diet components on GI tract microbial populations within an experimental farm and gastric model systems. The project involves an extensive collaboration that seeks to document GI tract microbiota of Tasmanian farmed salmon and to encourage improved productivity and fish welfare through dietary intervention, and to provide strategic information in relation to possible increased water temperature effects and diet usage on salmon mariculture productivity.

The experiments focus on sampling hindgut faeces since bacterial populations' peak at this point and represent the outgrowth within the digesta. The use of non destructive hind-gut samples gives a useful baseline for future comparative studies that may be undertaken by the Tasmanian industry. The study does not extend to particular components of the GI tract – such as what bacteria attach to or may dwell in the GI tract mucosa – this is beyond the scope of the project logistically. DNA was analysed to determine community structure since the GI tract community should exhibit rapid growth due to constant consumption, digestion and excretion processes. Next generation sequencing (NGS) will be used to profile the community structure from 16S rRNA gene amplicons since it offers high resolution, depth of analysis and taxonomic identifications that can also provide functional inferences, unlike standard fingerprinting methods (DGGE, TRFLP). The actual NGS techniques utilised (454 pyrosequencing, Illumina MiSeq) was determined on the basis of availability in terms of out-sourcing since University of Tasmania lacks NGS facilities.

Pyrosequencing was available as a commercial service as of 2011 and thus was utilised for Chapter 2 and 3 followed subsequently with Illumina Mi-Seq analysis for Chapter 4 (done in 2014). Due to the need for high replication older methods such as clone library analysis of 16S rRNA amplicons were deemed inadequate and non-cost effective. Rapid community fingerprinting that instigated in this thesis, automated ribosomal intergenic spacer analysis (ARISA), was utilised due to its in-house availability and low cost while being equally superior to more cumbersome and expensive techniques (DGGE, TRFLPs). The results of this work will provide a baseline analysis of the range of microbial composition of the GI tract of aquaculture salmon in Tasmania and be used in future efforts to improve farm productivity and sustainability.

This study is a co-funded collaboration between the Australian Seafood Cooperative Research Centre, Tasmanian Institute of Agriculture (TIA) University of Tasmania (UTAS), the Tasmanian-based Atlantic salmon farming company Tassal Limited, Skretting Australia, and CSIRO Food Future Flagship and CSIRO Marine and Atmosphere Research. The PhD supervisory team includes TIA and CSIRO representatives. The project undertaken by me is also as part of an initiative by the Malaysian Government and the Universiti Sains Malaysia to increase the number of trained scientists in Malaysia. The industry partner in this work, Tassal Limited is the largest producer of Atlantic salmon in Australia. Tassal Limited listed on the ASX, and produces around 25,000 tonnes of Atlantic salmon per-annum. The second industry partner Skretting Australia, is a subsidiary company of Nutrecco, a leading supplier of fish feed in Australasia. Globally Skretting produces more than 1.7 million tonnes of high quality aquaculture feeds annually. This project's primary goal is to achieve greater understanding of Atlantic salmon GI tract microbial communities in relation to the

environment and diets, and uses next generation sequencing and high levels of replication coupled to multivariate statistical analysis to obtain a better indication of microbial community dynamics.

These are four aims of this project as listed below:

a) Identify key gastrointestinal tract microbial species and overall community structure dynamics of farmed salmon in Tasmanian waters.

b) Identify the effects of environmental conditions on Atlantic salmon GI tract communities.

c) Determine how Atlantic salmon GI tract microbial communities respond to diets that have different protein/lipid ratios and fish meal levels.

d) Develop an *in vitro* model to stimulate salmonid gastrointestinal tract conditions and to determine effects of different dietary components on microbial populations.

The thesis has been written in such a way to be a "thesis by publication" thus resulting in a certain degree of reiteration of introductory material in each chapter, which consititutes a distinct but inter-related series of studies. The attribution for this research is described on page 4 and 5.

Chapter 2:

Pyrosequencing-based characterisation of gastrointestinal bacteria of Atlantic salmon (*Salmo salar* L.) within a commercial mariculture system

2.1 Abstract

The relationship of Atlantic salmon gastrointestinal tract bacteria to environmental factors, in particular water temperature within a commercial mariculture system, was investigated. Salmon gastrointestinal tract bacterial communities of commercially farmed in south-eastern Tasmania was analysed, over a 13 month period across a standard commercial production farm cycle, using 454 16S rRNA-based pyrosequencing. Faecal bacterial communities were highly dynamic but largely similar between randomly selected fish. In post-smolt, the faecal bacteria population was dominated by Gram-positive fermentative bacteria, however by mid-summer members of the family *Vibrionaceae* predominated. As fish progressed towards harvest, a range of different bacterial genera became more prominent corresponding to a decline in *Vibrionaceae*. The sampled fish were fed two different commercial diet series with slightly different protein, lipid and digestible energy level, however the effect of these differences was minimal. The overall data demonstrated dynamic

hind-gut communities in salmon that were related to season and fish growth phases but were less influence by differences in commercial diets used routinely within the farm system studied. This study provides understanding of farmed salmon gastrointestinal bacterial communities and describes the relative impact of diet, environmental and farm factors.

2.2 Introduction

Microorganisms present in fish gastrointestinal (GI) tracts are known to contribute to fish health (Olafsen 2001; Ringø *et al.* 2003; van Kessel *et al.* 2011). The fish GI tract is inhabited by many different microorganisms, and it is an important route for invasion of pathogenic bacteria and subject to microbial colonization (Hovda *et al.* 2007; van Kessel *et al.* 2011). There is a need to better understand how dietary regimes and the surrounding environment influence the GI tracts of maricultured fish species. Understanding changes in bacterial communities within the GI tract and their role in health of the fish is important within the broader agenda of mariculture productivity and sustainability.

Temperature, salinity and oxygen concentration strongly affect the health and growth rates of fish (Cahill 1990). These factors may also contribute to the diversity and community structures of fish GI tract microbiota. Pankhurst and King (2010) showed that increasing water temperature due to climate change may initially be beneficial in terms of increased growth rates, but there is a small thermal window beyond which temperature increase has detrimental effect upon growth, disease susceptibility and reproduction. Atlantic salmon (*Salmo salar* L.) are temperature sensitive (Oppedal *et al.* 2011) and show a strong dependence on temperature for their reproduction cycle and reproductive development (Pankhurst and King 2010). Furthermore, certain fish diseases such as amoebic gill disease

(AGD) become increasingly prevalent at higher temperatures (Adams and Nowak 2003; Steinum *et al.* 2008). Salmon growth may decline by 20% to 25% if water temperature increases from 16°C to 20°C (Oppedal *et al.* 2011), depending on the associated husbandry – based management of farmed populations.

A range of variables such as water temperature, oxygen availability, diet, and stocking levels may affect fish health and performance. The majority of Atlantic salmon production takes place in marine net cages, where the fish's ability to adjust to fluctuations in the natural environment is limited within the confines of the production enclosure. Thus changes to the physical environment may induce a stress response that incurs a physiological energy cost to the fish (Oppedal *et al.* 2011). Clark and Nowak (1999) reported that surface water temperatures in south-eastern Tasmania, Australia reach 18°C to 20°C in summer. Thus, further long-term surface water temperature increases could impact on productivity and economic sustainability of salmon mariculture in this region. At this stage, temperature effects are ameliorated by husbandry strategies, including lowered stocking density, optimized seasonal diets and by optimizing water flow and oxygen availability through net hygiene and aeration or oxygenation.

Previous studies indicate that the dominant culturable bacteria from the salmon GI tract include *Vibrio* spp., *Aliivibrio* spp., *Photobacterium* spp., *Lactobacillus* spp., *Enterobacteriaceae*, *Lactococcus* spp., *Flavobacterium* spp., and *Pseudomonas* spp. (Cahill 1990; Ringø *et al.* 1995). *Mycoplasma* spp., *Carnobacterium* spp., *Citrobacter* spp., and *Clostridium* spp. have also been identified using molecular-based methods (Holben *et al.* 2002; Hovda *et al.* 2007). *Lactobacillus* spp., *Lactococcus* spp. and others lactic acid bacteria have been indicated to be a major component of the gut microbiota of healthy salmon, and are presumed to provide benefits through immunomodulatory effects and pathogen

antagonistic interactions with the intestinal epithelial cell layer as well as providing nutrients by contribution to digestion (Ringø and Gatesoupe 1998; Balcázar *et al.* 2006; Balcázar *et al.* 2008; Ringø *et al.* 2009).

Conventional cultivation methods likely bias knowledge of the salmon GI tract bacterial community and may not accurately reflect the complete microbial composition (Suau *et al.* 1999; van Kessel *et al.* 2011). Therefore more recent investigations have applied molecular approaches (Hovda *et al.* 2007; Navarrete *et al.* 2009). Next generation sequencing (NGS) is a powerful technique allowing the investigation of the complex microbial community composition in different environments (Hong *et al.* 2010; Vahjen *et al.* 2010; van Kessel *et al.* 2011). The estimation of microbial diversity in the salmon GI tract by high throughput molecular screening of the 16S rRNA genes in multiple samples could provide an effective means to assess the diversity of microbiota in the GI tract of salmon and its changes over time in relation to environmental conditions and farm management.

The purpose of this study was to determine how salmon GI tract bacteria vary during the commercial marine growth cycle and to identify possible relationships with the environmental conditions. Furthermore, the study aimed to identify whether two different commercial diet series (in term of protein contains and energy levels) influenced the composition of GI tract bacteria in salmon.

2.3 Materials and methods

2.3.1 Fish diets

Two different commercial diet series incorporated in this study were those routinely used at the salmon farm investigated. Each diet series comprised three distinct parts referred to as transfer diets, summer diets and grower diets, respectively, implemented for optimization of feed conversion and associated fish growth. The diet series are referred to in this study as "diet group A" and "diet group B". The general basic composition of each of the diet group is shown in Table 2.1 with the differences in protein and lipid content and energy level indicated. The major ingredients with the diets include different proportions of fishmeal, fish oil, wheat flour, and vitamin and mineral premixes. Specific details of the diet formulations are however proprietary knowledge.

Table 2.1: Summarised features of diets group A and B utilised in this study.

Diet Type	Transfer		Summer		Grower	
Period	Input to late October		Late October to mid-March		Mid-March to harvest	
Diet Group	A	B	A	B	A	B
Protein (%)	46-50	47-49	42-46	45	38-41	40
Lipid (%)	22-28	23-24	28-29	25	33-36	30
Digestible energy (Mj/kg)	19.8-21.3	20.4-20.5	21.4	20.1	22.5	20.6
Protein to digestible energy ratio	2.34	2.35	2.06	2.24	1.76	1.94

2.3.2 Sample collection

The survey was conducted at Tassal Group Ltd Robert's Point lease located within the D'Entrecasteaux Channel, Bruny Island, Tasmania (43.18°S 147.30°E). The two aforementioned diet series (diet group A and diet group B) were fed to different pens initially stocked with 72,000 to 75,000 all-female smolt between 15th May 2011 and 6th June 2011 with an average weight at input ranging from 86 to 217 g. Feed was delivered by a centralized feeding system, fish were fed to satiation in regular meals and feed input rates and feed stop points were judged by underwater camera according to normal commercial standard. Faecal samples were obtained over nine sampling occasions ranging from 4 weeks (during warmer months) to 8 week intervals (during the cooler period of the year) from July 2011 until August 2012, as part of standard farm health checks from two pens representing each of the diet groups. In order to minimize the potential impact of freshwater bathing upon gut biota, faecal samples were not collected within 14 days following AGD treatment (Table 2.2). Since individual fish tracking was not logistically feasible as well as potentially imparting handling stress on the fish, samples were collected by randomly seining a large group of fish, crowding the fish in the seine to minimize bias and subsequently dip-netting a small batch of fish into 17 ppm Aqui-S anaesthetic (Aqui-S, Lower Hutt, New Zealand). Fish were assessed for the presence of AGD (Taylor *et al.* 2009), individually weighed and hind-gut contents obtained from 6 fish from each diet group (12 fish in total for each sampling time) by gently squeezing faecal samples into sterile plastic vessels. Six fish per group were chosen to account for variation within a population, in line with previous studies (Holben *et al.* 2002; Hovda *et al.* 2007). Faecal samples were then transported to the laboratory on ice and processed within three hours. Other farm data obtained included water temperature and oxygen concentration (collected at a water depth of 5 m).

Table 2.2: AGD treatment and freshwater bathing schedule and time of sampling. The faecal sampling was done at least 14 days after the freshwater bathing.

Diet group	Sample Date	Last AGD bath date	Days bath to sample
A	27/07/2011	15/05/2011	73
B	27/07/2011	26/05/2011	62
A	21/09/2011	5/09/2011	16
B	21/09/2011	6/09/2011	15
A	8/11/2011	17/10/2011	22
B	8/11/2011	10/10/2011	29
A	13/12/2011	11/11/2011	32
B	13/12/2011	15/11/2011	28
A	17/01/2012	30/12/2011	18
B	17/01/2012	30/12/2011	18
A	14/02/2012	23/01/2012	22
B	14/02/2012	24/01/2012	21
A	29/03/2012	20/02/2012	38
B	29/03/2012	22/02/2012	36
A	23/05/2012	14/03/2012	70
B	23/05/2012	30/03/2012	54
A	8/08/2012	N.A	N.A
B	8/08/2012	N.A	N.A

2.3.3 Total faecal DNA extraction

Total bacterial DNA was extracted directly from the faecal samples using the QIAamp DNA Stool Mini Kit (QIAGEN Sciences, Germantown, MD, US) following the manufacturer's instructions. The direct DNA extraction was performed soon after sampling or on samples that were maintained frozen at temperature -80°C.

2.3.4 16S rRNA gene pyro-sequencing

To examine the microbial communities present in the faecal samples 16S rRNA gene tag pyrosequencing was applied to each of the 108 samples collected during the study. Tag-encoded FLX amplicon pyrosequencing of the region covered by application of the 341F and 907R primers (Lane *et al.* 1985; Muyzer *et al.* 1993) was carried out by Research and Testing Laboratories (Lubbock, Texas) using a Roche 454 FLX instruments with titanium reagents as previously detailed by Dowd *et al.,* (2008). Approximately 3000 raw reads were obtained per sample. Sequences were denoised and chimera-filtered through a bioinformatics pipeline (Lanzén *et al.* 2011). Briefly, all sequences were organized by read length and de-replicated using USearch (Edgar 2010). The seed sequence for each cluster was then sorted by abundance and then clustered again with a 1% divergence cut-off to create consensus sequences for each cluster. Clusters containing only one sequence or <250 bp in length were then removed. Seed sequences were again clustered at a 5% divergence level using USearch to confirm whether any additional clusters appeared. Once this process was completed any reads that failed to have a similar or exact match to seed sequences (typically poor quality reads) were removed. Chimeras were also removed from the clustered sequences created during denoising by using UCHIME in the *de novo* mode (Edgar *et al.* 2011).

Each seed sequence with its tag removed was then queried against a database of high quality sequences derived from the NCBI database using a distributed .NET algorithm that utilized BLASTN+ (KrakenBLAST, www.krakenblast.com). High scores matches were grouped in terms of taxonomic hierarchy based on per cent similarity values (Suchodolski *et al.* 2009). Sequences that yielded high score matches of <75% were discarded.

2.3.5 Statistical analysis

The relative abundance of taxa at the genus level in the two different fish diet groups was compared using unsupervised hierarchical clustering using Cluster 3.0 (de Hoon *et al.* 2004). Clustering was based on complete linkage comparisons utilizing uncentred correlations. The derived cluster matrix was then used to create heat maps using Java Tree View version 1.1.6r2 (Saldanha 2004). To assess the influence of different factors on community compositions, PRIMER6 and PERMANOVA+ (version 6.1.12 and version 1.0.2; Primer-E, Ivybridge, UK) respectively were used to conduct permutation multivariate analysis of variance (PERMANOVA) (Anderson *et al.* 2005), and canonical analysis of principal coordinates (CAP) (Anderson and Willis 2003). For this analysis sequence read data organized at the genus–level was normalized as percentages, square root transformed and a resemblance matrix created by calculation of Bray-Curtis coefficients. PERMANOVA was conducted using default settings with 9999 permutations, while CAP was conducted using default settings. The PERMANOVA derived significance values were considered significant when $P < 0.01$, while $0.01 < P < 0.05$ were considered only marginally significant.

2.4 Results

2.4.1 Fish size and environmental conditions

Surface (5 m) water temperature changed seasonally throughout the 13 month survey (Table 2.3). Temperature exceeded 16°C for 4 months (mid-December to early April) with a peak at 19.9°C on 29th January 2012; the lowest temperature recorded was 9.2°C on 14th and 27th July 2011. The average weight of fish sampled from the different cages representing diet groups A and B increased throughout the study period with no significant differences between samples from the two diet groups (Table 2.3).

Table 2.3: Salmon farm water temperature and oxygen level (depth 5 m), and weight of salmon sampled for faeces (n=6 random for each diet group) during the sampling date.

Sampling date	Fish weight (gram, average per pen)	Fish weight (gram, average per pen)	Temperature (ºC)	Oxygen level (5m, average [ppm])
	Diet group A	Diet group B		
27/07/2011	390	401	10.1	7.85
21/09/2011	761	784	11.7	8.22
08/11/2011	793	1062	14.4	8.37
13/12/2011	1074	1089	16.2	7.85
17/01/2012	1131	1357	17.6	7.54
14/02/2012	1545	1614	18.1	7.37
29/03/2012	1741	1831	17.1	7.45
23/05/2012	2882	2803	12.7	7.89
08/08/2012	4089	4020	10.3	8.57

2.4.2 Salmon faecal communities are dominated by members of the family Vibrionaceae in salmon from spring and onwards

Cultivation independent means were used to provide a high resolution picture of salmon faecal microbial diversity across a large proportion of the marine production cycle. In all, 297,000 high quality sequences reads were obtained with 28 bacterial genera predominating within samples. The pyrotag read data indicated that faecal samples were dominated by bacterial genera belonging mainly to the class Gammaproteobacteria and the phyla Firmicutes and Bacteroidetes. In faecal samples obtained from post-smolt salmon (weight <1 kg), collected between July and September, the majority of reads grouped in the aerotolerant fermentative genera *Lactococcus*, *Weisella*, *Leuconostoc*, *Cloacibacterium*, and *Carnobacterium*, and also the facultative anaerobe *Diaphorobacter* (55.9-74.2% of total reads). Post-smolt faecal sample community structure was very similar across this time period (Fig. 2.1).
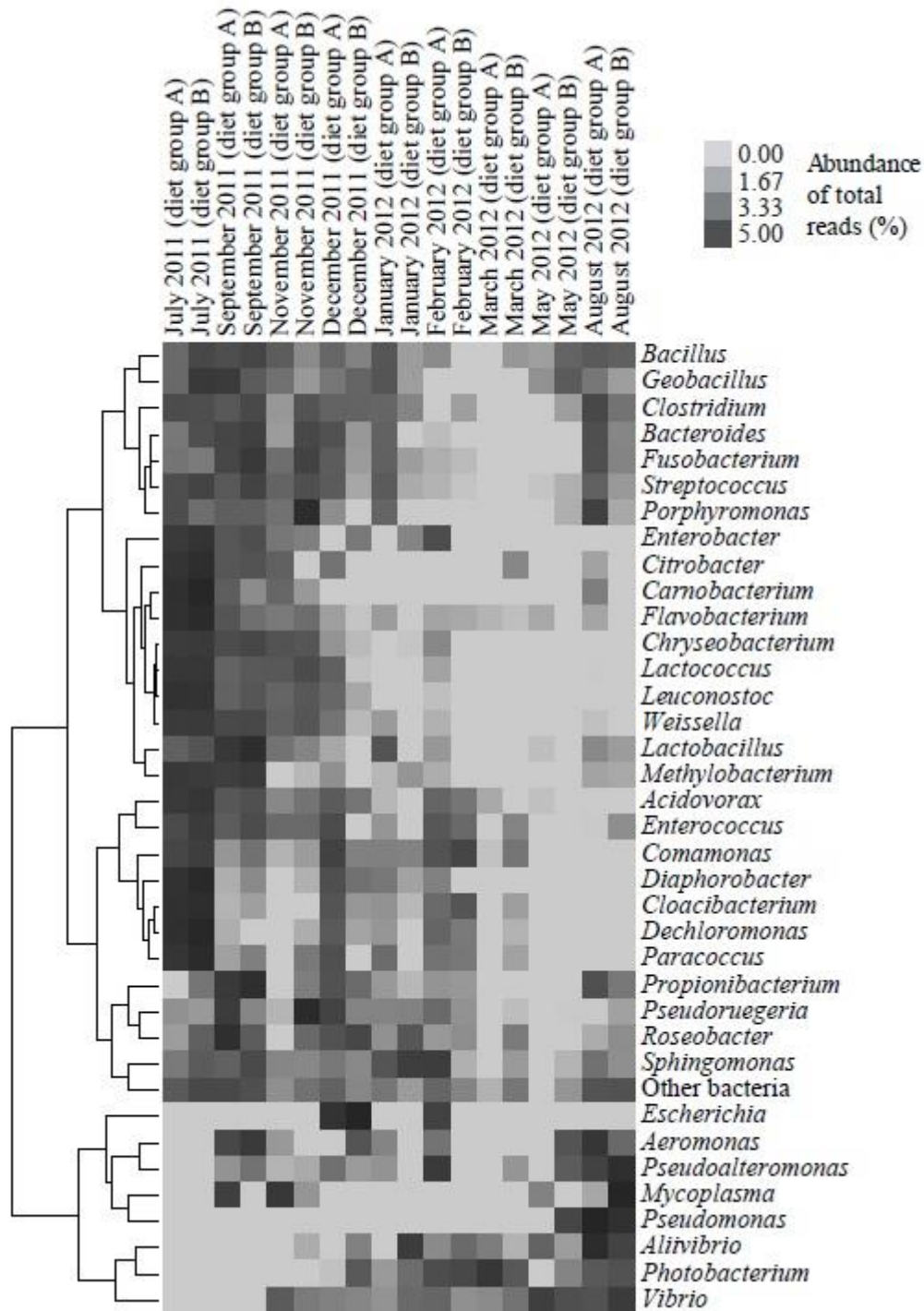
Figure 2.1: Heat map and hierarchical clustering plot of the 16S rRNA gene compositional

distribution of salmon faecal bacterial communities identified via pyrosequencing. Each

sample set includes 6 fish. Only taxa comprising >1% in at least one sample were included.

MDS plots of mean bacterial composition distribution according to month indicated that faecal community structure changed along a trajectory that was similar between the two commercial diet series used at the farm (Figure 2.2). However, as time progressed through summer and early autumn (January to March) these genera declined or became undetectable (Figure 2.1). Instead members of the family *Vibrionaceae,* including *Vibrio*, *Aliivibrio*, and *Photobacterium* became increasingly dominant making up the vast majority of sequence reads in most samples surveyed between December 2011 (late spring) and May 2012 (autumn) (Figure 2.1 and Figure 2.3). The main species of *Vibrionaceae* present include those that can be considered typical of fish GI tracts including *Vibrio ichthyoenteri, Vibrio fischeri, Aliivibrio wodanis*, and *Photobacterium phosphoreum*.
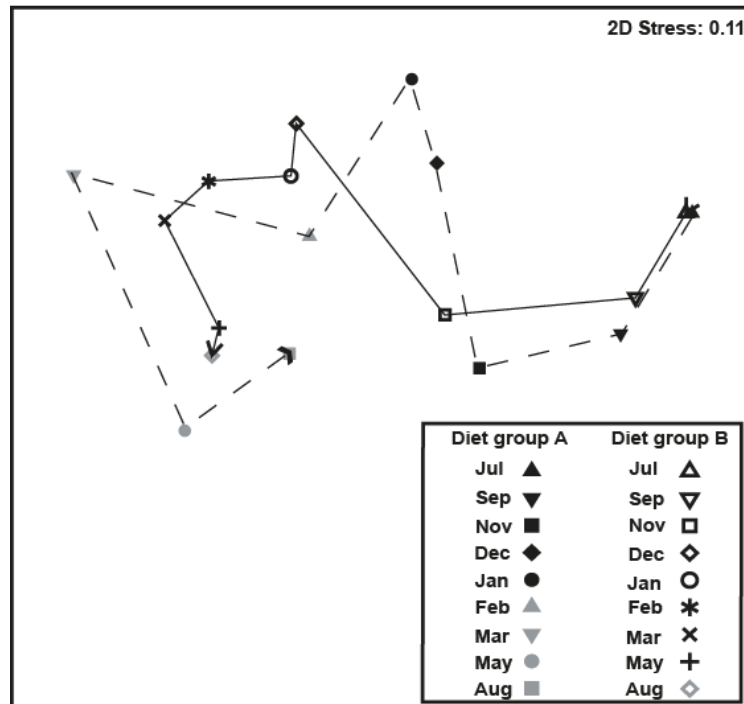
Figure 2.2: MDS plot showing average monthly faecal microbial community structure for each diet over the course of the sampling period with arrows indicating the order of monthly samples, illustrating the trajectory of community change.
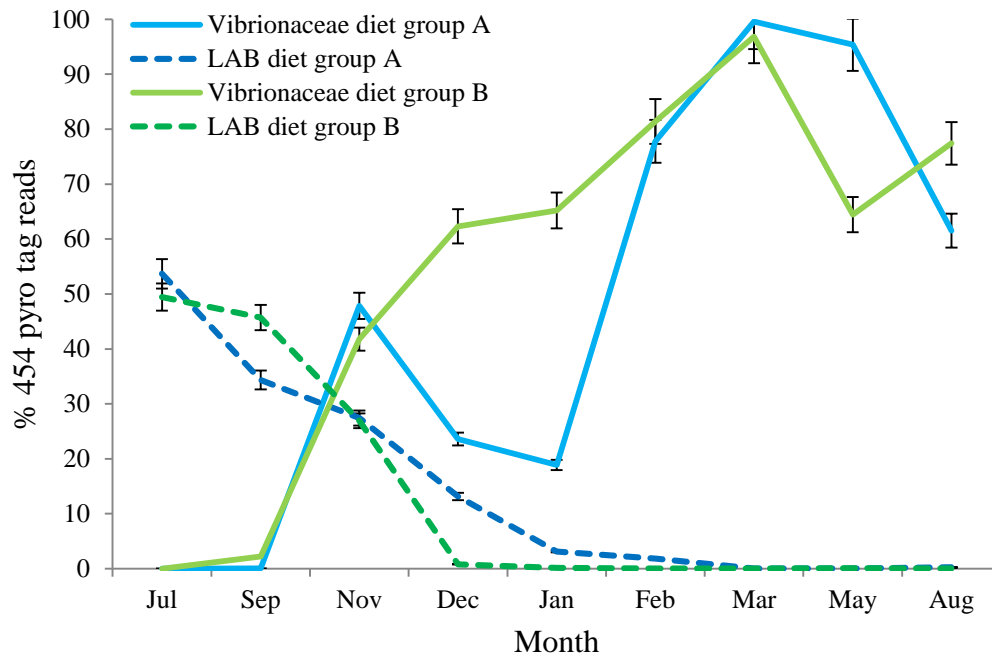
Figure 2.3: Proportional contribution of a) lactic acid bacterial (LAB, includes *Lactobacillus* spp., *Lactococcus* spp., *Leuconostoc* spp., and *Weisella* spp.) taxa and family *Vibrionaceae* taxa (includes *Vibrio* spp., *Photobacterium* spp., and *Aliivibrio* spp.). Data derived from bacterial 16S rRNA pyrosequencing analysis.

The dynamism in GI microbial community restructuring is underscored by high levels of plant-derived chloroplast 16S rRNA gene sequences appearing in faeces (up to 65% of reads, Figure 2.4), mainly for samples collected during the summer period. The chloroplast 16S rRNA sequences were mostly similar to those from leguminous plants, in particular *Lupini*. The enrichment of chloroplasts corresponds to increased lupin kernel meal (and the equivalent) addition to the "summer diet" (Table 2.1) balanced with fish meal to provide protein and other nutrients (Glencross 2008).
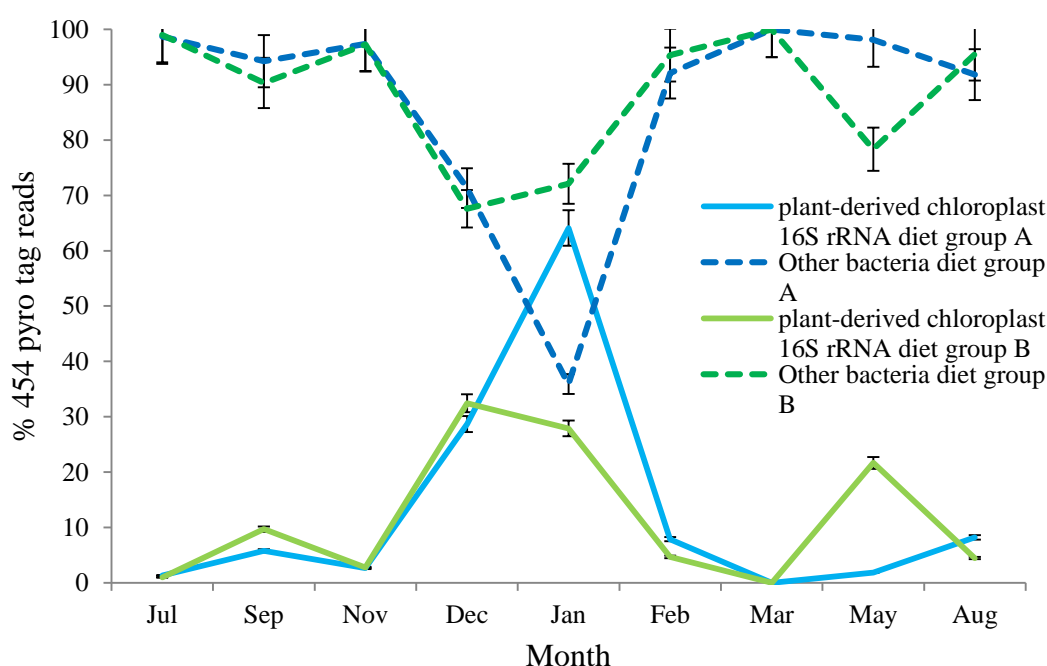
Figure 2.4: Proportional contribution of chloroplast 16S rRNA gene sequences (derived mainly from dietary lupin meal kernel) in all salmon faecal samples across the survey period for each diet group, demonstrating community shifts and overall salmon specimen variability.

2.4.3 Microbial community structure in farmed salmon is dynamic and community changes are acyclic

PERMANOVA analysis demonstrated that faecal populations changed as the seasons progressed. Faecal microbial communities were statistically different between the initial winter, summer and the second winter ($P<0.001$, Table 2.4a) with community structure dynamically changing over the course of the survey to be completely different to the starting point (Figure 2.2). There was no difference in bacterial community structure between commercial diet groups as suggested by PERMANOVA analysis ($P=0.2288$, Table 2.4a). However, PERMANOVA analysis of samples from each sample time point indicated commercial diet groups showed differences ($P=0.0098$, Table 2.4b) while the community structure differences were highly significant by comparison ($P<0.001$, Table 2.4b).

The distinctiveness of the faecal microbial communities as effected by diet groups were not evident for the July, November, February and March sampling times but were more separate for the September, December, January, May and August time points. These differences are illustrated in canonical analysis of principal coordinates (CAP) plots, which show clustering can be readily correlated on the basis of season and sampling time (Figure 2.5). Since communities change in a distinct trajectory over time, it is possible faecal communities are potentially predictable with lactic acid bacteria being abundant in post-smolt with subsequent adult fish populations being dominated by *Vibrionaceae*, while close to harvest the community shifts away from the *Vibrionaceae* predominance (Figure 2.5b).

Table 2.4: Comparison of microbial community structure with response to a) diet and season, b) diet and month using PERMANOVA.

a)

Source	df	MS	*F*	*P* (perm)
Diet	1	2092.2	1.3155	0.2288
Season	4	23440	14.738	0.0001
Diet (Season)	4	2751.2	1.7298	0.0305
Residual	88	1590.4		
Total	97			

b)

Source	df	MS	*F*	*P* (perm)
Diet	1	4034.2	3.435	0.0098
Month	8	16646	14.173	0.0001
Diet (Month)	8	2145.8	1.827	0.0036
Residual	80	1174.8		
Total	97			
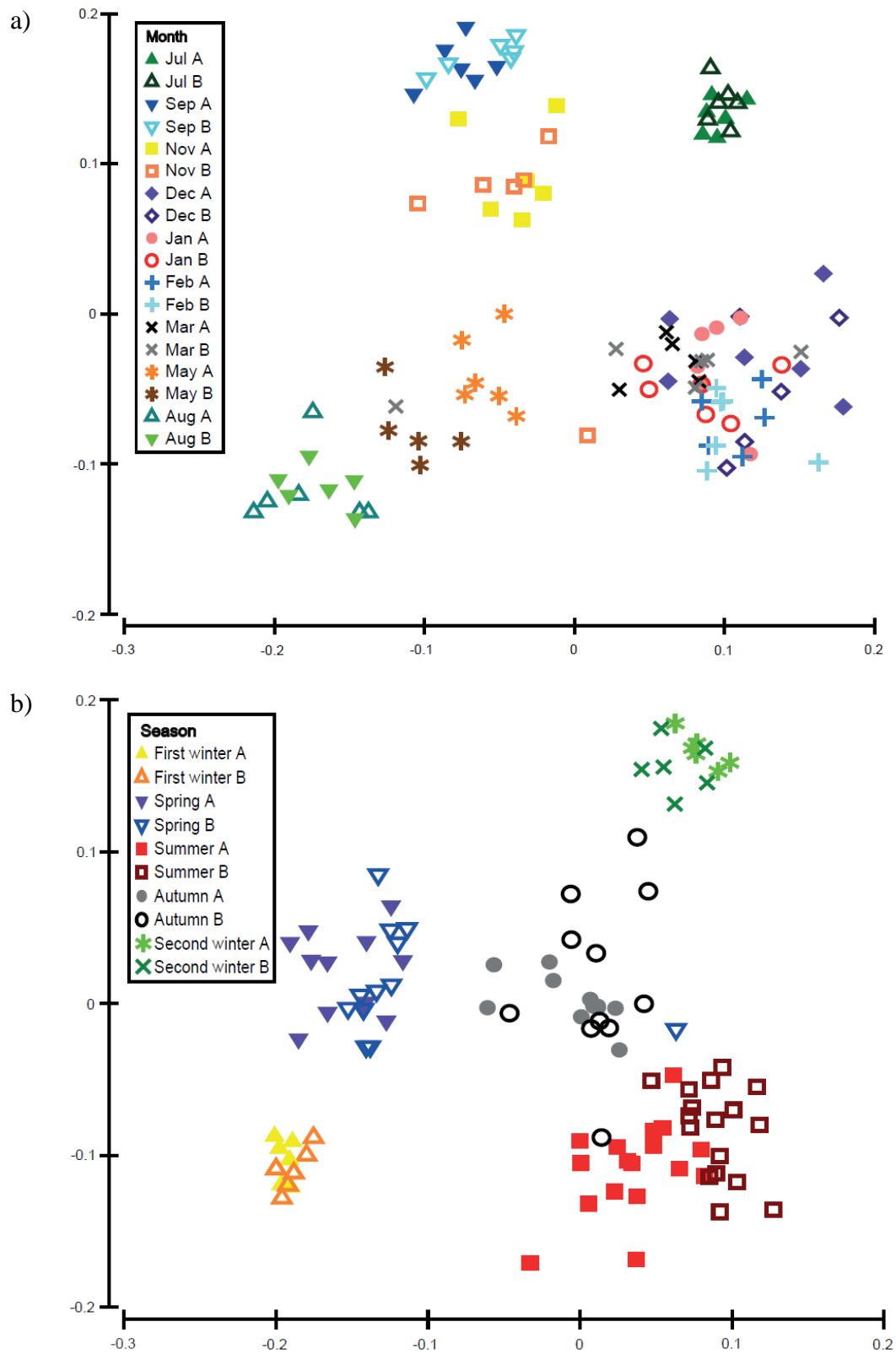
Figure 2.5: CAP plots showing faecal community similarity on the basis of (a) the time of sampling and (b) on the basis of season.

2.5 Discussion

This study describes hind-gut bacteria from sea cage-reared Atlantic salmon fed two commercial diets over a nearly complete farm production period. Although the summer was not considered extreme by Tasmanian standards, the fish experienced a continual four month period at above 16°C. Atlantic salmon (depending on genetic type) have higher tolerance to warm water temperatures compared to other salmon species (McCullough 1999). Studies have defined the temperature range for growth of Atlantic salmon as between 6°C and 22°C with an optimum temperature of 14°C (Elliott and Hurley 1997). The effect of high water temperature appears to be cumulative with thermal stress occurring following short periods of high temperature with insufficient recovery periods (Oppedal *et al.* 2011).

In this study the populations sampled were growing within normal commercial expectations for summer, thus the data here within do not involve salmon in poor health. It is likely that bacterial turnover in the gut is highest during summer. Most studies show that evacuation rate increases exponentially with temperature (Kawaguchi *et al.* 2007), though Handeland *et al*. (2008) found that the rate of change declines near the upper thermal tolerance of the species. This physiological response would likely have a profound effect on the nature of Atlantic salmon GI microbial communities during summer and the capacity to influence them via dietary manipulation.

The most abundant microbes in this study identified by pyrosequencing for both diet groups were *Vibrio* spp., *Aliivibrio* spp., and *Photobacterium* spp. in summer; *Lactococcus* spp., *Leuconostoc* spp., *Lactobacillus* spp. and *Weissella* spp. in post-smolt following marine transfer from hatcheries to stock salmon farms in the Australia winter (June). In addition, *Pseudomonas* and *Pseudoalteromonas* appeared to be the most predominant taxa that occur

in the wake of the *Vibrionaceae* domination during summer. The study of Hovda *et al.* (2007), which utilized DGGE analysis also showed adult salmon possessed bacterial populations of similar types observed here. Various bacteria such as *Aeromonas salmonicida, Piscirickettsia salmonis, Yersinia ruckeri* and *V. anguillarum* are serious salmonid pathogens.

Some related strains with low pathogenicity occur in Tasmania, however examination of the pyrosequencing data did not reveal any reads matching known pathogens. The farmed salmon examined in this study were only vaccinated against the non-Hagerman serotype of *Y. ruckeri*, which has relatively low virulence (Carson and Wilson 2002). *Mycoplasma* have been associated with wild adult salmon (Holben *et al.* 2002) and are suspected to be innocent saprophytes (Austin and Austin 2007). In this study, this taxon was only detected sporadically, but when present, it dominated the GI tract communities as was previously observed (up to 92% of reads in some specimens). The sporadic incidence of *Mycoplasma* in farmed salmon is interesting in that these taxa are quite fastidious and have a fermentative metabolism and presumably are ecologically restricted to the host fish. The sporadic nature may suggest host factors at play that may influence GI tract community structure and contribute to dynamic changes.

The bacterial colonization and growth within the salmon GI tract would be promoted due to the high nutrient content of salmon diet, which consists of a mix of proteins, lipids and the high energy level in the fish feed. Since *Vibrionaceae* are fast growing facultative anaerobic copiotrophs (Aiyar *et al.* 2002), we predicted this group is abundant within the salmon GI tract, because *Vibrionaceae* being typically marine and/or estuarine are abundant in the waters surrounding the salmon cages (Jones *et al.* 2007). This would be further enhanced by release of *Vibrionaceae*-rich faeces and thus intake of water by the fish during feeding may accelerate their predominance. In our study we initially considered that GI tract

microbial populations collected from faecal samples were allochthonous. However, it is possible *Vibrionaceae* and other bacteria are imbibed in large numbers and are capable of persistence, growth and colonization in the GI tract The prevailing view of bacterial colonization of fish GI tracts is that it reflects the bacterial composition of the surrounding water and the diet used (Ringø and Olsen 1999).

Temperature has a fundamental effect on bacterial growth, and all bacteria have specific minimum, optimum and maximum temperatures determined by inherent thermodynamic constraints (Corkrey *et al.* 2012). It is possible that rapid community changes are due to certain taxa having a competitive advantage over others in being fast growing. However as water temperature, diet and host physiology changes, competitiveness is lost by some taxa and gained by others resulting in both competition and colonization opportunities (Mouquet *et al.* 2005).

The rapid transit of feed through the salmon gut, together with seasonal temperature change and adjustment of commercial diet strategy undoubtedly has a dynamic impact upon colonization-competition processes at least through the winter to summer and summer to winter transitions, as supported by the large scale shifts in community structure observed at these times. It is probable that the rapid temperature change between spring and summer drives the change from lactic acid bacterial (*Lactococcu*s, *Weisella*, *Leuconostoc*, *Lactobacillus* and *Carnobacterium*) dominance to that of *Vibrionaceae* in summer, however the lactic acid bacterial populations do not regain predominance as temperatures decline into the second winter. Therefore it is likely that seasonal changes that affect gut microbiota are driven by the interplay between temperature, fish physiology (age, size, maturation) and dietary changes.

Our results considerably contrast with data from Hovda *et al.* (2012), who used fingerprinting analysis to show salmon GI tract bacterial communities, consisting of those presumed to be attached to the gut epithelial layer, were stable over seasonal periods. Besides methodological differences the different responses could be due to the regional differences since Tasmania water temperatures are generally 4°C warmer than that of southern Norwegian waters (for example around Stavanger, annual average range 4.5-16.8°C versus 9 to 20°C for the study site examined here). Such thermal differences could have strong effects on seawater taxa that might be present capable of colonizing the salmon GI tract. Farm management practices, diet regimes and general physiological stress levels also could potentially contribute to community structural differences and the community stability. Bacteria tightly adhered to the GI tract epithelial layer were not specifically analysed in this study and it is possible these populations are more stable than what is observed in the bulk faeces. Further work will be needed to assess these particular populations and determine their effect on salmon physiology.

External abiotic factors such as salinity, as well as the host physiology are influential on the structures of fish GI microbial communities (Sullam *et al.* 2012). The influence of host physiology could relate to the immune system and intestinal epithelial cell receptor interactions with bacteria. Diet however has been shown to impact, at least in a transient manner, fish GI tract microbiota. The age, health status, diet, season, development stage and eating patterns of farmed salmon likely also contribute to the variability in faecal microbial communities between individual fish. Previous studies have suggested that dietary composition is one of the most important factors when it comes to shaping the fish GI microbial community and switching diet regime seems to shift microbial diversity and/or

community structure (Ringø and Olsen 1999; Ringø *et al.* 2006b; Askarian *et al.* 2012; Sullam *et al.* 2012).

In our study, salmon GI bacteria were not significantly different between the two commercial diet groups. Differences in the levels of diet ingredients, such as protein to lipid ratios and digestible energy levels (Table 2.1) seem be too small to produce detectable impacts on the community structure. The dynamics in community structure variations could relate to the diet composition changes or alternatively to a salmon-associated physiology response or both, thus a much more focused study is needed to link community structural changes with specific dietary factors and salmon physiology.

2.6 Conclusions

In this study we describe the predominant lactic acid bacteria (in post-smolt over winter and spring) and *Vibrioneceae* (in larger fish from late spring onwards) within farmed Atlantic salmon hind-gut faecal communities. Results were largely corroborative with other studies though we show much greater detail in the community structures and also demonstrate the communities were strongly seasonally dynamic, which differs from other observations of GI tract communities (Hovda *et al.* 2012). In this respect the study also analysed sufficient samples to resolve community transitions over seasons despite variability between fish and diets and determined the effect of different commercial diet formulations had a relatively minor effect. The sequence data obtained could be used to compare salmon aquaculture management strategies as well as mariculture practiced in different regions that may have similar or different climactic conditions. A number of studies have recently used

pyrosequencing to investigate farmed fish GI tract microbiota (van Kessel *et al.* 2011; Wu *et al.* 2012) but have yet to compare them over a production cycle. Further studies of this nature could reveal important links between fish productivity, health, diet and husbandry strategies.

Since the study is naturally limited by the farm gate-related fate of the salmon, it is thus uncertain if the observed GI tract communities eventually would develop to form stable communities observed in wild animals and apparently wild fish species (Lozupone *et al.* 2012; Sullam *et al.* 2012), or is perpetually unstable representing an ecosystem that is partly exposed to the outside environment and thus readily affected by changes occurring there. This fact influences future directions in which maricultured fish species may have to be managed in the face of environmental changes as well as the economics associated with the cost of production in particular the value of fish feed components.

2.7 Acknowledgements

Chapter 3:

Atlantic salmon (*Salmo salar* **L.) gastrointestinal microbial community dynamics in relation to digesta properties and diet**

3.1 Abstract

Less productive Atlantic salmon growth performances have been linked to temperature stress, diet and indirectly linked to the incumbent GI tract microbial community. To obtain knowledge that may aid management of salmon production during abnormally warm summer periods as well as to better understand salmon GI tract microbial community dynamics a feeding trial was performed over a summer period. The diets were tested over a 5 month period in relation to a commercial standard diet that has intermediate protein levels (45:25 protein:lipid, 35% fishmeal, IntPro). Modified diets tested included a low (10% w/v) fish meal content diet (LFM diet); a diet with a high protein and energy content (50:20 ratio, 21.4 MJ/kg digestible energy, HiPro) and a low protein, low energy diet (40:30, 19.7 MJ/kg, LoPro). A six point categorical scoring system was developed to describe expressed digesta consistency, where a low score describes 'normal' faeces and a high score denotes casts (pseudofaeces), or empty hind gut. The "faecal score" was used as a proxy for gut function. Faster growing fish generally had lower faecal scores and this was due to accelerated growth of sexually immature subpopulations while the diet cohorts showed comparatively little difference in terms of faecal score though the overall lowest scores were observed after 5 months with the LoPro diet. The GI tract bacterial communities assessed with 16S rRNA amplicon pyrosequencing were dynamic over time with the LoPro diets most strongly shifting the community structure in relation to the commercial standard diet used. During the summer period the LoPro diet cohort and to a lesser extent all other cohorts with standard fish

meal content had transient increases in GI tract community diversity mainly represented by an increased abundance of anaerobic (*Bacteroidia* and *Clostridia*) and facultatively anaerobic (lactic acid bacteria) taxa. The digesta had enriched populations of these groups in relation to faecal casts. The majority of samples (60-86%) across all diet cohort faecal communities were eventually dominated by the marine-derived, bile-tolerant marine facultatively anaerobic genus *Aliivibrio*. The results suggest that time (incorporating seasonal changes in temperature) and diet is potentially related to faecal microbial community structure. Categorization of the digesta via the faecal score system revealed strict anaerobes were comparatively more abundant in firmer, normal faecal samples that are also rich in plant chloroplast material suggesting significant diet digestion had occurred therein. Anaerobes were comparatively much less populous in pseudofaeces, which is generally associated with poor feeding. These community shifts possibly through formation of different levels of metabolites and/or immune system stimulation could influence salmon physiology and farm-level performance outcomes.

3.2 Introduction

Aquaculture of Atlantic salmon (*Salmo salar* L.) in Tasmania is currently the highest volume and highest value fishery in Australia (ABARES, 2013) and is rapidly expanding. The South-eastern region of Tasmania (43°S 147°E) has been subject to intermittent unseasonably warm summers. This occurrence results in a still ill-defined problem that has been referred to as "Summer Gut Syndrome" (SGS) featuring salmon populations that have poor feed utilisation and poor or variable growth performance, especially when water temperatures exceed 17ºC (Green *et al.* 2013). The cause of SGS has been anecdotally linked to; i) water temperature, ii) diet and iii) husbandry and farm management. Though not

necessarily causally related, observations suggest changes in the gastrointestinal health of affected populations, suggesting the condition could be linked to GI tract homeostasis.

The rapid development of salmon aquaculture over the past three decades has required a parallel increase in global fish feed production (Tacon and Metian 2008) despite competition for pelagic sourced marine proteins and oils from terrestrial animal production, human consumption, industrial and pharmaceutical sectors. Salmon diets have changed markedly over this time, trending to higher energy and higher oil: protein ratios while incorporating an increasing range of alternative ingredients obtained from marine, animal and plant sources (Hardy 2010). The makeup of commercial diets is dictated by seasonal price and availability of alternative ingredients which are blended with the aim of meeting essential amino acid and fatty acid requirements. Although this approach continues to lower the dependence of farmed salmon on marine forage fish, there is potential to inadvertently impact fish health and performance as non-marine inclusion levels increase. Internal environmental factors, including the fish's nutritional state (Barton *et al.* 1988) may affect the magnitude of the stress response. Therefore, the susceptibility to limiting nutritional factors is likely to be expressed in response to seasonal environmental stress.

The Atlantic salmon gastrointestinal (GI) tract system like other fish is not as complex as mammalian systems and thus more influenced by external factors such as temperature (Sullam *et al.* 2012; Gomez *et al.* 2013). Understanding the connectivity between environment, diet, animal physiology and their GI tract microbiota has gained substantial attention in recent years due to the association of gut microbiomes with host health (De Cruz *et al.* 2012; Kostic *et al.* 2013; Hermes *et al.* 2014; Joyce and Gahan 2014). Though only limited knowledge is available on how GI tract microbes contribute to piscine physiology

(Ray *et al.* 2012; Clements *et al.* 2014) it can be assumed that there are interactions that can

be both beneficial and negative (Geraylou *et al.* 2013; Muñoz-Atienza *et al.* 2013).

The main interest in native GI tract microbial communities is that they likely maintain

GI tract homeostasis (Pérez *et al.* 2010; Cerezuela *et al.* 2013; Flint *et al.* 2014). Gut

homeostasis essentially refers to the role bacteria (amongst other influences) play in

stimulating the immune system and other physiological responses via end-products of

metabolism or through interactions with the epithelial layers. An appropriate level of

homeostasis would be assumed to be associated with optimal feed conversion efficiency.

Homeostatic changes to gut function could be influenced by probiotic bacteria, natural

bioactive products, and dietary components (Burrells *et al.* 2001; Burr and Gatlin 2005;

Balcázar *et al.* 2008; Ringø *et al.* 2009; Romarheim *et al.* 2011; Abid *et al.* 2013; Moldal *et al.* 2014). In this respect such an optimal state is required for maximal economic return in

mariculture operations since output is determined biomass production relative to the feed

input. To date the main focus of Atlantic salmon GI tract microbial communities and

physiological response research has been performed in relation to diet (Ringø and Olsen 1999;

Korsnes *et al.* 2006; Ringø *et al.* 2006a; Ringø *et al.* 2006b; Bakke-McKellep *et al.* 2007;

Kotzamanis *et al.* 2007; Askarian *et al.* 2012; Landeira-Dabarca *et al.* 2013).

Since feed costs are one of the important factors in the economic success of Atlantic

salmon mariculture, a focus has been placed on understanding how valuable feed resources

can be best used (Tacon and Metian 2008). Thus there is a need to better understand how

alternative protein and lipid substitutes and other feed additives potentially positive or

negative affect salmon health and productivity (Refstie *et al.* 2006; Bakke-McKellep *et al.*

2008; Krogdahl *et al.* 2010; Sahlmann *et al.* 2013; Moldal *et al.* 2014). Standard commercial

diets have been continually redesigned to maximize salmon growth in relation to

developmental and husbandry management regimes. Such manipulations to diets tend to impact on growth performance (Hartviksen *et al.* 2014) but there's a dearth of knowledge of whether there is a consistent effect on GI tract microbiota. An important question is whether GI tract microbiota can be linked to growth performance and whether manipulation of microbial communities can be achieved to optimise health and/or production.

This study explores the relationship between diet and GI tract community composition within the larger context of commercial mariculture diets. This study was performed between spring and autumn to cover the risk period of SGS and included the application of feeds of different protein:lipid ratios, digestible energy (DE) and fish meal levels to investigate the link between dietary energy, fishmeal replacement and GI tract microbial communities. As part of this study an assessment of the faecal digesta properties, using a ordinal 1 to 5 scoring system, was instigated to determine if it could be used as a measure of "gut health" since production of very liquid excreta and faecal casts (pseudofaeces), which consist of sloughed intestinal mucous cells and enterocytes, has been sometimes associated with GI tract immune system dysfunction (Turner Jr *et al.* 2003; Niklasson *et al.* 2014). This scoring system was assessed in tandem with community structure analysis to determine if GI tract microbial populations could be linked to a concept of "gut health" as well as to diet-linked changes.

3.3 Materials and methods

3.3.1 Caged fish trial design and feeding

Mixed-sex Atlantic salmon smolt (n=2359) that had previously been PIT (passive integrated tag, Sokymat, Switzerland) tagged in June 2010 were stocked within a single $10 \times 10m$ sea cage at Meads Creek, Dover, Tasmania on 4th August 2010 (mean weight 169 ± 48 g). The fish were fed with commercial feed (Skretting Australia) using an automated feeder (Aquasmart Pty. Ltd, Glenorchy, Australia) to ensure optimum growth rates. Three weeks after marine input the fish were treated under veterinary supervision with trimethoprim for seven days due to a low incidence of *Yersinia ruckeri* related mortalities in a neighbouring pen of fish.

At the commencement of the experimental trial in early November 2010 the fish population was split evenly (by random pre-allocation of PIT tag numbers) into four $5 \times 5m$ pens that were conjoined to a central walkway. Within two days the fish were provided 6 mm pellet trial diets representing a range of protein:energy ratios, one test diet per pen (Table 3.1). Four different diets has been tested, commercial standard (IntPro), low fish meal (LFM), high protein:DE ratio (HiPro) and low protein:DE ratio (LoPro). The low fish meal (LFM) diet included poultry meal with fish meal level reduced to 10% of the content. All feeds contained lupin kernel and faba bean meal. Feeds were supplied via a corner-mounted 50 kg spreader (AGK Kronawitter GmbH, Wallersdorf, Germany). Feeding reactions was routinely monitored to ensure fish were fully satiated.

Mortalities were removed every 5 days and the PIT tag number recorded. Throughout the trial, the populations were routinely assessed for sign of amoebic gill disease by fortnight

subsampling 40 fish and determining the range of gill score (Taylor *et al.* 2009). These checks triggered two freshwater baths at normal commercial bathing thresholds prior to the trial and a further three during the trial (Table 3.2). The trial was terminated in April 2011 by lethal anaesthesia (100 ppm Aqui-S). The maturation status of each surviving animal was assessed by inspection of the gonads.

Table 3.1: Diet specifications utilised in feeding trial*.

Ingredients:	commercial standard (**IntPro**)	low fish meal (**LFM**)	high protein:DE ratio (**HiPro**)	low protein:DE ratio (**LoPro**)
	(g/kg)			
Protein: lipid ratio	45:25	45:25	50:20	40:30
Fish meal (%)	35	10	35	35
Digestible energy (DE) (MJ)	20.4	20.4	19.7	21.4
Protein:DE ratio (g/MJ)	22.1	22.1	25.4	18.7

*More details are available in Table 4.1 (chapter 4).

Table 3.2: Sequence of freshwater bathing and faecal scoring events. Sampling order denotes the order in which cohorts were handled during each faecal measure event.

Date	Event	Comments	Cohort	Sampling order	Pop.	Faecal Score (%)					
						1	2	3	3.5	4	5
4 Aug 2010	Marine Input	10x10m pen, Spirit Feed			2359						
22 Sep 2010	AGD Bath 1	Bathed			2236						
21 Oct 10	AGD Bath 2	Split and bathed to two 5x5m pens			2215						
2-3 Nov 2010	Faecal Measure 1	Starved pre-sample; split to four 5x5m pens	IntPro	2	553	63.9	28.9	5.6	0	0.5	1.1
			LFM	1	553	74.6	18	5.6	0	0.5	1.3
			HiPro	3	550	51.1	34.7	8.9	0	1.8	3.5
			LoPro	4	557	48.9	33.8	11.9	0	1.3	4.2
1-2 Dec 2010	AGD Bath 3	Bathed									
14-15 Dec 2010	Faecal Measure 2	Starved from evening pre-sample	IntPro	3	526	13.1	15.2	12	8.2	20	31.6
			LFM	2	532	5.6	18.4	2.6	25.4	29.3	18.6
			HiPro	1	528	16.9	25.8	6.1	19.4	18.2	13.7
			LoPro	4	524	5.3	6.7	3.2	14.9	37.8	32.1
12-13Jan 2011	AGD Bath 4	Bathed									
25-27 Jan 2011	Faecal Measure 3	Fed until sample	IntPro	2	453	39.7	24.3	7.5	4.6	8.8	15
			LFM	3	470	22.6	24	21.7	3.2	15.1	13.4
			HiPro	4	465	23.7	27.1	12.9	7.7	13.8	14.8
			LoPro	1	485	24.7	29.2	12.3	12.8	14.2	6.8
23-25Feb 2011	AGD Bath 5	Bathed									
4-7 Mar 2011	Faecal Measure 4	Fed until sample	IntPro	3	424	17.1	21.6	28.6	7	16.4	9.2
			LFM	1	420	18.8	22.6	16.9	10	18.8	12.9
			HiPro	2	405	13.6	19.6	11.4	9.4	28.7	17.3
			LoPro	4	426	14.8	17.9	23.8	12.8	22.1	8.6
4-6 Apr 2011	Faecal Measure 5	Fed until sample	IntPro	3	384	20.2	23.9	17.8	9.4	26.2	2.4
			LFM	1	388	17.3	13.9	14.4	12.9	32.7	8.8
			HiPro	2	379	19.5	21.6	13.2	12.1	25.3	8.2
			LoPro	4	416	26	22.1	14.2	18.8	14.2	4.8

3.3.2 Faecal sample collection and assessment

Samples of hind gut faeces were obtained 5 times across a 5 month period (Table 3.2). Throughout the trial, faecal sampling was performed 10-14 days after each freshwater bathing process. This ensured AGD impact was minimised throughout the trial and reduced the direct effect of freshwater bathing on GI tract microbial communities. At each faecal sampling event fish were fed until crowding, then dip-netted in small batches into 17 ppm Aqui-S anaesthetic (Aqui-S, Lower Hutt, New Zealand). All fish individually weighed and hind-gut faecal content scored on a scale of 1 to 5 (solid faeces to no faeces, Table 3.3). Faeces were collected from 10 fish from each diet cohort by gently squeezing faecal samples into sterile plastic vessels. The process of taking faecal samples and ten fish per group were chosen to account for size variation within a population, in line with a previous study (Holben *et al.* 2002; Hovda *et al.* 2007). All faecal samples were frozen in liquid nitrogen, transported to the laboratory, and stored frozen at -80°C. Water temperature was obtained from data logger devices located at a depth of 5 m and compared against the long term average (LTA) temperature for the farm (LTA data since 1995).

3.3.3 Faecal score analysis

All faecal samples collected were qualitatively scored on the basis of appearance and consistency using the categorical system shown in Table 3.3. The distance and physiological significance between the individual score categories is undefined with the scores used purely as a relative comparison of performance. This follows the assumption a low score is considered favourable for performance and suggests normal GI tract function while a high score suggests poor feeding rates.

Table 3.3: Hind-gut faecal consistency and property categorisation

Score	Consistency	Description
1	Solid	Firm and holds its shape when expressed
2	Semi-solid	Runny to soft digesta that forms a low blob when expressed
3	Diarrhoea	Very runny fluid with feed digesta; typically squirts out
3.5	Mixed	Digesta containing casts
4	Casts	Yellow or non-white non-food matter; may be accompanied by blood when expressed
5	None	No feed in gut, either blood, clear fluid or nothing expressed

3.3.4 Total faecal DNA extraction and 16S rRNA gene sequencing

Total bacterial DNA was extracted directly from the faecal samples using the QIAamp DNA Stool Mini Kit (QIAGEN Sciences, Maryland, US) following the manufacturer's instructions. To examine the microbial communities present in the faecal samples, 16S rRNA gene tag pyrosequencing was applied to 217 samples collected during the study that yielded sufficient levels of faeces and subsequently DNA. Samples of each cohort and time included an even contribution of each faecal score (1 to 4), score 5 (empty gut) did not produce a sample. Tag-encoded FLX amplicon pyrosequencing of the region covered by application of the 341F and 907R primers (Soergel *et al.* 2012) was carried out by Research and Testing Laboratories (Lubbock, Texas) using a Roche 454. FLX instruments with Titanium reagents as previously detailed by Dowd *et al.,* (2008). Approximately 3000 raw reads were obtained per sample. Sequences were de-noised and chimera-filtered through a bioinformatics pipeline (Lanzén *et al.* 2011).

Briefly, all sequences were organised by read length and de-replicated using USearch (Edgar 2010). The seed sequence for each cluster was then sorted by abundance and then clustered again with a 1% divergence cut-off to create consensus sequences for each cluster. Clusters containing only one sequence or <250 bp in length were then removed. Seed sequences were again clustered at a 5% divergence level using USearch to confirm whether any additional clusters appeared. Once this process was completed any reads that failed to have a similar or exact match to seed sequences (typically poor quality reads) were removed. Chimeras were also removed from the clustered sequences created during denoising by using UCHIME in the *de novo* mode (Edgar *et al.* 2011). Sequences that yielded high score matches of <75% were discarded. Singleton sequences were not assessed.

3.3.5 Statistical analysis of faecal score

The primary variables accessed were body weight, Relative Growth Index (RGI) and Condition Factor (CF) in relation to factors "type" and "cohort". Gender (males, female, uncertain) and maturation level (mature, immature) were considered together as "type" (mature male, immature female etc.) with fish that died before the end of the trial removed from the data. The effect of pen and feed treatment (IntPro, LFM, HiPro and LoPro) was assessed together as "cohort" since due to the experimental design the independent effect of the pens could not be independently separated. Over the 5 time points stepwise regression analysis using Genstat v14.1 (VSN International) utilising 2-way interactions between variables and factors was used to rank the effect of the variables. Differences between variables in relation to the different factors were tested using one-way ANOVA with cohort, maturation and cohort × maturation representing fixed terms.

3.3.6 Diversity and multivariate analysis of GI tract microbial diversity

To assess GI tract microbial community compositions, PRIMER6 and PERMANOVA+ (version 6.1.12 and version 1.0.2; Primer-E, Ivybridge, UK), respectively were used to conduct permutation multivariate analysis of variance (PERMANOVA) (Anderson *et al.* 2005), and canonical analysis of principal coordinates (CAP) (Anderson and Willis 2003). For this analysis sequence read data organised at the genus-level was normalised as percentages, square root transformed and a resemblance matrix created by calculation of Bray-Curtis coefficients. PERMANOVA was conducted using an unrestricted permutation of the data (n=9999), fixed terms summed to zero, and utilizing partial sum of squares since the data is effectively an unbalanced data layout due to a high proportion of GI tract taxa frequently not observed owing to detection limitations, and the inherent nature of bacterial growth and eco-physiology. CAP was conducted using default settings. The PERMANOVA derived significance values were considered significant when $P < 0.01$, while $0.01 < P < 0.05$ were considered marginally significant. PRIMER-6 was used to calculate alpha diversity indices utilising Fisher's α-diversity and Pielou's evenness (J'). Overall species richness was calculated with Chao2 (Chao 1987). Beta diversity and multivariate dispersion (IMD) (Anderson 2006) was assessed using PERMDISP in Primer 6 and with the updated PERMDISP2 version (Anderson 2006) in order to determine whether trial sampling time, diets or faecal scores are important factors in determining community overlap and scatter.

3.4 Results

3.4.1 Fish environmental conditions

Temperatures experienced by experimental salmon populations were comparatively mild with a peak of 16.8°C and 16°C being exceeded from early January to early March 2011 (Fig 3.1). This was on average 1°C lower than the long term average (Spillman and Hobday 2014). Oxygen saturation was on average 91% (±6%) with only 3.2% of readings below <80%. AGD was effectively controlled by 5 freshwater baths (Table 3.2). The experimental series thus essentially assessed dietary responses in conditions where temperature stress and AGD pressures are not likely to be substantial, at least within the context of standard commercial operations.
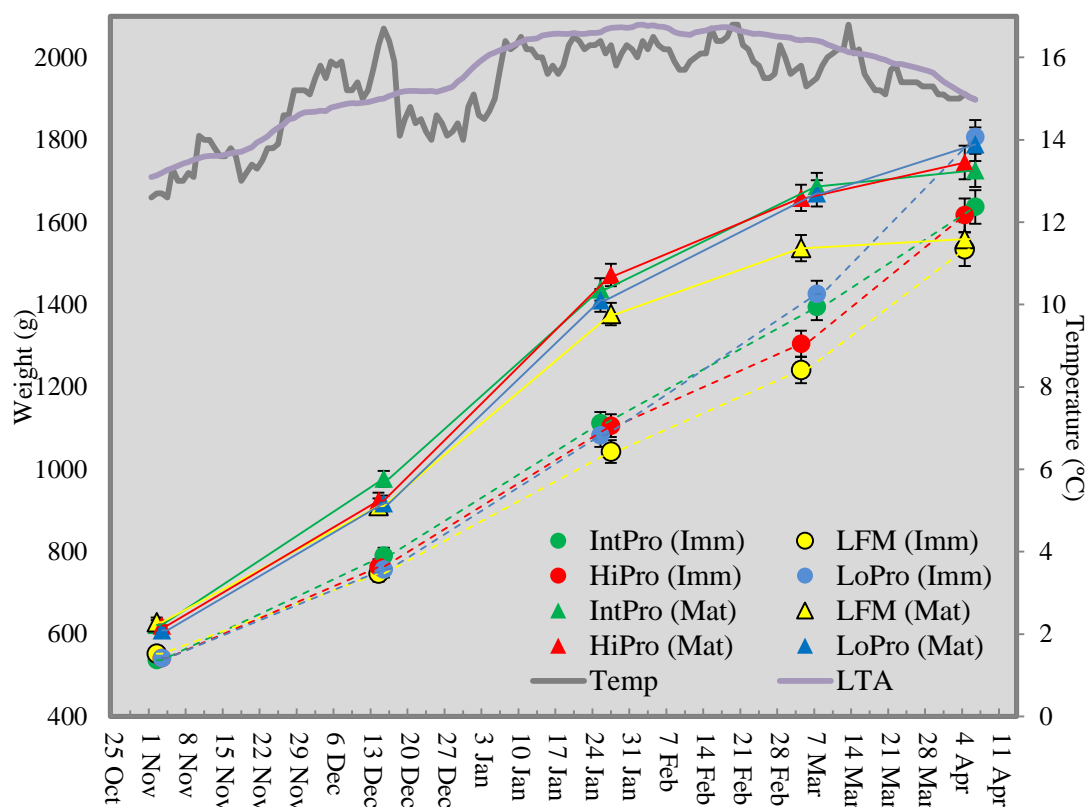
Figure 3.1: Salmon growth and water temperature during the feeding trial. Average weight at each measure (Measure1 in November 2010 to Measure5 in April 2011), showing immature (circles) and maturing (triangles) sub-populations within each 'Cohort'; IntPro (Green), LFM (Yellow), HiPro (Red), and LoPro (blue). Error bars show average least significant difference (0.5 LSD above and below each point). LSD bars that are not overlapping can be considered as depicting significantly different averages (P <0.001). Temperatures are daily 5 metre readings. Long term average (LTA) temperatures for Meads Creek are shown for comparison.

3.4.2 Faecal score trends

At the first faecal measure (December 2010) few fish produced casts or showed signs of empty hind-gut (scores 3.5, 4 and 5; 3.5% total of fish assessed, Table 3.2). As the trial progressed faecal scores rose substantially (Fig 3.2). Interpretation of this data is necessarily constrained due to the single cage per diet and the limitation of faecal scoring large numbers of fish. During a sampling day (two pens of fish) there was a tendency for faecal score to increase as feed progressed through the gut over time (data not shown). It became necessary to include at least a day of undisturbed feeding between sampling days because the presence of people on the cage walkway reduced feed intake on the two un-sampled pens (data not shown).

The primary variables of consequence affecting faecal scores included cohort, RGI, type, and to a lesser extent weight. The importance of these variables varied over time but in general the effect of fish type (gender and maturation status) and RGI strengthened while cohort declined as the trial progressed. This can be explained by immature fish having lower faecal scores by the end of the trial indicative of a greater level of overall feeding and weight gain compared to maturing progressing from mid-summer to early autumn. This trend can be attributed to the process of sexual development which is typified by faster growth in spring and early summer and lower feeding rate as gonads and secondary sexual characteristics develop (Hunt *et al.* 1982). Immature fish rapidly caught up in terms of body weight. Thus faecal scores must be interpreted in line with the maturation status of specimens.

The faecal scores associated with fish fed the LoPro ratio diet were significantly lower (on average 0.5) than those from the LFM (*p*=0.015) and HiPro ratio diets (p=0.019) at the final measure (April 2011). Overall the IntPro diet was slightly lower (0.34) compared to the LoPro diet (p=0.034).
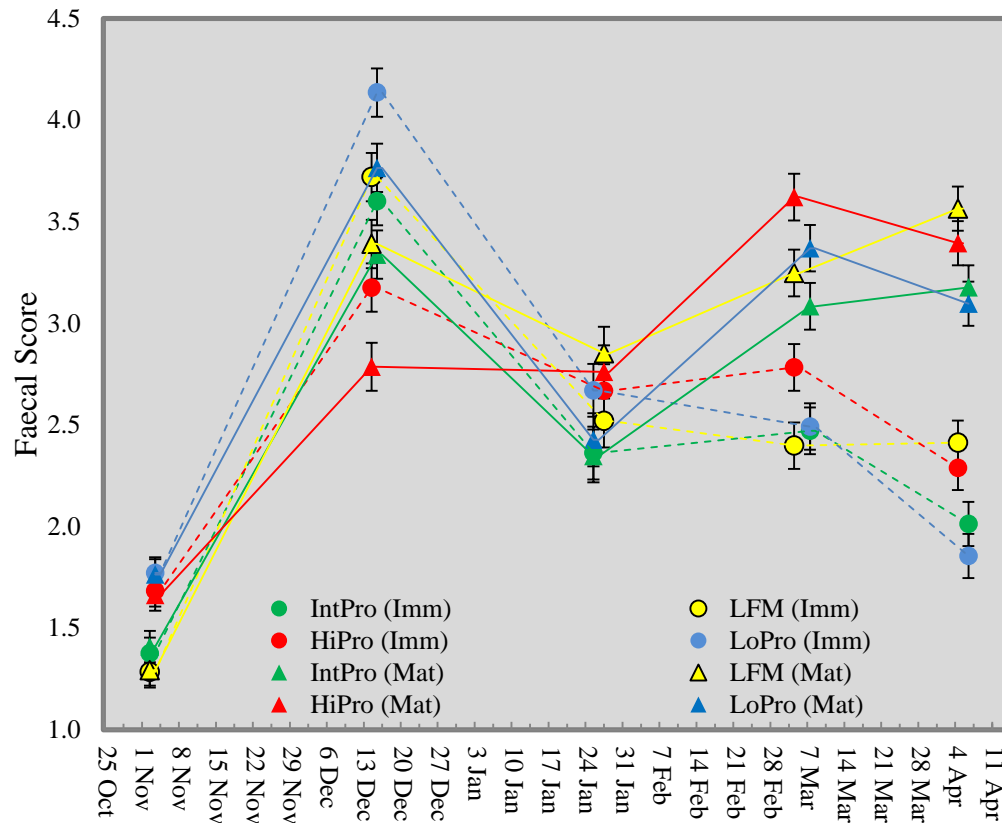
Figure 3.2: Faecal score progression during the feeding trial. Showing immature (circles) and maturing (triangles) sub-populations within each 'Cohort'; IntPro (Green), LFM (Yellow), HiPro (Red), and LoPro (blue). Error bars show average least significant difference (0.5 LSD above and below each point). LSD bars that are not overlapping can be considered as depicting significantly different averages ($P < 0.001$).

3.4.3 Diet cohort faecal microbial community dynamics and relation to faecal score

Faecal samples for community analysis were taken from sexually immature fish. A global view of faecal microbial populations and the effect of time, diet, and correlation to faecal score was analysed by PERMANOVA (Table 3.4) and illustrated by CAP (Fig 3.3). All of these factors have significant overall effects on GI tracts communities (Fig. 3.3a, 3.3b, 3.3c). Main (Table 3.4) and pair-wise (P <0.006) tests indicated the communities were ceaselessly dynamic with all times significantly different from each other and this was reflected in the CAP analysis (Fig. 3.3a) where 67-84% of samples were correctly categorised to a given sample time (m=50).

There was a significant shift in the community on the basis of diet (Table 3.4, Fig. 3.3b). The largest shifts from the trial start point were observed for the LFM, HiPro and LoPro ratio diets (Fig. 3.3b). From pair-wise analysis the HiPro ratio diet was not significantly different from either the LFM or LoPro diet (P=0.45) while the LFM diet showed greater separation from the LoPro ratio diet (P=0.008). Community structure separated between low scores (1 to 3) and samples containing casts (score 3.5, 4) (Fig. 3.3c) in the CAP analysis where 77-80% of samples were correctly classified (m=9) to the two main groups.

The overall temporal dynamism in community structure is consistent to that observed previously by Zarkasi *et al.,* (2014) in which samples were obtained through a farm production cycle. However, altering diet energy levels and the fish meal levels shifted the microbial community to a much greater degree than the communities achieved with diet regimes containing commercial standard levels of fish meal and DE as tested in the Zarkasi *et al.,* (2014) paper. From the statistical analysis faecal score-associated differences in the GI tract communities are largely independent of diet (P=0.406) while interactions are only

weakly associated with sampling time (P=0.045) suggesting the responses are associated with communities being consistently different between the digesta in comparison to excreted cast-rich material. This result was consistent with the findings related to faecal scores of immature fish, which had different feeding and growth rates throughout the trial whereby cohort groups did not otherwise have large differences in faecal scores.

Table 3.4: Multivariate Statistical comparison between Atlantic salmon GI tract microbial communities on the basis of sampling time, diet and categorised faecal properties.

Factor	df	MS	*F*	*P* (Perma-nova)	Unique permu-tations	P (Monte Carlo)
Time	3	14432	6.0811	0.0001	9872	0.0001
Diet	3	13051	5.4991	0.0001	9891	0.0001
Faecal Score	4	8464.2	3.5665	0.0001	9866	0.0001
Time × Diet	9	3910.6	1.6478	0.0008	9799	0.0010
Time × Faecal Score	12	2968.1	1.2506	0.0459	9783	0.0485
Diet × Faecal Score	12	2422.6	1.0208	0.4058	9805	0.4107
Time × Diet × Faecal Score	36	2661.6	1.1215	0.0798	9666	0.0924
Residuals	133	2373.3				
Total	216					
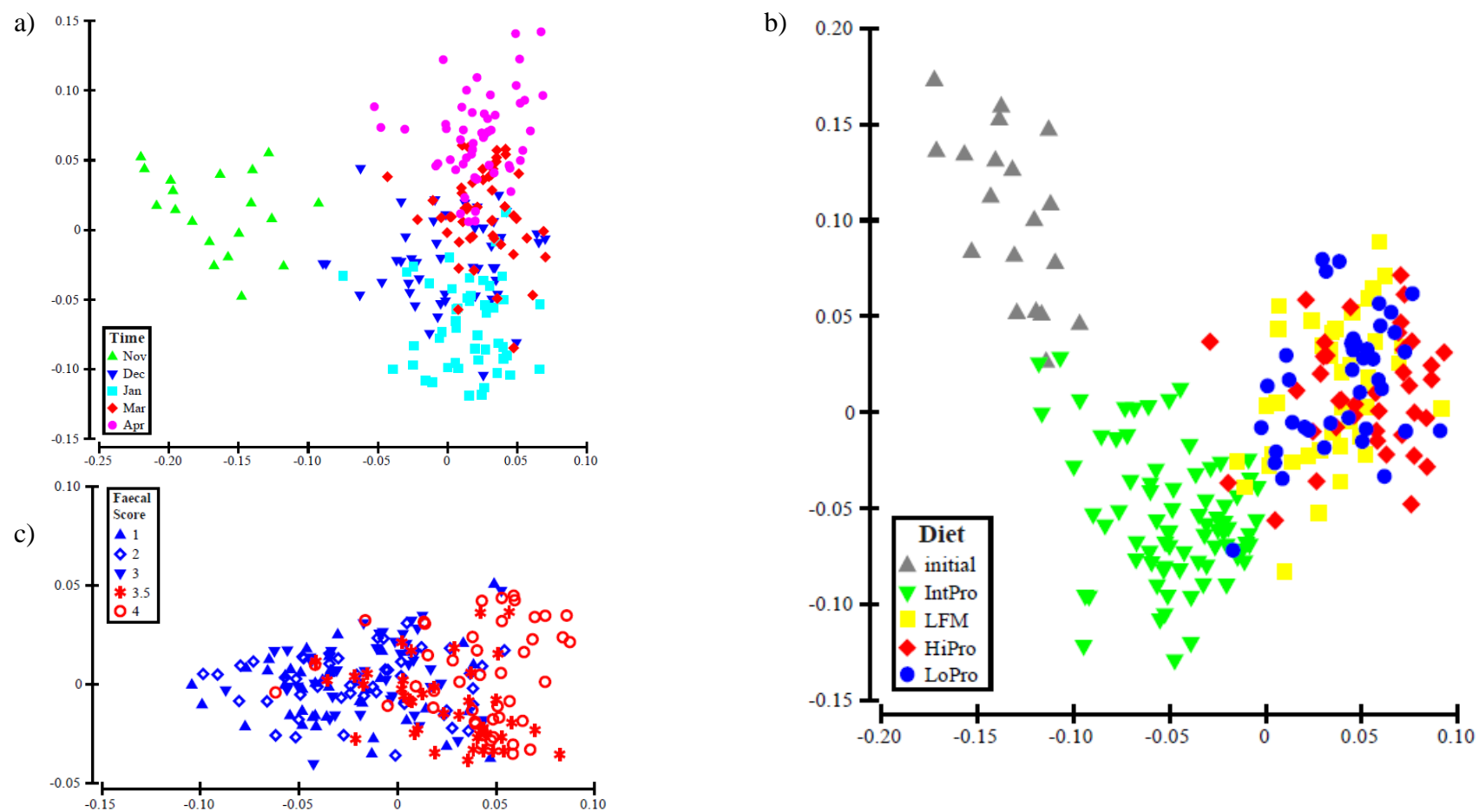
Figure 3.3: CAP plots comparing factors affecting GI tract community structure: a) time of sampling; b) diet cohorts (Table 3.1) with the first sampling time in November 2010 designated "initial" and representing prior starved specimens that only had recently fed; c) faecal scores (Table 3.3).

3.4.4 Diet influenced diversity changes in GI tract communities

From the 217 samples about 4100 filtered sequence reads were obtained on average per sample with 23.1% of all reads being *Salmo salar* 18S rRNA sequences, 11.4% chloroplasts or algae and the remainder bacteria. Chloroplast and algal sequences were excluded in the aforementioned statistical analysis though inclusion had virtually no effect on the outcomes (data not shown). Bacterial 16S rRNA reads were classified into 1507 OTUs mostly grouped in the phyla *Bacteroidetes, Firmicutes* and *Proteobacteria*. Chao-2 species richness across a conglomeration of all 217 samples was 3167 (±171) suggesting diversity on par with GI tracts of mammals though individual specimen diversity was much lower and highly variable (4 to 229 $OTU_{0.98}$ per individual, average 48). A relatively high proportion of reads could be ascribed to "species groups" since the 16S rRNA sequence region (V3 to V6) obtained was limited in its ability to demarcate species within certain clades.

Diversity was assessed to examine time, diet and faecal score influenced patterns. Fisher's α-diversity was affected over time with a transiently pronounced increased diversity observed for the LoPro cohort and similar weaker responses for the IntPro and HiPro cohorts (Fig 3.5). The LoPro ratio diet had the highest overall diversity and the IntPro cohort the lowest (68 vs 33 OTUs on average). The LFM diet showed a slow trend of increasing diversity while for the isoenergetic IntPro cohort it declined. A sharp reduction in evenness occurs after the first month of the trial though this was temporarily reversed during the summer period.
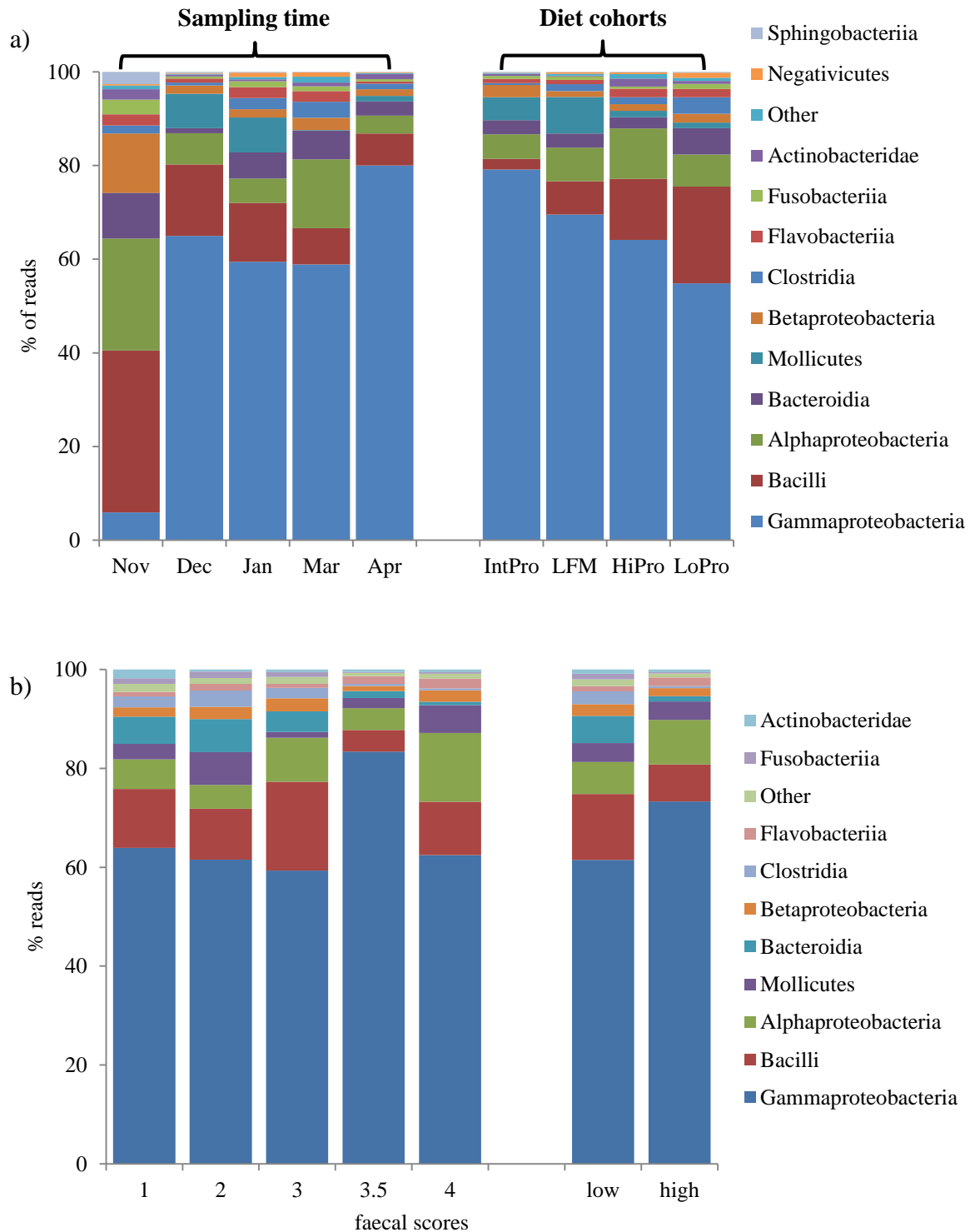
Figure 3.4: Bacterial diversity at the class level as defined by a) sampling time and diet cohort; and b) faecal score factors. Faecal scores that are low (1 to 3) and high (3.5 and 4) are shown for comparison since communities were statistically distinguishable.

PERMDISP/PERMDIP2 provided an assessment of multivariate spread in the datasets and via pairwise analysis in Primer 6 an indication of overlap by measuring relative distances to a group centroid value (the point that minimises the sum of squared distances to the points within a given group) (Ratkowsky 2008). The sampling time showed a trend of increased dispersion in summer (F=5.87, df=4, 212, P=0.001) (Fig. 3.6), which correlates with the increased of diversity from the December to March sampling periods. The trend was weaker for cohort (F=4.18, P=0.02) and negligible for faecal score. The LoPro cohort stood out in being distinctly different (Fig. 3.6) likely related to the higher species diversity and to other cohorts (Fig. 3.6, P<0.02).
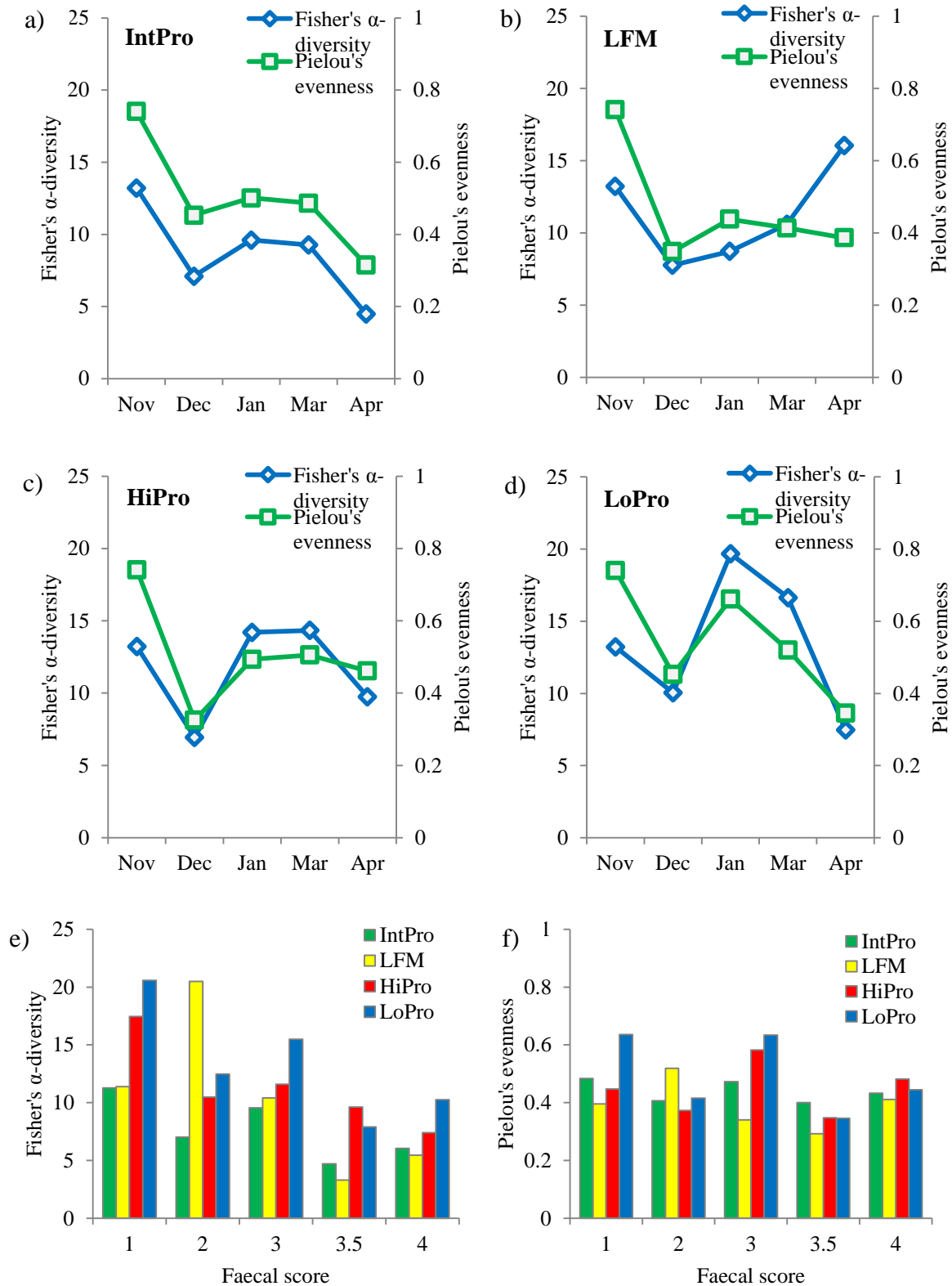
Figure 3.5: Diversity (based on Fisher's α-diversity) and evenness (Pielou's J) index trends for diet cohorts over time, a) IntPro; b) LFM; c) HiPro; d) LoPro. The same information is provided for faecal score categories for each of the diet cohorts, e) Fisher's α-diversity; and f) Pielou's evenness.
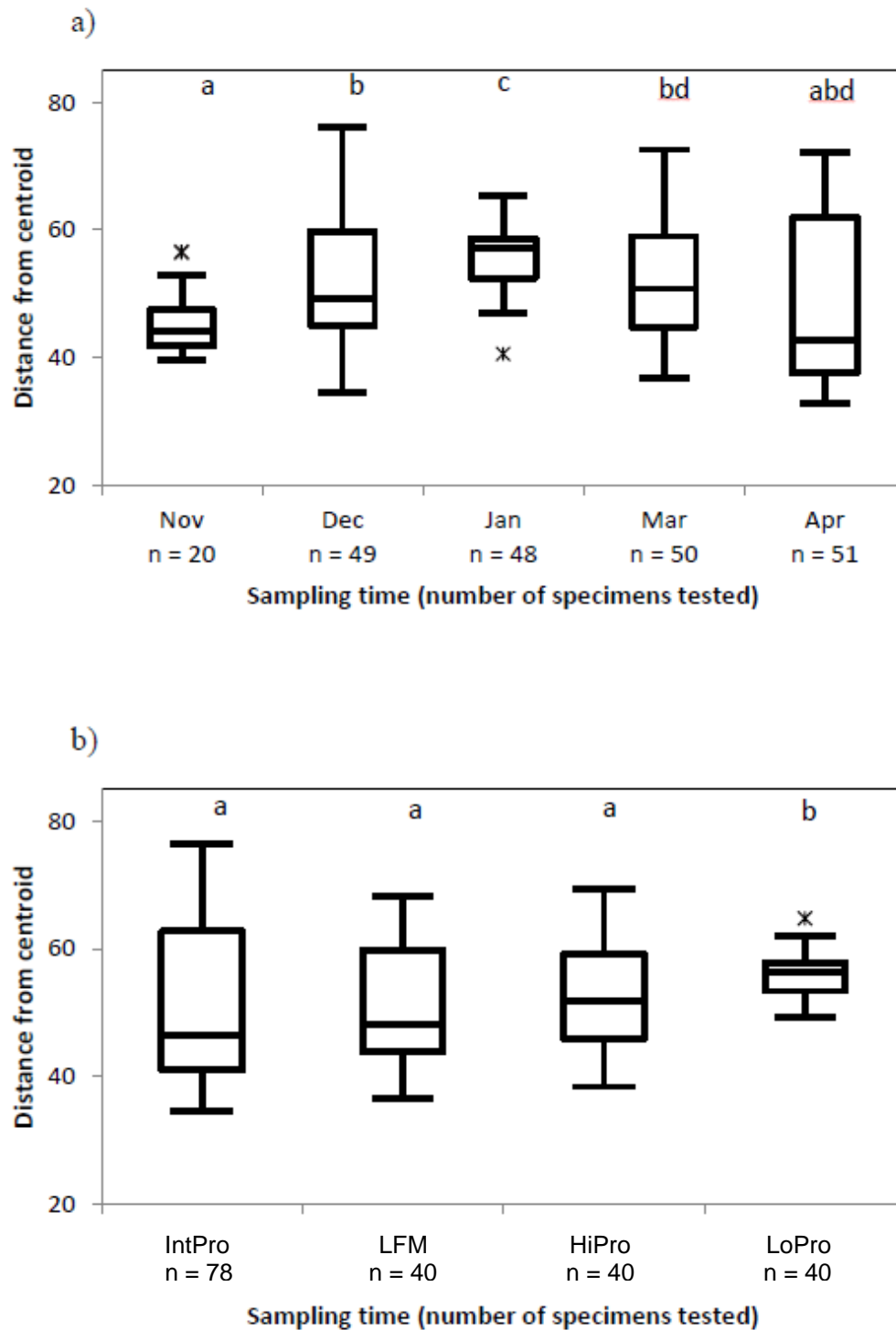
Figure 3.6: Boxplots showing distances of a) time, and b) treatment subgroups from the centroid inferred from PERMDISP2. Asterisks indicate outlier values. The line in the box indicated the mean centroid distance value for the subgroup. The letters above the box indicate significance ($P<0.02$) determined by permutation in PERMDISP2 (n= 999) with the designation of the same letter denoting non significance.

3.4.5 Ecogroup dissection to assess GI tract β-diversity

Given the salmon gut is an open system and thus readily influenced by environmental factors an approach was used to dissect community dynamics and β-diversity by examining the GI microbiota in relation to oxygen requirement and to a predicted ecosystem source, the latter defined by the typical habitats the identified species are most often associated. This is based on the types of taxa observed which ranged from anaerobic to aerobic and had either marine or non-marine origins.

This breakdown resulted in six major "ecogroups": i) GI tract anaerobes (abbreviated as GAN) typically associated with GI tracts, primarily class *Bacteroidia*, class *Clostridia* and class *Fusobacteria*); ii) GI tract facultative anaerobes (GFA), mainly non-spore-forming lactic acid bacteria (class *Bacilli*) and class *Mollicutes*; iii) facultatively anaerobic marine species (MFA) dominated by members of the family *Vibrionaceae* of class *Gammaproteobacteria*; iv) marine aerobes (MA) mainly belonging to major seawater clades including the *Roseobacter* clade (class *Alphaproteobacteria*) and class *Flavobacteriia*; v) facultatively terrestrial anaerobic (TFA), and vi) aerobic bacteria (TA) normally associated with plants, fresh water, and soil.

Respectively, the terrestrial originated groups consisted primarily of genus *Bacillus* and its spore-forming relatives (class *Bacilli*) and members of the proteobacterial families *Comamonadaceae*, *Sphingomondaceae*, and *Methylobacteriaceae*. Minor levels of anaerobes bacteria from marine and terrestrial ecosystems were also observed though abundances were 1 to 3-orders of magnitude lower than the other groups.

3.4.6 Diet and seawater derived bacteria transiting the Atlantic salmon hindgut

The proportions of marine aerobes and the terrestrial facultative anaerobes both declined from initially high levels in all four cohorts (Fig. 3.7). Based on the taxa present the marine species are derived from seawater imbibed during feeding and the predominance at the beginning of the trial owes to low bacterial numbers present initially. Thus the marine bacteria passing into the hind gut seem to become less predominant as other bacteria grow and colonise digesta in the GI tract. Spore-forming bacilli which made up most of the terrestrial facultative anaerobes included substantial presence of the thermophilic genera *Aeribacillus*, *Anoxybacillu*s and *Geobacillus*.

These bacteria are diet derived, present as "contaminants" since minimum and optimum growth temperatures of these taxa (minimum temperature for growth 30-35°C, optimum rate at 55-65°C) are well above that of the *in situ* temperature (≤17°C). Analysis of feeds using an initial heat exposure of 80°C for 10 minutes followed by plating onto Brain-Heart infusion agar and incubation at 25, 37 and 55°C revealed 10^3 to 10^5 CFU of putative spore forming bacteria per gram of feed. The kilning treatment used in feed manufacture likely selects for these bacteria which are able to survive on stored feeds.

3.4.7 Salmon GI tract anaerobes are mostly confined to the digesta

The proportional level of salmon 18S rRNA reads was doubled in high faecal score samples consistent with casts including sloughed intestinal cellular material. Chloroplast sequence levels were markedly more abundant (7-10-fold) in low faecal score samples and thus associated with the digesta (Fig. 3.8). The vast majority of chloroplast 16S rRNA sequences were derived from the diets (11.1%) and included sequences identified as lupin (*Lupinus*), faba bean (*Vicia*), wheat flour (*Sativa*/*Triticum*) and pea (*Pisum*). *Nannochloropsis*

was the source of most of the algae-derived chloroplasts. Based on distributions between high and low faecal scores anaerobic taxa typically associated with GI tracts seem to be predominant in the digesta while the proportion of reads is 6-fold lower in samples containing casts (Fig 3.7). This phenomenon did not apply to the marine (and non-marine) associated facultative anaerobic taxa consistent with the concept that the bulk of the digesta represents an anoxic domain allowing strictly anaerobic bacteria to grow. There was a high diversity observed amongst the anaerobic taxa, contributing about one-third of observed OTUs, and populations were very dynamic (Fig. 3.8). Thus changes in anaerobe populations seem influential on the diversity within the faecal samples since the shape of the anaerobic diversity curves (Fig. 3.7a) matches overall diversity curves (Fig. 3.6).

In all diets, most obviously for the LoPro diet, anaerobes peaked in the summer months and then declined in cooler months of March-April. This change contributed strongly to the increase and then subsequent decrease in α-diversity observed for this cohort and correlated to sampling time dispersion as indicated above. Community structure appraisal suggests that initially members of genus *Bacteroides* predominate amongst the anaerobes (>50% of GI tract anaerobe reads) but are progressively replaced by members of genus *Porphyromonas* towards the end of the trial. *Clostridium*, *Veillonella* and *Fusobacterium* spp. were also quite common. *Bacteroides* spp. typically are saccharolytic while porphyromonads are proteolytic mainly fermenting amino acids (Krieg 2011) thus the progressive enrichment of *Porphyromonas* may occur due to the high protein content of the diets.
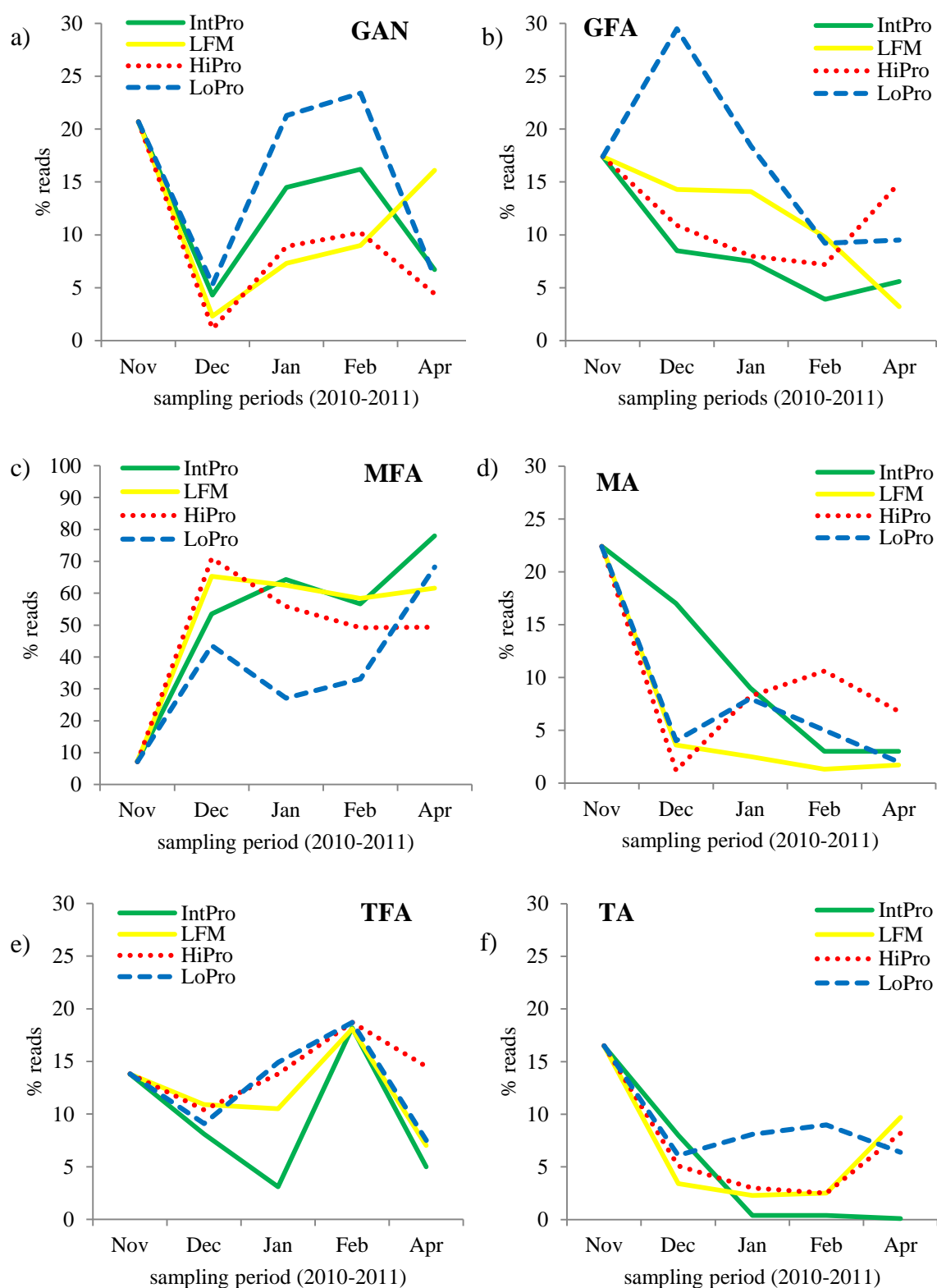
Figure 3.7: Changes in the relative abundance of GI tract microbial components within each diet cohort categorised as "ecogroups". The taxonomic groups a) GI tract anaerobes (GAN); b) GI tract facultative anaerobes (GFA); c) facultatively anaerobic marine species (MFA); d) marine anaerobes (MA); e) facultatively anaerobic (TFA); f) aerobic bacteria (TA).

3.4.8 The LoPro diet transiently promotes growth of facultative anaerobes including lactic acid bacteria.

The community component consisting of GI tract facultative anaerobes consisted (in order of predominance) of the Gram-positive genera *Brochothrix*, *Streptococcus*, *Carnobacterium*, *Lactobacillus* and *Lactococcus.* All of these genera are classic aerotolerant strictly fermentative anaerobes that are grouped collectively as the "lactic acid bacteria", forming lactic acid as a major end-product of metabolism. Members of the genus *Cloacibacterium,* which belongs to the *Chryseobacterium* clade in family *Flavobacteriaceae,* were also comparatively abundant. An unclassified *Mycoplasma* OTU was the predominant community member in 5% of specimens by the end of the trial similar to observations made by Zarkasi *et al.,* (2014).

As with the anaerobic taxa, populations of facultative anaerobes peak in the summer in the LoPro diet and then decline. The levels in the other diets by comparison show a gradual downward trend. These results suggest that the availability of substrates and lack of oxygen is potentially most optimal when the LoPro diet is applied. However, the reasons for this are unclear, but could be associated with diet: metabolic interactions within the GI tract. Many of the lactic acid bacterial taxa are known to form bacteriocins (Dobson *et al.* 2012) that potentially provide them a temporary advantage against the growth of other bacteria, however if this is the case the effect is clearly only transient.

Distribution between samples lacking casts and cast-rich faecal samples shows that lactic acid bacteria are slightly more enriched in the digesta (Fig. 3.8). Lactic acid bacteria could also be present as adherent populations on the gut epithelium (Hovda *et al.* 2007; Ringø *et al.* 2009) but the exact localisation of these bacteria is still poorly defined.

3.4.9 Marine facultative anaerobes eventually predominate regardless of diet.

All diets by the end of the trials became dominated by marine-derived facultatively anaerobic species (Fig. 3.7), especially those belonging to the genus *Aliivibrio*. The primary *Aliivibrio* taxon (making up 33.2% of reads) was nearly identical to the species *A. finisterrensis*, originally isolated from the Manilla clam (*Ruditapes phillipinarum*) (Beaz-Hidalgo *et al.* 2010). In some samples it made up >95% of reads. Other members of the family *Vibrionaceae* were also significant, especially *A. fischeri* (3.3% of reads), *A. sifiae* (0.9%), an unclassified *Aliivibrio* species (0.2%), *Vibrio atypicus* (2.1%), *V. vulnificus* species complex (0.6%), *V. ichthyoenteri* (0.2%), and *V. scophthalmi* (0.4%).

In the first month of the trial rapid growth of these species occurs in all cohorts. In the IntPro and HoPro diet the levels reach a peak of 65-70% of bacterial reads before slightly declining to 55-65%. In the LFM diet the level reached an average of 78% of reads at the end of the trial. The LoPro diet showed a pronounced lag before the proportion of the marine facultative read group rose to approximately 70% at the end of the trial.

3.4.10 High abundance of non-halophilic aerobes in the salmon intestinal tract

It was observed that taxa typically of non-marine origin with a strictly aerobic oxidative metabolism made up 5-20% of reads throughout the trial peaking in the March 2011 samples. The major taxa include *Sphingomonas paucimobilis*, *Acidovorax ebreus*, and *Methylobacterium* spp. (mainly *M. extorquens*). This surprising result could suggest these taxa are diet "contaminants", however the populations persist at significant levels suggesting they may have footholds in the salmon GI tract. Diet did not have much effect on their distribution nor was their any form of digesta distribution (Fig. 3.7 and 3.8) consistent with anaerobes preferring the largely anoxic digesta.
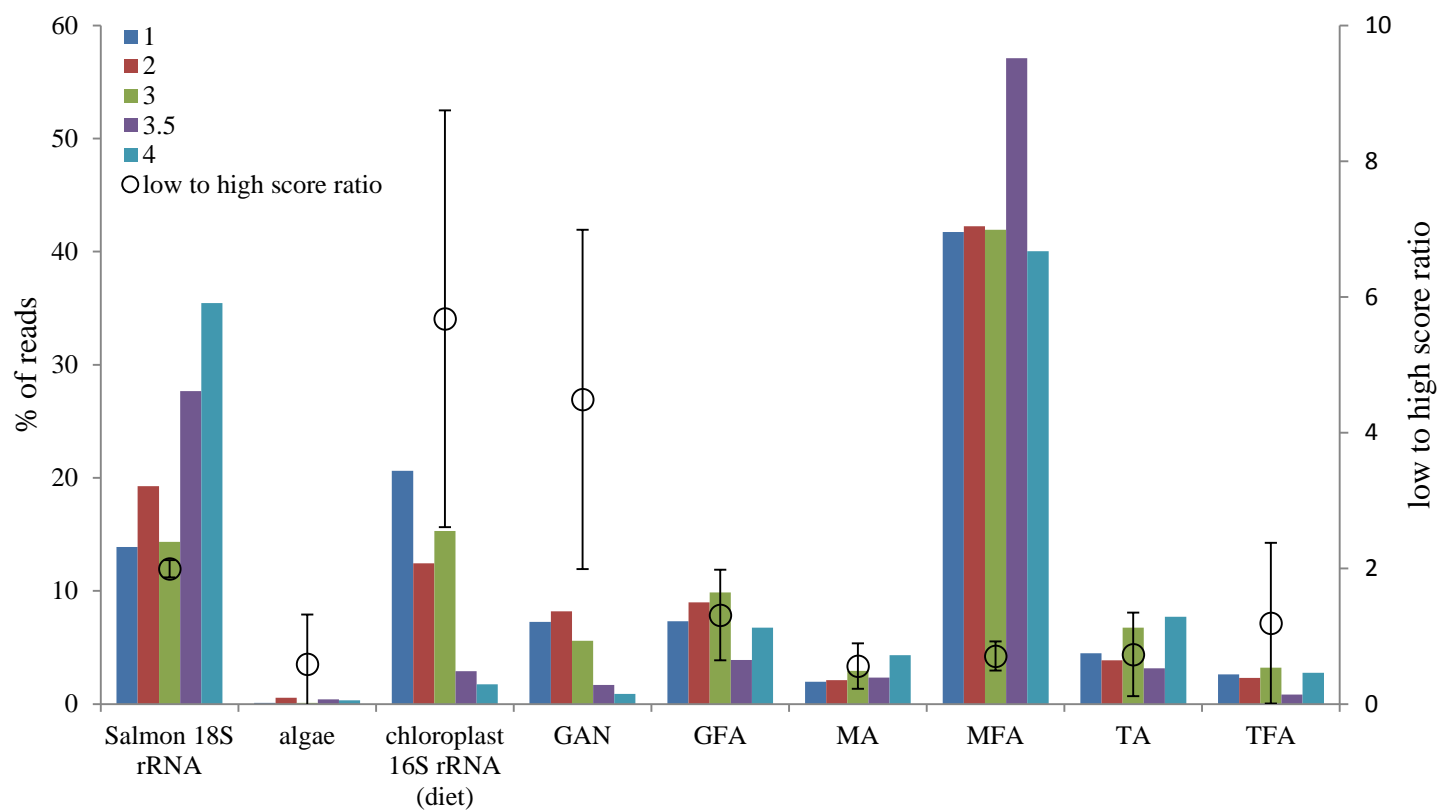
Figure 3.8: The enrichment of sequence groups on the basis of faecal scores. Bars indicate the proportion of reads making up all reads accumulated for the community or sequence component. The symbols above the bar indicate the proportional ratio of reads between low (1 to 3) and high (3.5 and 4) scores for each community or sequence subcomponent.

3.5 Discussion

Manipulations of diet attributes such as protein and oil, supplementation with prebiotic additives, plant extracts and probiotic agents can potentially stimulate immune system responses possible as a consequence of the alteration of GI tract microbial populations (Tacchi *et al.* 2011; Abid *et al.* 2013; Grammes *et al.* 2013). However determination of how microbial community adjustments generate a farm level effect such as increased SGR or body weight is challenging due to experimental system complexities and lack of knowledge on what constitutes a salmon core GI tract microbiome and how it connects with salmon physiology. Many factors impact what that the core species suite could be composed of including ecosystem inputs and influences (Sullam *et al.* 2012); scale and methods of sampling communities (Hamady and Knight 2009); the nature of the specimen cohorts being tested (wild; domesticated; maturation stage; sex; ploidy) and their management; and the dietary regimes relative to what is considered a commercial standard being tested. That said, the process to realise this form of knowledge within commercial production system settings requires first some clear concepts of what can be achieved from observations of GI tract community changes.

In this study we used a relatively high resolution community analysis approach, substantial replication (10-20 samples per treatment,) and also examined faecal samples in an ordination system as way to study GI tract community patterns that might be subsequently related to performance outcomes. In chapter 2, where 6 specimens each from two separate diet groups per time point were analysed we observed considerable specimen-to specimen variability and though we could discern temporal trends differences between diets was more difficult to discern if different community responses were indeed present. To increase the power of the analyses we increased replication to 10-20 specimens.

From the 4 diet cohorts tested we could observe different community structure dynamics and a range of faecal scores. This was done at the *fish specimen level* only though such experiments would also be needed at whole farm level to better account for environmental and temporal variables. Nevertheless, we could show that the LoPro diet, which resulted in growth performances at the end of the trial distinguishable within non-mature and mature subpopulations also possessed pronounced albeit transient differences in the abundance and diversity of strict anaerobe, lactic acid bacteria, and *Vibrionaceae* community components. However, it is still unknown if these transient changes in the communities relative to the other diets have casual links to final growth performance outcomes, for example via differential effects on immune system modulation or via more efficient digestion. Also how reproducible these transient phenomena are is unknown and for greater confidence would require instigation of experiments where there is tighter control on the community structure. The open nature of the salmon GI tract challenges this proposition especially within a commercial or experimental farm setting since local conditions are potentially highly influential and could potentially change over the long term affecting all facets of the biology, for example changes in surface water temperatures (Spillman and Hobday 2014).

We observed a wide variety of microbes in the faecal samples and could show conclusively differences in community structure relative to the nature of the samples, diets and on a temporal scale as well as the inherent variability within treatment groups. Based on the typical bacterial densities in digesta (10^5 to 10^9 cells/g) bacterial taxa could be resolved with pyrosequencing with a 1000-fold dynamic range (effectively 10^2 to 10^6 cells per gram). We observed high specimen to specimen variability, however this was not entirely consistent.

At the commencement of the trial the individual variability was relatively constrained (49 ± 15 OTUs, n=19) however as the trial progressed through summer it seems GI tract communities of individual fish diverge as confirmed by dispersion analysis. A similar situation has been observed in wild Atlantic cod populations sampled in the same region. Individuals within these wild cohorts were found to have very poorly conserved β-diversity (Star *et al.* 2013). In order to adequately assess salmon GI tract microbial communities high resolution procedures seem necessary, coupled to sufficient biological replication. The variability between specimens could be to some extent be partly understood by applying the faecal scoring system since variations in some community components could be observed systematically. In human microbiome studies large datasets have been used in order to link metabolic dysfunctions to GI tract diversity as well as to make correlations with marker metabolites (Le Chatelier *et al.* 2013). In order to make better links between diets or other treatment criteria with farm level outcomes replicated cohorts would need to be studied since individual fish variation may mask subtle trends.

Rapid fingerprinting procedures for community structure analysis (for example ARISA analysis (Sadet-Bourgeteau *et al.* 2014) could be useful for delineating large numbers of specimen samples followed by more focused sequencing and other forms of analysis. Older methods such as DGGE and TRFLP seem to be less appropriate being costly, labour intensive, non-quantitative and given to gel-based and PCR-based biases (Powell *et al.* 2013) and only with limited means for sequence interrogation (e.g. DGGE gel band analysis). Statistically testing community structure changes with multivariate approaches seems to be also an essential requirement to understand community structure changes in relation to other factors. Doing this made it possible to show populations are dynamic overtime as well as affected by diet leading to an interaction effect (Fig. 3.3, Table 3.4) and observations that

digesta has high chloroplast content correlates to high strict anaerobe population levels suggesting these bacteria can play a substantial role in feed digestive processes even in the open fish digestive tract (Fig. 3.8). The data suggests that the microbes that predominate in digestion roles changes over time and may comprise anaerobes, *Vibrionaceae*, *Mycoplasma* and/or lactic acid bacteria. This implies the types of metabolites formed during feed metabolism plus other benefits (vitamin synthesis) could also change and thus impact on fish growth performance.

The function of temperature in this dynamic situation seems to be also highly influential. Bacterial growth is fundamentally controlled by temperature and can be accurately modelled (Ratkowsky *et al.* 1983). The fact *Vibrionaceae* predominates in most samples could be related to the relatively fast-growing, psychrotolerant (Soto *et al.* 2009; Beaz-Hidalgo *et al.* 2010) and bile tolerant (Chen *et al.* 2010) nature of these species. Since many *Vibrionaceae* share similar ecophysiological traits it is likely specific species can get supplanted by other species. In other Tasmanian salmon surveys it was observed *A. fischeri*, *Photobacterium phosphoreum*, *Vibrio scophthalmi* and *V. ichthyoenteri* could predominate (Green *et al.* 2013; Hatje *et al.* 2014; Neuman *et al.* 2014; Zarkasi *et al.* 2014) instead of *A. finisterrensis*. Further analysis of salmon strains may reveal biological facets that could have significance to salmon physiology and health, though data suggests that *A. finisterrensis* and its relatives seem to be neutral in the relation to fish growth rates and CF as they are comparatively predominant across all diets. Furthermore their abundance is equivalent between low and high faecal score samples suggesting that this group is not specifically predominant in digesta and may also congregate or interact with the intestinal epithelium.

It must be noted the predominance of *Vibrionaceae* did not apply to all specimens. By the end of the trial 14-40% (dependent on the cohort) of fish had different predominant OTUs

present including species belonging to the genera *Bacteroides*, *Sphingomonas*, *Litoreibacter*, *Mycoplasma* and *Brochothrix*. The reasons for this lack of homogeneity between fish is unknown but could relate to a host of factors such as hierarchical dominance and maturation resulting in different fish body size and feeding rates; stochastic colonisation processes; and/or differential affects caused by the farm conditions and husbandry including anti-AGD baths destabilizing microbial communities.

One might speculate that these differences in dominant microbiota and overall individual variability could collectively comprise the population wide effects influencing averaged performance outcomes. However, greater understanding of how GI tract microbiota functionally influence salmon physiology including the redundancy (Qin *et al.* 2010) of such influences is needed to determine this. This would require utilisation of metagenomic technologies for assessing salmon gene expression and protein abundances (Dantas *et al.* 2013) coupled to bacterial-mediated processes including enzymatic and metabolomic characteristics (Ray *et al.* 2012).

Our data also suggests aerobic microbes are found in the digesta and also associated with sloughed off intestinal cast material suggesting possibly some level of GI tract epithelium association or interaction though this remains to be clearly defined. The presence of aerobes in GI tract systems is not unprecedented as the mouse colon has a substantial population of aerobic taxa colonizing the depths of epithelium crypts (Pédron *et al.* 2012), with oxygen supplied by the cell layer. The population most enriched in crypts versus the gut lumen were members of classes *Betaproteobacteria* and *Gammaproteobacteria*. It was hypothesized these bacteria may contribute to gut biology in homeostatic and protective capacities based on their inherent ability to consume oxygen radicals and xenobiotic compounds though this remains to be determined in any detail.

The types of non-marine aerobic taxa detected in this study mainly belonged to classes *Alphaproteobacteria* and *Betaproteobacteria* could also have capacity for detoxification since the main genera found (*Sphingomonas*, *Methylobacterium* and *Acidovorax*) are strong catalase producers, have diverse metabolic pathways for xenobiotic catabolism and in the case of *Methylobacterium* spp. can utilise methanol and convert toxic formaldehyde to CO_2. The fact that an array of aerobic bacteria are present in the salmon GI tract, which unlike mammals lacks crypts raises the possibilities of some bacterial role in providing protection and homeostasis as observed in the mouse model. Confirmatory analysis of the epithelium layer is required to determine if stable populations are indeed present and also determine their biological activities *in situ*. Higher resolution studies of the GI tract epithelial layer via careful biopsies coupled with other methods such as fluorescent in situ hybridisation could confirm spatial localisation of these and other important bacteria that can be potentially linked to gut homeostasis and immune system modulation (Ivanov and Littman 2010). This would be important in connecting GI tract communities with salmon physiological and immune system responses and eventually broader performance outcomes.

3.6 Conclusions

We conclude that time (incorporating seasonal changes in temperature) and diets contribute to how faecal microbial communities are structured. Categorization of the digesta also revealed that microbes are different in relation to digesta properties, especially anaerobic bacteria. We hypothesize these community shifts could lead to the formation of different levels of metabolites and/or immune system stimulation that and could influence overall salmon physiology though we are still far from establishing mechanistic links between microbial communities and farm-level performance outcomes.

3.7 Acknowledgements

Chapter 4:

In vitro growth characteristics of dynamic Atlantic salmon (*Salmo salar* L.) gastrointestinal microbial community in relation to different diet formulations

4.1 Abstract

In order to better understand microbial changes within the salmon GI tract as determined at the dietary level, the microbial community dynamics were assessed within a simple *in vitro* growth model system. In this system the growth and composition of bacteria were monitored within diet slurries held under anaerobic conditions inoculated with salmon faecal samples. This system was assessed using total viable bacteria counts (TVC), automated ribosomal intergenic spacer analysis (ARISA), and 16S rRNA pair-end Illumina-based sequence analysis. A total of 5 complete diets were tested including low fish meal (LM), low protein (LP), high protein (HP), a commercial standard (CS) diet with intermediate protein and lipid content and a CS diet version where fish oil was completely replaced with poultry oil (PO). In addition plant meals (lupin kernel meal and pea extract, referred to as the LK and PE diets) were tested in isolation to determine if plant-derived material promotes the growth of specific bacteria. The *in vitro* model cultures were incubated at 20ºC to simulate warm summer temperatures. The microbial growth in the diet slurries after a lag phase of ~3 h grew over a 24 h period with a progressive decline in pH. TVC counts indicated growth on MA and TCBS plates were equivalent indicating most bacteria that grew were bile salts tolerant. ARISA and Illumina sequencing data revealed there was very clear separation between the complete diets and the LK and PE plant meal diets suggesting bacteria that grew were distinct. The sequencing analysis showed in the case of the complete diets members of the genera *Aliivibrio*, *Vibrio* and *Photobacterium* became greatly predominant. However, based on

replicated experiments there was evident stochasticity of what exact species became dominant. *Vibrionaceae* may have become predominant due to their rapid growth capacity, relatively high abundance within the starting faecal material and salt tolerance though several other bacterial taxa were also present in great abundance initially. The LK and PE diets only allowed the growth of the aerobic genus *Sphingomonas*, no other faecal-associated bacterial grew including *Vibrionaceae* suggesting a combination of protein and lipid diet components structure the salmon GI tract community.

4.2 Introduction

Gastrointestinal (GI) microorganisms serve a variety of functions in the nutrition and health of fish by promoting nutrient supply, preventing the colonisation of pathogens and maintenance of gut mucosal immunity (Nayak 2010). Thus the intestinal flora plays an important role in fish health and growth rates (Ringø *et al.* 1995; Olafsen 2001; Meziti *et al.* 2010; van Kessel *et al.* 2011; Zarkasi *et al.* 2014). These microbial communities can be influenced by diet and this has been the basis of substantial research with the goal to improve farmed finfish productivity (Korsnes *et al.* 2006; Bakke-McKellep *et al.* 2007; Askarian *et al.* 2012; Green *et al.* 2013).

Understanding fish GI tract microbiota and how fish physiology is influenced potentially can lead to improvements to the efficiency of aquaculture systems and aid in industry sustainability. It has been found that Atlantic salmon GI tract bacterial communities are highly dynamic (Holben *et al.* 2002; Hovda *et al.* 2007; Ringø *et al.* 2008; Hovda *et al.* 2012; Zarkasi *et al.* 2014), sensitive to diet and dietary changes (Ringø *et al.* 2008; Merrifield *et al.* 2010; Landeira-Dabarca *et al.* 2013), antibiotic application (Burridge *et al.* 2010), and

the impact of environment conditions such as temperature (Cahill 1990; Pankhurst and King 2010; Neuman *et al.* 2014; Zarkasi *et al.* 2014), salinity (Sullam *et al.* 2012), geographical location and ecosystems (Holben *et al.* 2002).

In general, farmed salmonids require a diet containing fishmeal and fish oil, which contains high-quality protein and is rich in essential amino acids and lipids (Sargent and Tacon 1999). Other nutritive ingredients such as wheat, poultry meal and vitamins can also been added. Numerous studies have supplemented diets and/or manipulate composition to observe responses in fish growth performance (Hillestad and Johnsen 1994; Einen and Roem 1997; Peres and Oliva-Teles 1999; Karalazos *et al.* 2011; Sørensen *et al.* 2011). Comparatively recently there has also been a focus on using diets to improve or assist the stability of gastrointestinal health, which may improve overall fish health status, feed utilization, growth performance and productivity (Dimitroglou *et al.* 2011; Askarian *et al.* 2012). However, environmental and management complexity in farm systems represents a challenge in devising diet formulations that have predictable and stable effects since elements of randomness and functional redundancy could lead to different taxa predominating.

Due to global limitations on the availability of marine sourced ingredients (fish meals and fish oils) and the need to minimise feed production costs several studies have investigated the modification of the level of valuable fishmeal and fish oil resources with exploring substitute nutrients such as lupin kernel, inulin, nucleotide, chitin, soybean, cellulose and microalgae (Hillestad and Johnsen 1994; Carter and Hauler 2000; Burrells *et al.* 2001; Burr and Gatlin 2005; Korsnes *et al.* 2006; Spolaore *et al.* 2006; Bakke-McKellep *et al.* 2007; Glencross *et al.* 2008; Ringø *et al.* 2008; Merrifield *et al.* 2010; Askarian *et al.* 2012; Molina-Poveda *et al.* 2013; Salini and Adams 2014). The actual effects on the GI tract microbiota of these changes have received relatively limited attention so far.

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Amongst a range of nutritive feed supplements increasingly routinely included in fish diets is lupin kernel meals, which offers well-balanced amino acid, high crude protein content, and palatability amongst a range of farmed finfish species including salmonids (Carter and Hauler 2000; Glencross *et al.* 2011; Salini and Adams 2014). Lupin kernel is extracted from members of the leguminous flowering genus *Lupinus* spp., commonly known as lupin, and native to North and South America. *Lupinus* species which have been used as lupin kernel meals in fish diets includes *Lupin angustifolius* (Glencross *et al.* 2008) the main species grown commercially in Australia, *Lupin albus* (Salini and Adams 2014), *Lupin luteus* (Glencross *et al.* 2011), and *Lupin mutabilis* (Molina-Poveda *et al.* 2013).

Despite its popularity, no specific studies about the effect of lupin on salmon GI tract microbial communities has been performed in terms of determining potential impacts on community structure. However, a study has been performed on the faecal/GI contents in gilthead sea beam and goldfish (Silva *et al.* 2011). Other plant derived nutritive meals are also used including faba beans (*Vicia* spp.). *Pisum sativa* meal is also used in diets as a binding agent at low levels but despite its high level of carbohydrates and protein contains substantial anti-nutritional factor levels such as trypsin inhibitors, which hinders protein digestion.

The aim of this study was to investigate whether different diet compositions potentially influence the GI tract bacterial community growth. This included diet formulations where fishmeal and fish oil had been largely substituted with poultry and plant meal products. The primary question being asked is whether different diets favour different bacterial species with these reformulations. However, another important goal was to study this within an artificial in-vitro system that may provide a testbed to inexpensively test diet formulations in terms of how they might affect GI tract microbiota. For this purpose, faecal

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samples were collected from a salmon farm and investigated in a simple culture-based system where diet slurries served as the medium of growth. More specifically we were interested in determining what species grew most rapidly and become predominant when the temperature is at a level typical of extreme summer conditions (~20ºC) in south-eastern Tasmanian waters. At this temperature farmed Atlantic salmon can demonstrate inconsistent or poor feeding levels leading to suboptimal growth performance (Zarkasi *et al.* 2014). This issue is believed to be compounded when high energy diets are used where fish meal and oil levels have been substituted with lower cost or seasonally available alternatives. This study provides potentially useful insights into the capability of potential diet manipulation to lead to improve salmon production but also tests a simple system that may provide an avenue to do initial diet trials to determine if specific diet formulations can lead to GI tract community shifts or provide stable platforms for implementation of probiotic agents.

4.3 Materials and methods

4.3.1 Fish diets

Five different diet formulations were used in this study, including diets with low fish meal (LM), low protein (LP), high protein (HP), a commercial standard (CS) diet with intermediate protein and lipid content, and a diet containing poultry oil. The diets used also included protein: lipid ratio modified diets LP and HP which were produced to broadly match the diets tested in Chapter 3. More specifically the CS diet was modified to yield two different diets. The first formulation had fish meal largely replaced with poultry meal and lupin kernel meal and is referred to as the LM diet. In the second formulation fish oil was replaced with poultry oil and is referred here as the PO diet. In addition to these complete diet formulations lupin kernel meal and *Pisum sativa* meals were also tested independently to determine if they are capable of supporting microbial growth. The general basic composition of each of the diet group is shown in Table 4.1 where differences in protein and lipid content and digestible energy level are also indicated. Yttrium oxide represents a tracer compound used for digestibility assessments.

To manufacture the experimental diets a laboratory-scale, twin-screw extruder (MPF24:25; Perkins-Baker, Peterborough, United Kingdom), with intermeshing, co-rotating screws was used. The methodology is based on that reported in Glencross *et al.* (2012). Each of the raw materials used was milled using a Retsch rotor mill with a 750 μm screen to create flour prior to incorporation in the diet mashes. Each mash was then mixed in a Hobart mixer (Hobart, Ohio, USA). Each dry mash was separately delivered into the barrel at a feed rate of

around 360 g/min. Barrel temperatures were set for each of the four zones from drive to die at 50ºC, 80ºC, 100ºC and 120ºC, respectively. The barrel of the extruder was a smooth-walled, open-clam design with twin-screws each with dimensions of 24 x 600 mm (diameter x length). The screw configuration was composed of a series of intermeshing feed screws (FS), forwarding paddles (FP) and lead screws (LS) arranged according to defined barrel diameters (D) such that overall configuration from the drive end was: 16D FS, 2D FP, 1D FS, 2D FP, 1D LS, 1D FP, 2D LS: to the die. A single 4.0 mm diameter cylindrical die tapered at a 67° angle with a land length of 3 mm was used. Each diet was extruded using the same temperature parameters.

Water was peristaltically pumped (Watson-Marlow 504U, Falmouth, England) into the barrel at around 100 mL/min based on optimising the expansion of the pellet. Pre-conditioning and steam injection were not used during the process. Pellets were cut into 6 to 7 mm lengths using a four-bladed variable speed cutter and collected on large aluminium oven trays (650 x 450 x 25 mm, L x W x D), which were subsequently used for drying of the pellets at 60°C for 12 h. Where required, following drying the pellets was vacuum infused with their formulated allocation of oil. To infuse the oil an allocation (~5kg) of the warm, dried uncoated pellets were weighed into the mixing bowl of a Hobart mixer (Hobart, Ohio, USA) and the formulated allocation of warmed (60ºC) oil slowly poured over the pellets whilst they were being mixed. Once all the pellets were evenly coated, the bowl was removed, a lid applied and the bowl chamber evacuated of air using vacuum pump. The vacuum was maintained until all signs of air escaping from the pellets were seen to stop. At this point the air pressure was slowly re-equilibrated, the lid removed and the pellets removed, bagged and stored at 4ºC ready for use.

Table 4.1: The composition of diet formulations and ingredients utilised in this study.

Diet group	HP	CS	LP	PO	LM	LK	PE
Composition and energy:							
Protein (%)	50	45	40	45	45	37	24
Lipid (%)	20	25	30	25	25	8	1
Digestible energy (Mj/kg)	18.0	18.8	19.6	18.8	18.4	13.2	ND
Protein to digestible energy ratio	25.0	21.5	18.3	21.5	22.0	22.0	ND
Ingredients:							
Fishmeal (%)	71.2	63.5	55.8	63.5	16	0	0
Fish oil (%)	13.4	63.5	24.8	0	18.1	0	0
Wheat flour (%)	14.8	19.1	18.8	16.8	12.3	0	0
Wheat gluten (%)	0	16.8	0	0	0	0	0
Lupin kernel meal (%)	0	0	0	0	10	100	0
Poultry meal (%)	0	0	0	0	40	0	0
Poultry oil (%)	0	0	0	19.1	0	0	0
Vitamin/minerals premix (%)	0.5	0.5	0.5	0.5	0.5	0	0
Yttrium oxide (%)	0.1	0.1	0.1	0.1	0.1	0	0
Pisum sativa meal (%)	0	0	0	0	0	0	100

ND: no data available for Atlantic salmon.

4.3.2 Atlantic salmon faecal collection

The faecal samples were collected during November 2013 and April 2014 from Tassal Group Ltd Robert's Point lease located within the D'Entrecasteaux Channel, Bruny Island, Tasmania (43.18°S 147.30°E). Samples were collected by randomly seining a large group of fish, crowding the fish in the seine to minimise bias and subsequently dip-netting 5 to 10 fish into 17 ppm Aqui-S anaesthetic (Aqui-S, Lower Hutt, New Zealand). The fish were approximately 3 – 4 kg average weight on both occasions. The faecal samples were collected by gently squeezing the hind gut into individual sterile plastic zip-lock bags. After collection, excess air was squeezed from the bags before closing. The sample bags were immediately transferred on ice to the laboratory and processed within three hours.

4.3.3 *In vitro* gut model

In vitro fermentation was conducted in replicates for each of the diets shown in Table 4.1. The diets were crushed and dissolved, and 10 g/L of the diets were added into the basal growth medium. The basal growth medium contained the following ingredients: $NaHCO_3$, 4 g/L; K_2HPO_4, 0.5 g/L; KH_2PO_4, 0.5 g/L; $MgSO_4.7H_2O$, 0.09 g/L; $CaCl_2$, 0.09 g/L; sea salts 30 g/L (Sigma, St Louis, US); Resazurin, 0.5 mg/L; hemin, 10 mg/L (MP Biomedicals, Santa Ana, US); and sterile water, 1L. The faecal samples collected from several individual fish were pooled with equal contributions per fish to avoid erroneous conclusions due to individual variation (Hatje *et al.,* 2014; Neuman *et al.,* 2014; Spanggaard *et al.,* 2000). The focus was to determine the influence of individual diets and faecal sample variability (based on data shown in chapter 2 and 3 of this thesis) needed to be cancelled out since the experiments required a substantial amount of material. The faecal samples collected from several fish samples were pooled, homogenized, and then diluted 1:2 (wt/vol) in marine broth.

A faecal slurry sample of 1 ml was then aseptically inoculated into the growth medium and incubated anaerobically using the AnaeroGen bag system (Oxoid, Basingstoke, England) at 20ºC, with mixing periodic during the incubation. The Anaerogen system produces an atmosphere containing approximately 90:10 N_2:CO_2 with O_2 content reduced below 0.1% within 1 h. The sampling time points of 0, 3, 6, 12 and 24 hours were determined by prior analysis of pH in a trial run where pH was found to decline and stabilise at the 24 h time point. Samples (5 mL) were taken from the growth medium and processed for microbial enumeration and DNA extraction.

4.3.4 Microbial evaluation

Samples collected from the *in vitro* fermentation at 0, 3, 6, 12 and 24 hours were serially diluted using marine broth (Oxoid, Basingstoke, England) and plated onto marine agar (MA), thiosulfate-citrate-bile salts-sucrose (TCBS) agar and De Man-Rogosa-Sharpe (MRS) agar (Oxoid, Basingstoke, England) (Hovda *et al.* 2007). The plates were incubated at 20°C for 48-72 hours in order to determine the total viable counts. TCBS is a selective media for members of the family *Vibrionaceae*. The plates that possessed between 30 and 300 colonies were counted manually to obtain estimates of bacterial numbers (colony forming units/gram wet weight).

4.3.5 DNA extraction

DNA extraction was performed using the QIAamp DNA Mini Kit (Qiagen Sciences, Germantown, MD, US) following the manufacturer's instruction and standard protocols. The extraction of DNA was performed soon after samples collected from the in-vitro process or on samples frozen at temperature -80°C. The DNA samples were stored at -20°C and used for

further analysis using automated ribosomal intergenic spacer analysis (ARISA) and 16S rRNA gene pyrosequencing.

4.3.6 Automated ribosomal intergenic spacer analysis (ARISA)

The bacterial community structure was fingerprinted using ARISA (Fisher and Triplett 1999). Polymerase chain reaction (PCR) amplification was performed using primers 1392F (5'-GYACACACCGCCCGT-3') and 23SR (5'-GGGTTBCCCCATTCRG-3') (Brown *et al.* 2005). The PCR conditions consisted of an initial denaturation step at 95°C for 10 minutes, followed by 34 cycles of 94°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes and final extension at 72°C for 10 minutes and soaking at 15°C. PCR products were purified using UltraClean PCR Clean-Up Kit (MO BIO Laboratories, Inc., Carlsbad, CA, US). PCR-amplified fragments were performed by the preparation for capillary electrophoresis separation using dsDNA Reagent Kit, 35-1,500 bp (Analytical Technologies, Ames, IA, US) mixed with 2 µL of DNA samples.

Capillary electrophoresis was performed using a Fragment Analyzer™ (Advanced Analytical Technologies, Inc., Ames, IA, US) following the manufacturer's standard protocols. Electrophoretograms with peaks of different sizes were obtained and each peak represented an operational taxonomic unit (OTU) and was identified by its fragment size. Fragment Analyzer output files were further analysed by PROSize (Advanced Analytical Technologies, Inc., Ames, IA, US) using a systematic binning procedure.

4.3.7 MiSeq Illumina-based 16S rRNA gene sequencing

16S rRNA gene amplicon sequencing was applied to examine the microbial communities present in the *in vitro* system samples, which were collected at the initial time point of 0 h and at 24 h. Sequencing was carried out by Research and Testing Laboratories (Lubbock, Texas, USA) using the Illumina MiSeq platform. Pair-ended PCR amplification of the 16S rRNA gene V3 region was carried using 341F and 519R primers that possessed 12 bp barcode tags. FASTQ files generated were merged using PEAR (Zhang *et al.* 2012) that were then trimmed to remove primer, barcode and adapter regions using an internally developed algorithm at Research and Testing Labs (Lubbock, Texas, USA). The seed sequence for each cluster was then sorted by length and clustered with a 4% divergence cut-off to create centroid clusters. Clusters containing only <2 sequences or <100 bp in length were then removed. These seed sequences were again clustered at a 4% divergence level using USearch to confirm whether any additional clusters appeared. Consensus sequences from these clusters were then accurately obtained using UPARSE (Edgar 2013).

Each consensus sequence and its clustered centroid of reads was then analysed to remove chimeras utilising UCHIME in the *de novo* mode (Edgar *et al.* 2011). After chimera removal each consensus sequence and its centroid cluster were denoised in UCHIME in which base position quality scores of >30 acted as the denoising criterion. Sequence dereplication and OTU demarcation was further performed in USEARCH and UPARSE to yield OTUs that were aligned using MUSCLE (Edgar 2004) and FastTree (Price *et al.* 2010) that infers approximate maximum likelihood phylogenetic trees. OTUs were then classified using the RDP Classifier (Wang *et al.* 2007) against the curated GreenGenes 16S rRNA gene database (DeSantis *et al.* 2006) utilising the May, 2013 database update.

4.3.8 Statistical analysis

PRIMER6 and PERMANOVA+ (version 6.1.12 and version 1.0.2; Primer-E, Ivybridge, UK) respectively were used to conduct permutation multivariate analysis of variance (PERMANOVA) (Anderson *et al.* 2005), and canonical analysis of principal coordinates (CAP) (Anderson and Willis 2003) to assess the influence of different factors on community compositions. For this analysis, results data collected from the ARISA was tabulated with the size bins combined across the samples, square root transformed and a resemblance matrix created by calculation of Bray-Curtis coefficients. PERMANOVA was conducted using default settings with 9999 permutations, while CAP was conducted using default settings. Multiple pairwise comparisons were also performed. The PERMANOVA derived significance values were considered significant when $P < 0.01$, while $0.01 < P < 0.05$ were considered only marginally significant (Zarkasi *et al.* 2014). For relative abundance of taxa at the genus level in the seven different fish diet groups was compared using BaseSpace 16S metagenomics application.

4.4 Results

4.4.1 Isolation and growth

Bacterial growth on marine agar and TCBS agar is visualised in Fig. 4.1. The TVC progression over time was consistent across all diets.
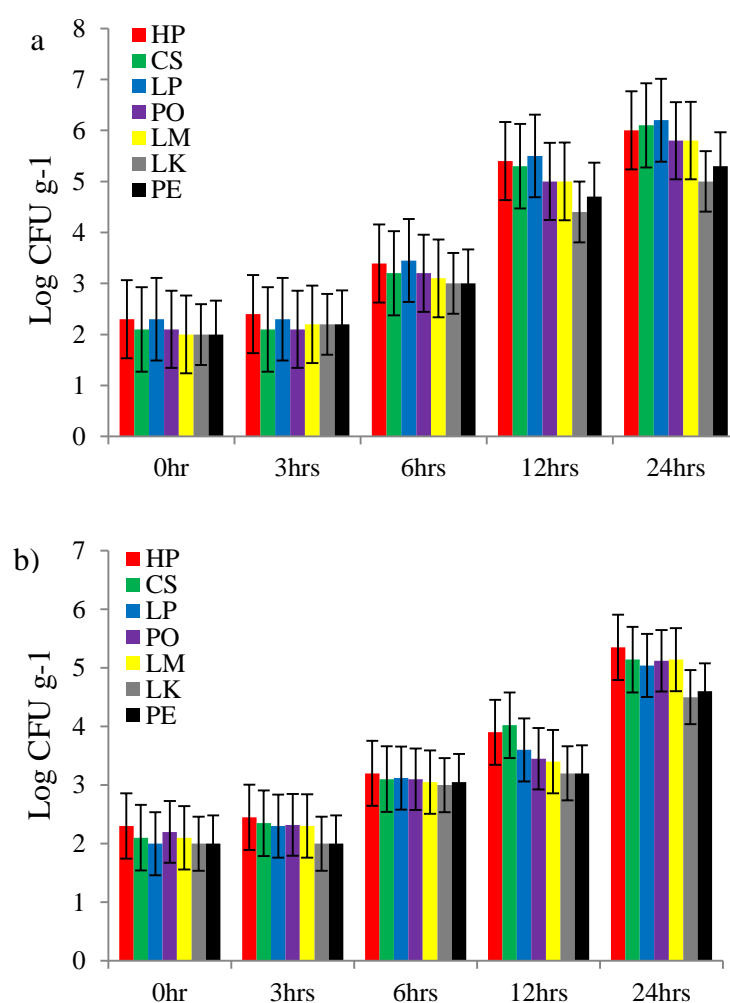
Figure 4.1: Total viable counts in the *in vitro* growth experiment (2 replicates for each diet group) according to the time of sampling. TVC are derived from the colony numbers appearing on a) marine agar and b) TCBS agar.

4.4.2 Analysis of community structure among different diets

The ARISA profiles generated from the bacteria occurring within the *in vitro* growth system showed that initial starting material was bacterially diverse with no binned ARISA fragments present in all samples (data not shown). Different diet formulations produced different patterns of ARISA fragments ($P < 0.01$) but the results also indicated the significance of sampling time (0 h vs 24 h, $P < 0.01$), however there was no interaction between diet and sampling time ($P = 0.15$, Table 4.2) indicating bacteria growing within the system inevitably become predominant. Further analysis using pairwise tests showed that populations varied either significantly ($P < 0.01$) or marginally significantly ($0.01 < P < 0.05$) between several diets tested (Table 4.3). No separation was observed between the HP and CS diets ($P = 0.75$), CS and LP diets ($P = 0.27$), CS and LM diets ($P = 0.08$) or between the LP and LM diets ($P = 0.08$). These differences are illustrated in canonical analysis of principal coordinates (CAP) plots which show clustering can be readily correlated on the basis of diets. The microbial profiles emerging from cultures based on purely plant meal based material LK and PE are clearly separated from the others diet (Fig. 4.2).

Table 4.2: Comparison of microbial community structure with response to diet and sampling time using PERMANOVA.

Source	df	MS	*F*	*P* (perm)
Diet	6	13132	5.6200	0.0001
Sampling time	4	4876.7	2.0870	0.0012
Diet x Sampling time	24	2426.7	1.1327	0.1558
Residual	35	2336.7		
Total	69			

Table 4.3: Pair-wise tests between diets for response of microbial community structure using PERMANOVA.

Groups	t	P(perm)
HP, CS	0.8189	0.7541
HP, LP	1.6532	0.0144
HP, PO	1.6377	**0.0042**
HP, LM	1.4425	0.0393
HP, LK	2.9358	**0.0001**
HP, PE	3.1419	**0.0001**
CS, LP	1.1259	0.2656
CS, PO	1.4904	0.0138
CS, LM	1.3253	0.0804
CS, LK	2.8221	**0.0002**
CS, PE	2.9258	**0.0001**
LP, PO	1.4837	0.0451
LP, LM	1.3715	0.0816
LP, LK	3.1607	**0.0001**
LP, PE	3.3105	**0.0001**
PO, LM	1.5342	0.0425
PO, LK	3.7173	**0.0001**
PO, PE	3.9213	**0.0001**
LM, LK	3.2075	**0.0001**
LM, PE	3.3108	**0.0001**
LK, PE	1.5664	0.0191
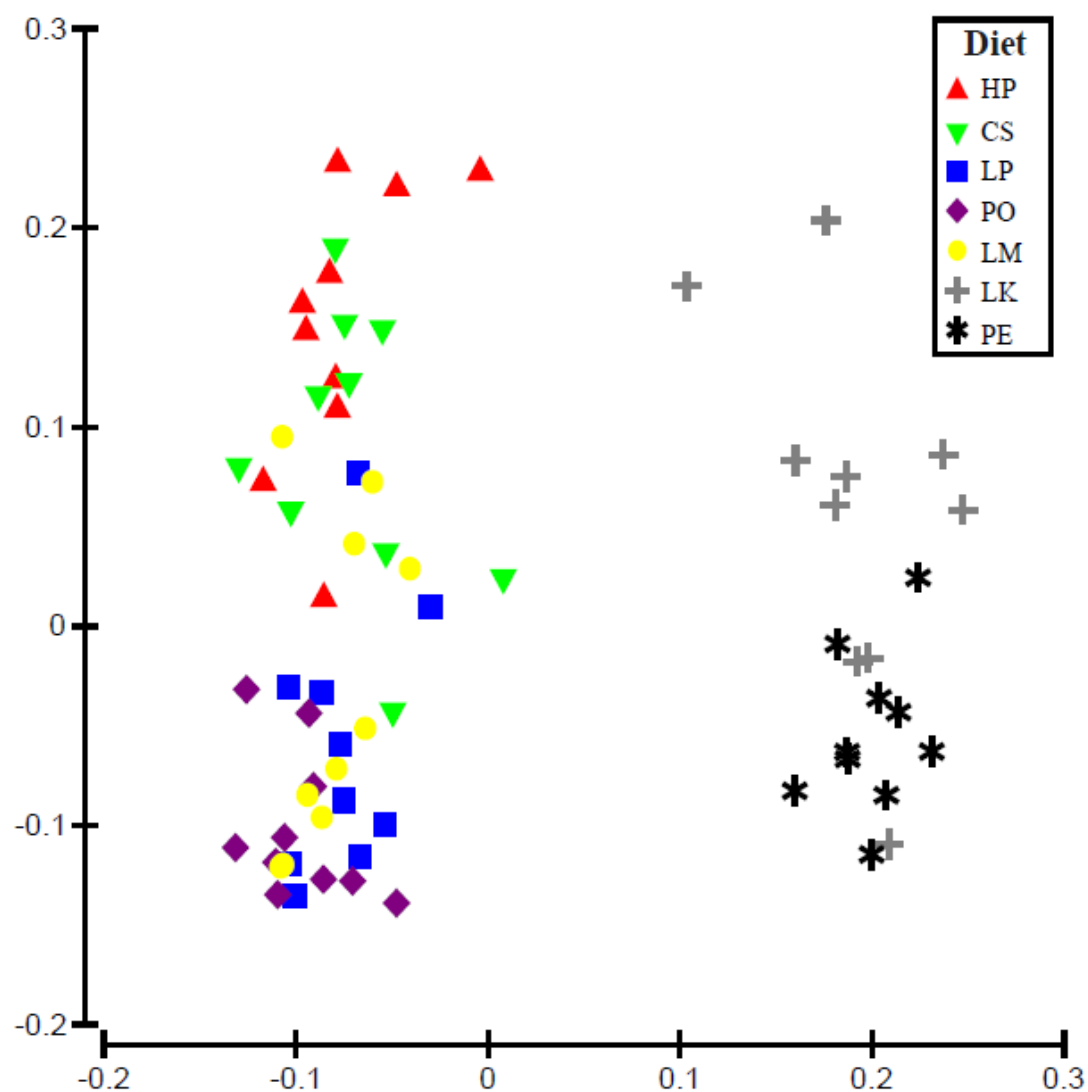
Figure 4.2: Canonical analysis of principal coordinates plots showing faecal community similarity on the basis of diet.

4.4.3 Composition, diversity and similarity of salmon faecal communities

Samples from different diets were further analysed for bacterial composition and diversity. The data indicated all samples regardless of diets were dominated by sequences affiliated with the family *Vibrionaceae* (*Aliivibrio, Photobacterium* and *Vibrio*) after 24 hours *in vitro* fermentation (making up >90% of total reads), except for the 100% plant meal diets (Fig. 4.3). The CS diet formulation supported mainly growth of *Aliivibrio finisterrensis* (66% of reads), *Vibrio tasmaniensis* (15%), *Photobacterium phosphoreum* (6%), and *Aliivibrio fischeri* (6% of total reads, Fig. 4.3). The LP diet cultures were dominated by *Aliivibrio finisterrensis* (85% of total reads) and also contained unclassified *Vibrio* spp. (7%), and *Aliivibrio fischeri* (5%, Fig. 4.3). Diet HP, however, was dominated by the salmon derived species *Vibrio tasmaniensis* (39% of total reads) and contained *Aliivibrio finisterrensis* (23%), *Photobacterium phosphoreum* (14%), unclassified *Vibrio* sp. (13%), and *Aliivibrio fischeri* (5% of total reads, Fig. 4.3).By comparison, diet PO and LM differed in containing mainly unclassified *Vibrio* spp. (making up 36-50% of reads), *Aliivibrio finisterrensis* (33-44% of reads), and *Vibrio tasmaniensis* (8-14% of reads, Fig. 4.3). Other bacterial species that grew in the HP, CS, LP, PO and LM diets included *Pseudoalteromonas* spp., *Sphingomonas* spp., *Pseudomonas* spp., *Vibrio aestuarianus*, *Photobacterium leiognathi*, and unclassified *Photobacterium* spp. (<5% of reads depending on the diet, Fig. 4.3). CAP analysis of the sequence data (Fig. 4.2) reiterated the outcomes of ARISA analysis showing essentially similar statistical relationships between samples.
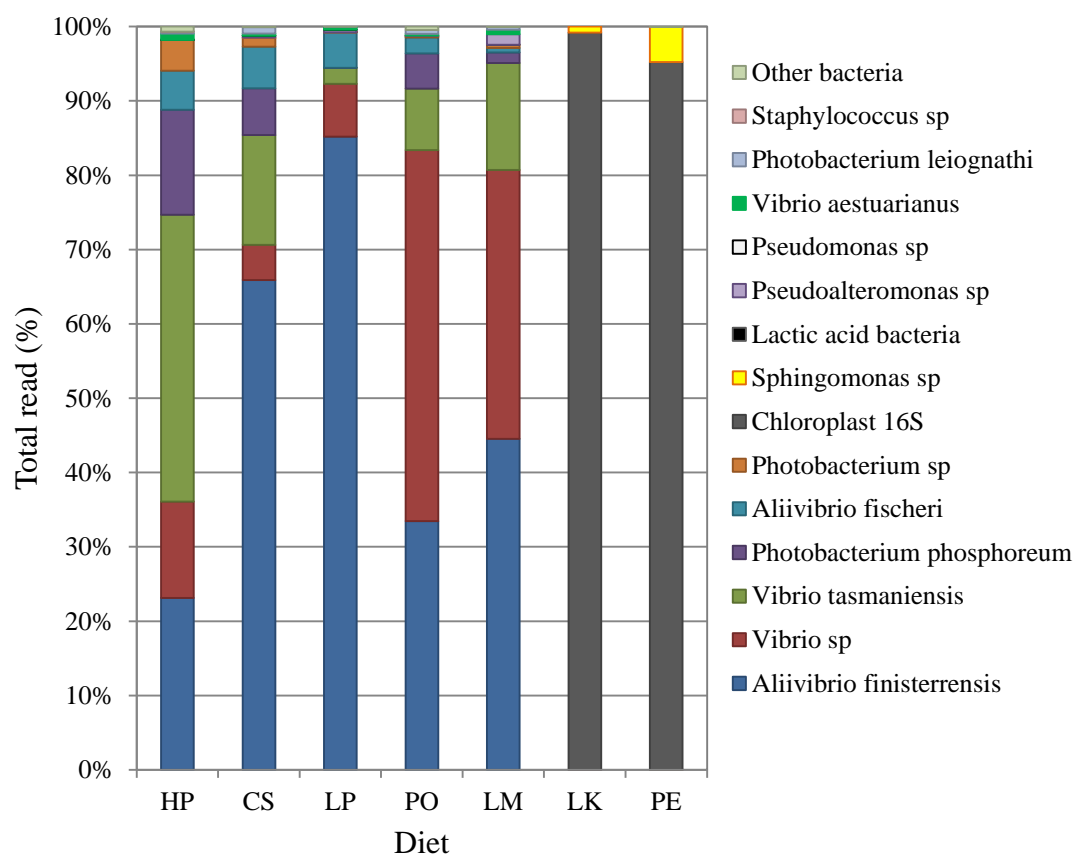
Figure 4.3: Relative abundance of the bacterial species rising on a range of diets and dietary ingredients within an anaerobic *in vitro* system at 20°C after 24 h. The relative abundance was calculated on the proportion of reads obtained by 16S rRNA gene sequencing utilising the Illumina MiSeq platform.
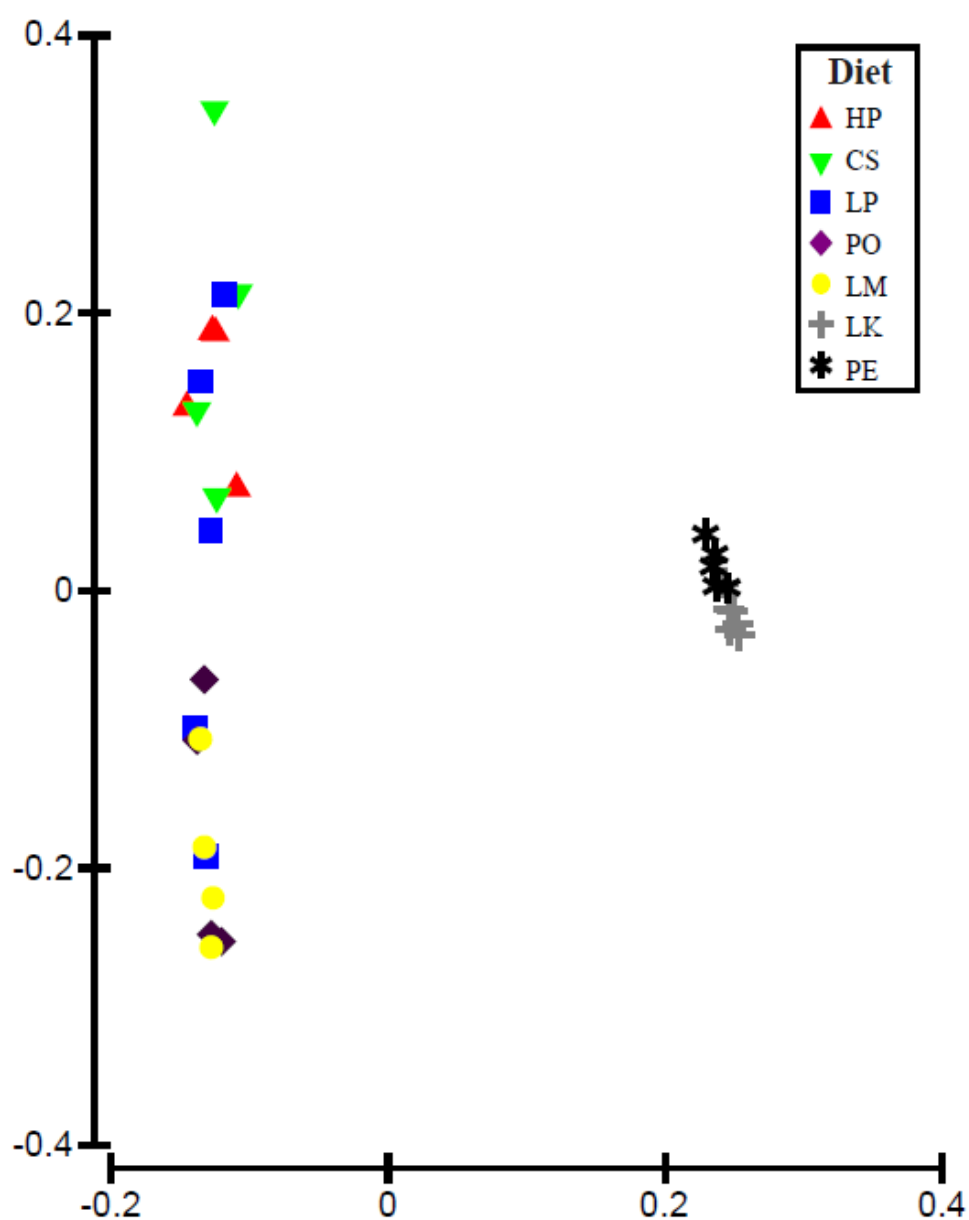
Figure 4.4: CAP plot of showing comparisons of salmon faeces-derived bacterial assemblages analysed by 16S rRNA amplicon sequencing arising on a range of diets and dietary ingredients within an anaerobic *in vitro* system at 20°C.

4.4.4 *In vitro* fermentation of plant meal based ingredients

The 100% plant meal diets (LK and PE) did not support the growth of most bacteria originating in the faecal inoculum including species of the family of *Vibrionaceae*. Due to the overwhelming plant DNA background most reads classified as chloroplast 16S rRNA (grouped within phylum *Cyanobacteria*) including 95% (diet PE) and 99% (diet LK) of total reads (Fig. 4.3). Most of the remaining reads were classified as undefined *Sphingomonas* species. This result correlates with the finding from the PERMANOVA and CAP analysis of the (Table 4.3, Fig. 4.2 and Fig. 4.4).

4.4.5 Response of other bacteria detected during *in vitro* system lag phase.

Bacterial species identified from the initiation of the *in vitro* system likely those are observed within the perceived adaptation lag phase (Fig. 4.1), however plate counts are grossly lower than the bacterial populations in the inocula (typically 10^7 to 10^9 CFU/ml). This is due to dilution and bacteria having to adapt to the diet slurries within the lag phase. The bacterial composition was much more diverse than what was subsequently observed after 24 h (Fig. 4.5). Results showed *Vibrionaceae*, *Sphingomonas, Paenibacillus, Pseudomonas, Methylobacterium, Pectobacterium, Obesumbacterium, Propionibacterium, Staphylococcus* and *Carnobacterium* (a member of the LAB group) were the main taxa present in the samples at the beginning of the experiment and in the starting faecal material. However, the proportional representation of most of these bacteria at 24 hour sampling period was drastically reduced indicating most did not grow immediately under the conditions used and likely were competitively excluded by *Vibrionaceae*, which grew rapidly.
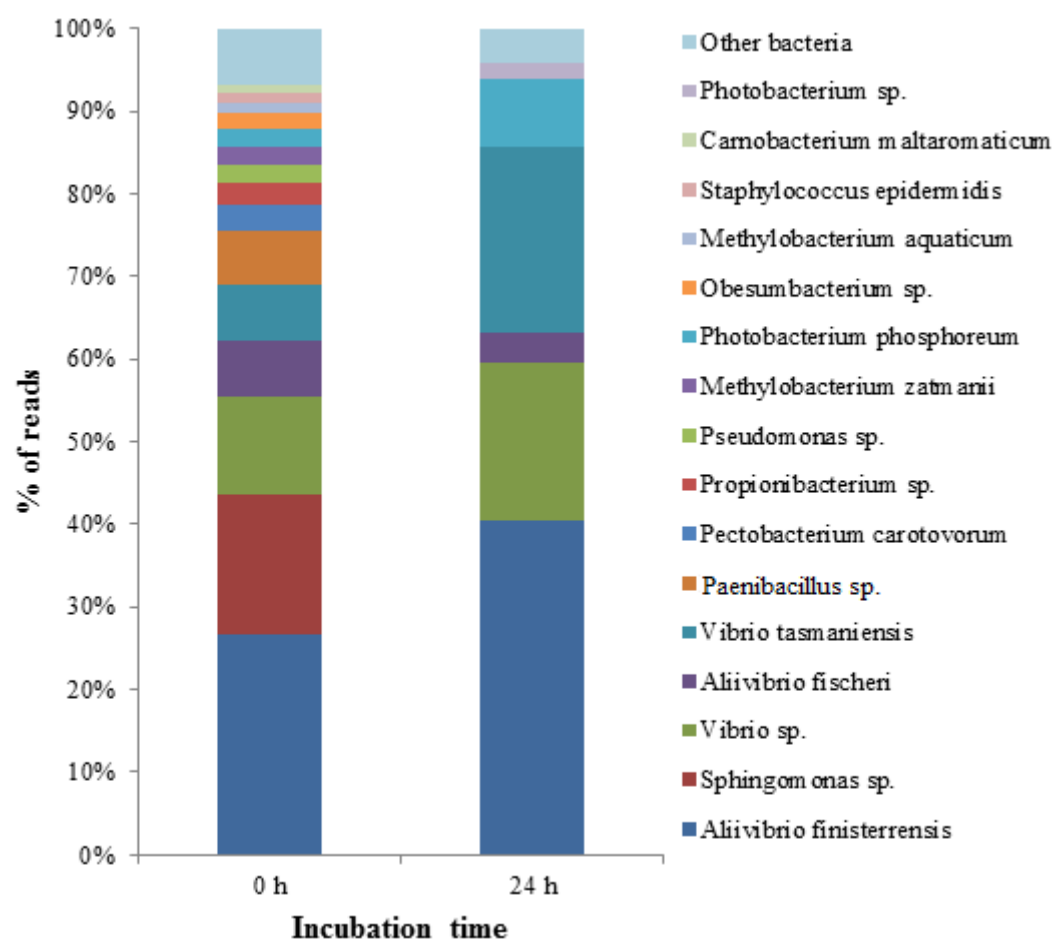
Figure 4.5: Relative abundance of salmon faeces derived bacteria present at the commencement of the experiment within the in-vitro model system and after 24 h incubation at 20°C under anaerobic conditions (90:10 N_2:CO_2)

4.5 Discussion

This study investigated and analyzed the direct growth responses of Atlantic salmon gastrointestinal tract associated bacteria within diet formulations within a simple *in vitro* fermentation system. Though this system does not attempt to replicate the salmon GI tract the experiments are based on the principal bacterial growth is controlled largely by basic criteria: temperature, nutrient availability, O_2 availability and pH. We assume that GI microbial communities in this system would show dynamic growth as observed occurring with salmon specimens as described in Chapters 2 (Zarkasi *et al.* 2014) and Chapter 3. This system is, however, limited because it cannot fully model changes in the salmon gut environment that may impact the gut microbiota.

The fish GI tract is a dynamic environment which responds metabolically to environmental stressors or may express enzymatic changes in response to diet (Askarian *et al.* 2013). Nevertheless, the results show faecal bacterial can instigate a rapid growth response directly on diets. The endpoint (24 h) microbial community is influenced to a degree by specific diets according to the CAP and PERMANOVA analysis (Table 4.2, Fig. 4.2). Furthermore the rapid growth of *Vibrionaceae* in complete diet slurries reflects summer time samples of Tasmanian Atlantic salmon (Chapter 2 and 3) (Green *et al.* 2013; Hatje *et al.* 2014; Neuman *et al.* 2014; Zarkasi *et al.* 2014) where *Vibrionaceae* predominate in most faecal samples.

Amongst the complete diets the HP, CS and LP diets produce similar outcomes suggesting that the differences in protein to lipid ratio were too subtle to produce a marked effect in the *in vitro* system. It is possible these components promote the growth of

Vibrionaceae since the species most promoted in the *in vitro* system are all inhabitants of marine fauna GI tracts and would likely be adapted to marine faunal sources of proteins and lipids. The LM and PO diets that have low fish meal and low fish oil contents respectively have a qualitatively similar species structure though individual species abundances change resulting in the statistical separation on the CAP plots. The alteration of these components though disparate appears to lead to a similar outcome that could be coincidental.

Among replicates there is some variation suggesting a degree of stochasticity in the responses. The results are partly affected by the high level of *Vibrionaceae* (mean 54%) in the starting faecal inocula. This level is typical of the *Vibrionaceae* composition observed in the previous thesis chapters and published studies (Green *et al.* 2013; Hatje *et al.* 2014; Neuman *et al.* 2014; Zarkasi *et al.* 2014) but would inevitably provide a large advantage to this group of species given they have fast growth rates. The inocula is also prepared and diluted under aerobic conditions and the oxic shock could have diminished the growth potential of some O_2 sensitive species though none of the more populous species in the initial samples are strict anaerobes.

Aliivibrio finisterrensis was one of the most abundant bacterial species through all the complete diet culture results (especially in the CS and LP diets). This bacterial species was originally isolated from the Manilla clam (*Ruditapes phillipinarum*) (Beaz-Hidalgo *et al.* 2010), and also isolated from the intestinal tract of Tasmanian farmed Atlantic salmon (Hatje *et al.* 2014) and fish obtained in coastal Japan (Sugita *et al.* 2012). *Aliivibrio finisterrensis* may not be as persistent in the Atlantic salmon in comparison to other GI tract associated species, such as *Vibrio ichthyoenteri*, and mainly occurs during the warmer months based on data obtained to date (Hatje et al., 2014). In the HP, PO and LM diet formulations the most

abundant bacterial species were unclassified *Vibrio* spp., together with *Aliivibrio*

finisterrensis. The prevalence of potentially novel *Vibrio* and *Aliivibrio* spp. in salmon GI

tract has been reported in previous studies (Hatje *et al.* 2014) and is noted in Chapter 3.

Vibrio spp. such as *Vibrio tasmaniensis*, *Vibrio ichthyoenteri*, *Vibrio aestuarianus*, and

Vibrio splendidus, also observed to grow in the diet slurries appear to be normal flora in the

salmon GI tract, since they have also been observed in the Northern hemisphere (Hovda *et al.*

2007). Other bacterial species detected in this study *Photobacterium phosphoreum*,

Photobacterium spp., *Pseudomonas* spp., *Pseudoalteromonas* spp., *Sphingomonas* spp., and

Aliivibrio fischeri are common bacteria that can be found and previously isolated from the

salmon GI tract (Holben *et al.* 2002; Ringø *et al.* 2008; Hatje *et al.* 2014; Neuman *et al.* 2014;

Zarkasi *et al.* 2014).

Since diet LK and PE is purely plant based derived material the lack of response by

most of the detected microbes in the 0 h samples suggests nutrients in the lupin kernel and

pea meals are either not accessible or the meals contain inhibitory substances. These could

include phytogenic substances, mainly essential oils and flavonoids that usually have

generalised antimicrobial properties (Ganguly 2013). The phytogenicity of the plant meals, if

any, used here is uncertain, however since the slurry only consisted of the meals the effect

would have been concentrated relative to what would be a typical situation where the lupins

are merely supplements (e.g. in diet PO).

Only *Sphingomonas*, present initially at high levels in the inocula (16% of reads) was

able to grow suggesting it possesses defenses against possible inhibitory compounds. This

aerobic genus was common in faecal samples analysed in Chapter 3 is known to possess

extensive detoxification and xenobiotic degradative capabilities as well as an ability to grow

under conditions of nutrient stress (Balkwill *et al.* 2006). It is unclear whether plant meal diet

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supplements have any capacity to select for this particular genus of bacteria in complete diets though the data raises this possibility.

4.6 Conclusions

According to Kotzamanis *et al.,* (2007) manipulation of fish meal by replacement with fish protein hydrolysates (FPH) seems to boost bacterial proliferation, and bacteria such as *Vibrio* spp., can be favoured when high doses of FPH are applied. Thus thermal or chemical processing of diet components such as during preparation of the diet slurries used here (which were heat sterilised) could be one reason for the *Vibrionaceae* predominance. Factors that also potentially favour *Vibrionaceae* included the high salt content (3% w/v) of the basal medium, which was implemented since the goal was to investigate a system that is relevant to finfish mariculture. Future experiments would need to examine alternatives to the design of the *in vitro* system including the anaerobic atmosphere CO_2 and H_2 content, inoculum preparation (which may need to be done anaerobically) and amount added, application of mixing, overall culture volumes, additives such as bile salts, and pH control. Furthermore, the experiments performed here are preliminary, limited by funds and time, many improvements and experiments could be implemented testing different forms of diets and diet additives including probiotics, prebiotics, phytogenic additives, activated carbon and different forms of the core ingredients (non-heat treated versus heat-treated).

4.7 Acknowledgements

Chapter 5:
General discussion and conclusions: Research benefits, adaptation, further development and future research

5.1 Research benefits and adaptation improving Tasmania's salmon industry productivity

5.1.1 The SGS problem

Tasmania's salmon farming industry is the highest value ($513 million) and largest volume (43,989 tonnes) fishery in Australia (ABARES, 2013). The contributions into the Tasmanian economy make the salmon industry a vital and critical agribusiness, and considerable research and development (R&D) investment has been made in the areas of environmental management, stock management, disease control, nutrition, physiology and selective breeding to sustain and improve the industry. Following rapidly increased production, the use of higher energy feeds and replacement of fish meals and oils with plant and vegetable alternatives, Tasmanian farmers have experienced production issues seemingly not associated with infectious disease but rather an issue that has become labelled as "Summer Gut Syndrome". The name of course implies the problem occurs in summer and from accumulated data can be linked to GI tract functional changes leading to anorexia (Glencross and Taylor 2012; Green *et al.* 2013).

Early Tasmanian salmon farmed fish diets were largely fish based and were high protein/low energy (steam cooked pellets), as extrusion technology developed (Glencross *et al.* 2010) expanded pellet structure allowed the inclusion of higher oil levels which produced a marked growth increase, this technology was led by the cold water producers

(Norway/Scotland/Chile). Then, Tasmania began using extruded pellets around 1998 and energy levels rose into the early 2000's. More recently availability of fish oil/meal is more limited globally and thus has become more costly, therefore feeds have tended to have increased animal and vegetable replacements. From the early 2000's it was anecdotally noted that salmon summer growth was quite poor with high proportions of thin/failing fish and excess production of pseudofaeces (casts). By 2008 this was known as either 'irritable gut syndrome' or more popularly 'summer gut syndrome' (SGS), and became the subject of a three year commercial-in-confidence research effort.

The SGS problem was the focus of an industry report produced by CSIRO for Tassal Ltd and Skretting Australia, and including work done by members of the supervision team (Richard Taylor, Guy Abell and John Bowman) and coordinated by Brett Glencross. Information collated on SGS indicated it manifests in a segment of a given salmon population during summer, it can be correlated with poor growth performance (20% or more reduced in overall SGR), reduced feed intake (by up to 60% for the whole cohort), general inappetance and poor feed utilisation. There is a coincident increase in morbidity and mortality (up to 0.2%/day). Specimens impacted by SGS typically display poor condition, possess a reddened GI tract especially the hind gut and often produce pale yellow faecal casts (Glencross & Taylor, 2012). The situation seems to have become prominent during very warm summers, especially for the period of 2003-2007, when SGS was noticed for the first time, causing lowered harvest weight, delayed production schedules and reduced opportunity to fallow leases between year classes.

5.1.2 What are the pre-disposing factors associated with SGS and its connection to GI tract microbiology?

The aetiology of SGS is unknown; however it has been suggested to be a multi-factorial problem linked to the diet regime and environmental factors such as water temperature and water recirculation, especially when the water temperature exceeds 17ºC (Green *et al.* 2013). Other factors also suggested include the use of high energy feeds, reduced levels of fish oils and fish meal, overall gastrointestinal health, husbandry and farm management limitations (Glencross and Taylor 2012). Medication of fish showing poor feeding due to presumed SGS exhibited temporary improvement in feed intake after short term application of tylosin or oxytetracycline. This response presumed a connection to gastrointestinal microbiota though the association is not necessarily causal or even directly relevant. However, voluntary anorexia as well as the opposite condition bulimia in humans (Tennoune *et al.* 2014) have been recently tentatively connected to gut microbiota and could involve interaction of proteins of bacteria that are mimetic to alpha-melanocyte-stimulating hormone which is involved in food intake, body weight, anxiety, and melanocortin receptor signalling.

Studies of temperature stressed salmon in Norway also suggest a connection between poor feeding and the control of appetite via changes in the level of the neuropeptide ghrelin/obestatin (found in mammals, birds, fish, and amphibians) which regulates the feeling of hunger and directs utilisation of energy in the body (Hevrøy *et al.* 2012). Lack of ghrelin product leads to increased anxiety and social disconnection in mice and thus in salmon could result in poorer ability to cope in crowded pens. This leads to the interesting concept that SGS could involve a disruption of appetite control and general feeding behaviour and is induced by a combination of factors that could also incorporate the activity of bacteria. The

SGS condition also coincides with GI tract sloughing leading to faecal casts and reddened (inflamed) hind gut or in severe cases inflammation of the entire GI tract. Faecal casts readily produced by starving fish could also be linked to apparent GI tract irritation. Fish feed less at high temperature and are more prone to stress. It is logical to assume that these conditions would lead to a higher expression of casts (Ringø *et al.* 2014). The actual mechanism of cast formation and how it relates to diet, thermal stress, activity of microbes and possibly other unforeseen factors is unclear. Microarray analysis suggests connections to immune responses, lipid metabolism, stress responses and other levels of metabolism based on tissue samples from fish with presumed SGS (Glencross and Taylor 2012).

5.1.3 Current SGS mitigation strategies

Currently, there are no known simple specific or direct management practices beyond antimicrobial treatment and existing stress-reduction husbandry approaches, which includes importantly circulating water effectively reducing some of the thermal stress, cleaning bio-fouled nets, minimising predator interactions, and improved fish handling procedures. Veterinary approved antibiotic treatment is a last resort necessary for fish welfare, but is not favoured by the industry due to negative environment and product quality concerns. Management husbandry practices have successfully contained SGS since 2008 possibly partly due to the fact sea surface temperatures have been at or below the long term average. Control of SGS has otherwise has been controlled by optimised summer diets that contain a balanced digestible energy and fish meal levels. The application of high protein low lipid diets (50:20 ratios versus 45:25) seems to result in greater feeding during thermal stress (Glencross and Taylor 2012) though more experiments are desirable in more extreme summers. The application of other feed additives such as prebiotics, alternative oils, and

phytogenic compounds could have promise but is largely an empirical and expensive process of discovery of what works best.

5.1.4 Connection to gut microbiology

The understanding of salmon GI microbial communities can help improve knowledge on how to best maintain optimal salmon health, provide background information on better salmon farm management practices, and also contribute to understanding on whether fish diet formulation can be performed to increase growth performance. Although fish health depends upon the immune status of the fish, which is directed by multiple factors including nutrition, genetics, stress and environmental factors, the aquatic environment and their open GI tract system causes fish to be constantly exposed to a wide variety of microorganisms. Microbes may colonise fish internally and externally (Gómez and Balcázar 2008; Zarkasi *et al.* 2014). Moreover, GI tract bacteria clearly contributes to gut health by enhancing host nutrition absorption, digestion, metabolism and disease resistance (Guarner and Malagelada 2003). On the other hand, the colonisation of certain GI tract bacteria is also possible for numerous negative impacts to the host such as parasitism and infectious disease (Harris 1993).

5.2 Planned Outcomes - Contribution to the understanding of salmon GI tract microbiology within Tasmanian farm systems

Based on data generated in this thesis elevated levels of members of the family *Vibrionaceae* occurs within the salmon GI tract starting in the late spring and summer and persisting thereon in production. It can be hypothesized these bacteria by their sheer number

not due to any inherent virulence (Neuman *et al.* 2014) potentially reduce salmon GI health status though a mechanistic connection to the SGS condition is still to be made. Several *Vibrionaceae* species are associated with serious fish diseases (Austin and Zhang 2006; Higuera *et al.* 2013) though no evidence that these species are prevalent in farmed Tasmanian salmon occurs at any time based on data presented in this thesis. An increased level of lactic acid bacteria that are suggested by previous researches to have probiotic properties can potentially increase salmon productivity and improve their health status (Balcázar *et al.* 2006; Navarrete *et al.* 2013). The lowered population of this group coincident with increased *Vibrionaceae* was observed in this study following from the SGS report where this relationship was first observed. The connection of imbalance of microbiota in the salmon GI tract is a compelling possibility, however we still know very little of about the dynamics and heterogeneity of microbiota in fish GI tract systems and relationship to environment, diet and management practices. In order to control problems such as SGS more knowledge in this area is clearly required. In order to better direct the development of diet formulations as well as the impact of better management practices more fundamental knowledge is needed on salmon physiology and also their microbiome, the microbes that salmon host.

5.2.1 Characterisation of GI bacteria of Atlantic salmon.

The initial 13 month farm survey described in chapter 2 (and published as Zarkasi *et al.,* 2014) attempted to assess the relative impact of diet, environmental and farm factors, and provides an improved understanding of salmon GI tract bacterial community diversity dynamics. The study was performed within Tasmanian waters where surface water temperatures ranged between 9.2ºC to 19.9ºC, and generally exceeded 16ºC between December and early April. These temperatures would normally be conducive for SGS,

however due to the improved husbandry practices used SGS affected salmon populations were not overtly observed and growth performance was within commercial expectations. Nevertheless the study provided useful findings in relation to salmon gastrointestinal microbiology that can be summarised as follows:

i) Salmon GI tract bacterial communities are highly dynamic and community changes are acyclic, but largely similar between randomly selected fish.

ii) GI tract bacterial population changed over time through the growout cycle of the farmed salmon. These changes appeared to be driven by life stage (smolt to grower post marine stocking) and season. Gram-positive fermentative bacteria dominated in post-smolt (weight ~0.3kg), followed by predominance of members of the family of *Vibrionaceae* starting mid-summer and onwards. Towards the end of the survey, the relative proportion of *Vibrionaceae* declined towards harvest time, leading to prominence of other bacterial genera. Further studies of life stage and season effects would require further studies of different smolt types (input seasons) running through to harvest (1518 months post input).

iii) The differences observed between two commercial diet series (sourced from Skretting Australia and Ridleys Agriproducts) were minimal, likely because the protein: lipid ratios and digestible energy contents were largely similar.

iv) Multivariate statistics indicated the effect of salmon life stage and sampling season influencing the dynamism of hind guts microbial communities. Initial levels of LABs present in post-smolt in winter declined through summer but did not substantially come back in the second sea-winter. *Vibrionaceae* began to decline as temperatures declined in late autumn but there was no sign of LAB levels improving.

The overall data suggests a number of key findings. Salmon GI tract communities are extremely dynamic unlike the communities in mammalian systems that are compartmentalised and stable (Hatje *et al.* 2014). This sole finding means experiments require high replication and that systematic differences must take into account temporal effects (fish size, fish lifecycle and seasonal temperature). Another key fact is the predominance of *Vibrionaceae*. The most compelling reason is that these taxa are fast growing, bile tolerant and facultative anaerobes capable of readily using diet nutrients as shown in data presented in this thesis. Indeed *Vibrio* species are known to be amongst the fastest growing known bacteria under optimal conditions (10 min generation time for *V. natriegens*) (Eagon 1962). However, other bacteria such as *Mycoplasma* can become predominant in a fraction of specimens suggesting other factors are at play that we do not understand.

5.2.2 GI microbial community dynamics relation to digesta properties and diet.

The knowledge of how salmon GI tract bacterial communities are structured, their inherent heterogeneity and the influence of diet was deeply studied in chapter 3. This was done to determine whether community dynamics could be influenced by diet since SGS or the lack of it has been connected with diet energy and lipid content (Glencross and Taylor 2012). The digesta faecal consistency and appearance was also categorised as done in the SGS report (Glencross and Taylor 2012) generating the 5-point faecal score. This score was correlated to the microbial community present. Low scoring samples (considered relatively normal faeces) had communities much richer in anaerobes and slightly richer in lactic acid bacteria. Low score samples also were enriched in chloroplast 16S rRNA sequences suggesting digestion of plant meal in the diet. High scoring samples that essentially include some or mainly cast material had lower anaerobe and lactic acid bacterial abundances relative

abundances. High faecal scores are associated with poor feeding though the exact reason, beit hunger, maturation and physiological status, stress or the presence of a SGS condition was not established in this study. The surface water temperatures during the trials were always <17ºC and systematic SGS was never observed so we assume the data obtained could be normal within a typical salmon farming context. The faecal score digesta analysis is a potential tool to gain knowledge on fish that may be performing poorly in the population and the data could aid management of salmon production during future abnormally warm summer periods. A higher proportion of speciemens producing sustained high faecal scores would require intervention of some form before obvious issues of thining and mortality appear. The study's findings can be summarised as follows:

i) Results suggest that sampling time from spring to summer and then to early autumn, diet (within single cohorts of salmon) and digesta properties contribute to how faecal microbial communities are structured. Diet and time of sampling clearly interact suggesting diet specific transient shifts in the community occur.

ii) Although the experimental design lacked replicates to properly assess growth effect by diet, this initial study indicates that the feed formulation with a low protein: DE ratio (LoPro diet) gave the best growth performance outcome, and a low fish meal diet formulation gave the poorest performance.

iii) The overall data indicated a low protein: DE ratio diet have the lowest faecal score than any other diets though this was statistically marginal and specimen sexual maturity status showed a much greater correlation to faecal scores. This suggests that maturation affects feeding rates, which could explain the presence of some fish showing poorer performance. This study focused on non-maturing fish thus the

heterogeneity within the same salmon cohorts is another factor that must be kept in mind for future experiments.

iv) The most dynamic and strongly shifting community structure over time can be observed in "extreme" diets, relative to a commercial standard diet. There were more subtle differences between the extreme diets that were teased out from the effect of sampling time.

v) During the summer period the LoPro diet cohort and to a lesser extent all other cohorts with standard fish meal content had transient increases in GI tract community diversity mainly represented by an increased abundance of anaerobic (classes of *Bacteroidia* and *Clostridia*) and facultative anaerobic (lactic acid bacteria) taxa. The digesta had enriched populations of these groups in relation to cast-rich samples.

vi) The marine-derived, bile-tolerant marine facultative anaerobic bacteria genus *Aliivibrio* was dominant in the majority samples across all diet cohort faecal communities.

vii) High levels of aerobic bacteria of the genera *Sphingomonas*, *Methylobacterium*, and *Acidovorax* were observed. It was hypothesized these bacteria could have GI tract homeostatic roles such as removal of toxic compounds.

viii) A hypothesis was also developed that the faecal microbial community shifts could result in different levels of metabolite and/or immune system stimulation potentially influencing salmon physiology that is not observed straight away but is reflected in growth performance trends over time.

Much diversity data was obtained in this larger survey. The community was exhaustively analysed (217 samples, approximately 1500 OTUs classified) and carefully

classified avoiding the issues typically associated with pyrosequencing noise. A high proportion of bacteria could be identified to species level within the limitation of the sequence resolution. Heterogeneity was clearly observed within populations since sampling was more controlled than in chapter 2. Breaking the community down into functional subsets was an extremely useful process in better understanding the dynamics especially in relation to the concentration of anaerobes in the digesta and making the correlation of these bacteria with low (presumed healthy) faecal scores.

Secondly, the realisation that strictly marine taxa are likely just visitors passing through the salmon gut was made obvious by their greater predominance in the initial starved salmon at the beginning of the trial but declining to a base level of roughly 5%. This suggests colonisation in the GI tract may occur but only early on by microbes best suited for the GI tract conditions. This could be potentially exploited when transferring fish from freshwater to saltwater but would require exposure to bacteria that are able to maintain stable populations over time. Thus, the appearance of certain taxa predominating in the specimens is not always *Vibrionaceae*, which predominated in 60-70% of samples but also included other taxa such as *Mycoplasma*, *Bacteroides*, and *Brochothrix*. The results also imply that diets can induce a transient effect on the community whether this transience translates to growth performance later in time, due to delays from the metabolic and immunogenic effects of the transient populations, remains to be determined. Finally, the interesting discovery of aerobic bacteria being common was unexpected, and further work to determine their role and the determinants of their presence is an important future research direction.

Environmental sustainability is a prime metric of the Tasmanian salmon farming industry. Quite recently in the scientific literature there has emerged the desire to have a more ecological theory based approach to connecting biological facets to an end goal of

aquaculture production success and sustainability (Costa-Pierce 2008). This theory can be applied to managing gut biota to promote fish production (De Schryver and Vadstein 2014). Such conceptualisation is directed at controlling disease to prevent direct fish losses. More subtle conditions such as SGS have not been explored because growth loss is a more subtle effect, leading to reduced or delayed harvest production. In an ecological sense SGS has a more profound impact due to poor feed conversion, reduced lease fallowing opportunity and animal welfare considerations. Ecologically oriented ideas have been discussed in a recent review (De Schryver and Vadstein 2014) where 'join them' and not the traditional 'beat them'" microbial management strategies for aquaculture are seen to be "the future". Interestingly many of the ideas of that review are the same as the concepts explored in this thesis. However, the authors fail to give credence to fact that physiological aspect of the host and the interaction with the microbiome present complicates the picture enormously. SGS is presumed to be the manifestation of a dysfunctional interaction. The host biology aspect is not addressed in this thesis but certainly will play an increasingly important role in terms of making future progress in solving complex management issues in aquaculture.

5.2.3 *In vitro* characteristics of dynamic salmon GI microbial community.

The final research performed in the thesis explored whether we can predict the effects of diet formulations on salmon GI microbial communities using a simple *in-vitro* system. Manipulating microbiota is a strategy that could prevent pathogenic infection and improve fish nutrition. An *in vitro* model could be useful in testing diet formulations initially to see if there are able to significantly influence GI tract community structure and offers a rapid, cost effective method to screen diets or ingredients. The effect of diets in this system was

examined using ARISA fingerprinting and Illumina MiSeq next generation sequencing of 16S rRNA gene amplicons. The results included the following outcomes:

i) *In-vitro* growth characteristics of salmon GI microbial communities indicated the communities were highly dynamic between samples growing to a plateau in 24 h, indicating that feeds have distinct short term effects on bacterial communities that are sourced in season from local marine farmed salmon.

ii) In a warm summer (20°C) model, members of the family *Vibrionaceae* always became predominant *in vitro*, including *Aliivibrio finisterrensis, A. fischeri, Vibrio tasmaniensis*, unclassified *Vibrio* spp. and *Photobacterium* spp. This was also shown by plate counts since colony numbers on TCBS agar was higher than marine agar. The reasons for this dominance could be due to the faecal inocula having high levels of *Vibrionaceae* and the high salt content of the media.

iii) Statistical analysis confirmed that fish meal and oil content of different diet formulations were factors in determining bacterial communities within the in-vitro system though the effects were quantitative than qualitative.

iv) The testing of lupin kernel meal and *Pisum sativa* (pea) meal resulted clear separation from the communities arising from the other complete diets indicating the strong influence of lipid content. Only strains of the largely aerobic genus *Sphingomonas* was observed suggesting the plant meals do not support much, if any, bacterial growth without some form of enzymatic or chemical pre-processing.

The experiments though preliminary provide a basis to test diet formulations to support bacterial populations that may have desirable GI tract homeostatic traits. This would

allow testing of supplements, such as prebiotics that could be added that may support growth of LABs since degradation of oligosaccharide prebiotics is more common amongst that group of bacteria. Addition of salmon-associated LABs with probiotic potential (Neuman *et al.*, 2015) and determining if their populations are stable would be another useful line of investigation. Previous studies have described the potential of lactic acid bacteria as probionts in the fish. In early research there was found substantial evidence that lactic acid bacteria provided a significant protection against pathogenic bacteria in fish (Gatesoupe, 1994). The possible involvement of lactic acid bacteria as probiotics in aquaculture is discussed by Ringø & Gatesoupe (1998). Since then many researches have studied the potential of LABs in aquacultural systems. According to Balcazar *et al.,* (2006), Korsnes *et al.,* (2006) and Verschuere *et al.,* (2000), lactic acid bacteria (*Pediococcus*, *Lactobacillus* and *Carnobacterium*) have been proposed as biological control agents in aquaculture. Moreover, lactic acid bacterial potential could be different from the human since the human gastrointestinal tract is more complex than the open system in fish. We do not however yet understand whether metabolism in LABs actually has any impact on fish growth performance. A focus on LABs is however simplistic and studies should also examine major groups of bacteria (such as strict anaerobes) that transit the salmon GI tract since they may have unexpected benefits. Limitations of time and budget prevented these experiments being completed within this thesis but the system set-up, implementation and analysis can be performed quite inexpensively now that the basic concept has been ground truthed.

5.3 Further research development

5.3.1 How could we create healthy salmon GI tract communities?

Salmon farmers strive to sustainably produce a consistent high quality product in a dynamic production system. Environmental conditions, husbandry, pathogens and nutrition all affect fish performance in short (seconds to days) or long (weeks to seasons) time frames. Producers must manage these potential stressors to maintain homeostasis and optimise fish performance. Management of intestinal microbiota to support fish health and functionality is currently in its infancy but offers a holistic and sustainable option for the aquaculture industry. Understanding of fish gut health is particularly important because fish live in an aquatic environment characterised by high organic loads supporting microbial growth (Vadstein *et al.* 2004). The information of salmon GI tract microbial communities from this thesis provides a framework that will assist the Tasmanian salmon industry in managing gut health to promote sustainable salmon production. An understanding of 'healthy' or 'optimal' microbial balance for fish at a particular life-stage or season is the basis to cost effectively manages the microbiota using diet manipulation or addition of probiotics.

The rationale that diet can be used to straight forwardly select and promote the lactic acid bacterial population is questionable based on the findings here. However, the experiments need to be performed using faecal samples richer in non-*Vibrionaceae* bacteria, such as lactic acid bacteria and strict anaerobes. There is also the need to consider more environmental variables when applying live feed additives (probiotics). For example, commercial probiotic cultures used for livestock and poultry are likely unsuitable for salmon due to it possessing high temperature growth optima (e.g. *Pediococcus acidilactici* 35ºC)

despite its ability to grow down to 6ºC. The growth rate and bacteriocin synthesis reduction between 35ºC and 15-20ºC (Zhang *et al.* 2012) could be sufficient for the microbe to cease to be competitive in a salmonid GI tract. The species is though appropriate for avian cultivation (Mikulski *et al.* 2012). The dynamism in the GI tract is also so great and the fact conditions favour the growth of *Vibrionaceae* and other bacteria that the reliable success of an off-the-shelf probiotic or simple traditional diet-level manipulation is not likely to succeed without a new strategy being utilised.

In this respect it is proposed inoculation with probiotics is likely time critical and should instead juxtapose with immersion-based vaccination, which is commonly used in the Tasmanian industry to prevent enteric red mouth disease (ERM) caused by atypical biotypes of *Yersinia ruckeri* (Bridle *et al.* 2012) prior to transfer of fish from freshwater to saltwater. Smoltification and marine transfer is likely the time when salmon are most open to colonisation and are also subject to considerable stress. Rather than passive colonisation from external seawater microbes salmon could be bathed in water that contains an inoculating culture that is able to not only successfully colonise the salmon gut, but is also able to persist there. It is likely the culture will need to be a consortium not a single species since the probiotic traits are unlikely contained in a single strain. Obviously the inoculating consortium would need to possess beneficial GI tract homeostatic properties. At this stage none have been definitively identified. Another means of inoculation could be microbial transplantation (Rawls *et al.,* 2006) where gut microbes are incorporated in the diet itself in freeze dried form. This approach is already seen as more palatable approach to faecal transplant therapy in humans, which is being used more frequently to solve GI tract dysfunction issues, following antibiotic disruption of the GI tract microbial community (Youngster *et al.* 2014). Some initial research by others including work in Tasmania (Neuman *et al.,* 2015) have identified

possible probiotic candidates isolated from the salmon GI tract that possess adhesive qualities, no cytotoxicity to salmonid tissue culture cells, and ability to exclude several different pathogens via production of bacteriocins. Such putative probionts would need to be assessed in experimental farm experiments but overall there is potential promise here.

It is recognised the focus of this thesis is biased towards community structure rather than function. To obtain greater clarity about the role of GI tract microbiota additional research in the area of proteomics, metabolic pathways and processes, metabolites and metabolomics is also required. Such knowledge will inform feed-oriented and salmon physiology research. To obtaine successful outcomes from new approaches such as microbiota transplantation (Rawls *et al.,* 2006) substantial investment into new research is clearly required.

5.3.2 Are diets the way to promote appropriate GI tract communities?

Gross variation of protein to energy ratio offers the most pragmatic way to vary seasonal diet options as studied in chapter 3 and chapter 4. Modified energy ratios could be used in future project to better understand it potential in salmon health and productivity since we found it generated the strongest impact on microbial communities and at least within the limitations of the trial experiments. However, with such studies reproducibility is critical and thus further experiments are needed to determine if modifications to diet yield consistent outcomes. For this work to be furthered, a set of replicated research tanks or trial cages is required so that impacts upon fish production can be effectively measured in tandem with gut microbiota studies. To avoid temporal bias in faecal sampling it is preferable that these trial units are not grouped around a central walkway. In that respect extensive research is still needed on whether diet re-formulation and controlling GI tract microbiota assembly (as

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advocated by De Schryver & Vadstein, 2014) can face the challenge of changing environmental factors and economic considerations.

Building up more information about what is normal for farmed salmon GI tract bacteria and the relationship with environmental factors and diet formulation would be useful for developing better management approaches. We still are not certain if geography plays a strong role in what microbes predominate and what the inherent differences are. Would one strategy work in once place but not somewhere else because of this? It is presumed that largely the communities are similar since the types of microbes found in the studies are generally familiar or at least described recently, such as *Aliivibrio finisterrensis*. Future research could include comparison with other salmon farms located in different geographical locations such as in other parts of Tasmania and in Norway, Chile or Scotland. This will connect the rather piecemeal studies done to data (Holben *et al.* 2002; Hovda *et al.* 2007; Skrodenyte-Arbaciauskiene *et al.* 2008; Hatje *et al.* 2014; Zarkasi *et al.* 2014) into something more informative. More data on wild salmon would be also useful especially in regards to what might be indigenous stably present species that could be tapped as a probiotic resource.

Ideally there is the desire to do more fish trial research where different strategies of GI tract manipulation can be performed utilising novel dietary approaches and salmon-sourced probiotics agents and to achieve a stabilised gut microbiota populations that can help fish withstand better summer conditions. The role of possible detoxifying bacteria as mentioned above is another interesting angle forming the concept the probiotic approach will involve a culture that could contain several microbes all with roles to play in GI tract homeostasis and health. The research could proceed with the option of using a model gastric system (using a mechanical bioreactor system) or the simple version explored here in chapter 4. Such an approach would be useful testing to examine whether *Vibrionaceae* that occur

potentially produce protein products (virulence factors) that may stress fish in vivo. *In vitro* testing determining efficacy in salmon tissue culture, biological aspects such as pathogen exclusion and adhesion followed by establishing whether the strain has potential persistence potential in a GI tract system also offers the option of selecting probiotic agents efficiently rather than with expensive and empirical fish feeding trials. In conclusion, the improved knowledge of salmon GI tract microbial communities provides the salmon industry new options in developing sustainable practices in a changing environment and economy.

Many studies have demonstrated inter-individual phenotypic variation of gut microbiota in salmonids (Navarrete *et al.* 2012; Boutin *et al.* 2014). It is therefore likely that the host environment plays a part in shaping the microbiota. The host genotype is expected to influence inter-individual variation in the intestinal microbiota (Rawls *et al.* 2006; Navarrete *et al.* 2013) or external microbiota (Boutin *et al.* 2014), suggesting that genetic selection may be a long-term approach to altering microbial communities inhabiting Tasmanian salmon. The Tasmanian industry has developed a selective breeding program since 2004, this program selects for a number of commercial traits (Elliott and Kube 2009; Kube *et al.* 2012) based upon marine performance of fish of known pedigree. Although microbial characterisation of large numbers of individuals may be impractical, a focussed study of microbiota in pedigreed individuals characterised by their summer growth performance in different nutritional environments may be a useful first step in assessing the likely success of this approach.

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Appendix A

## Relative major abundance of bacterial species in the salmon GI tract (Chapter 2)

Figure A1: Relative major abundance of bacterial species in the gastrointestinal tract of Atlantic salmon from five different seasons; a) first winter; b) spring; c) summer; d) autumn; e) second winter. Species level identification is based on bacterial number of reads of 454 pyrosequencing analysis.
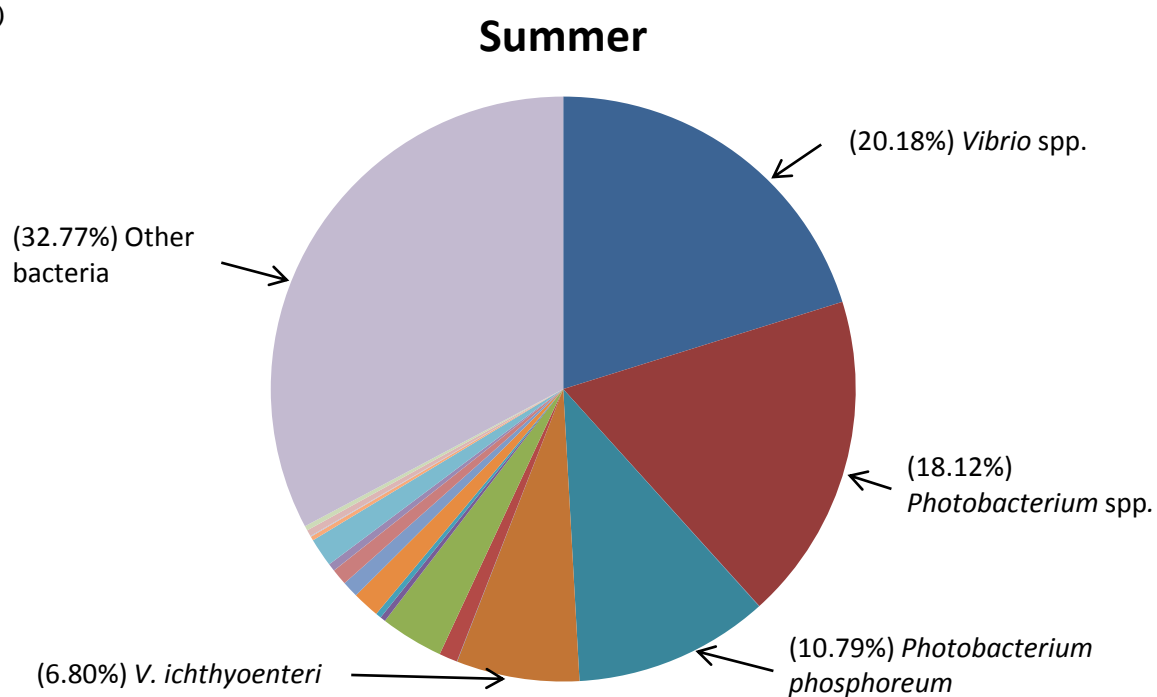
a)
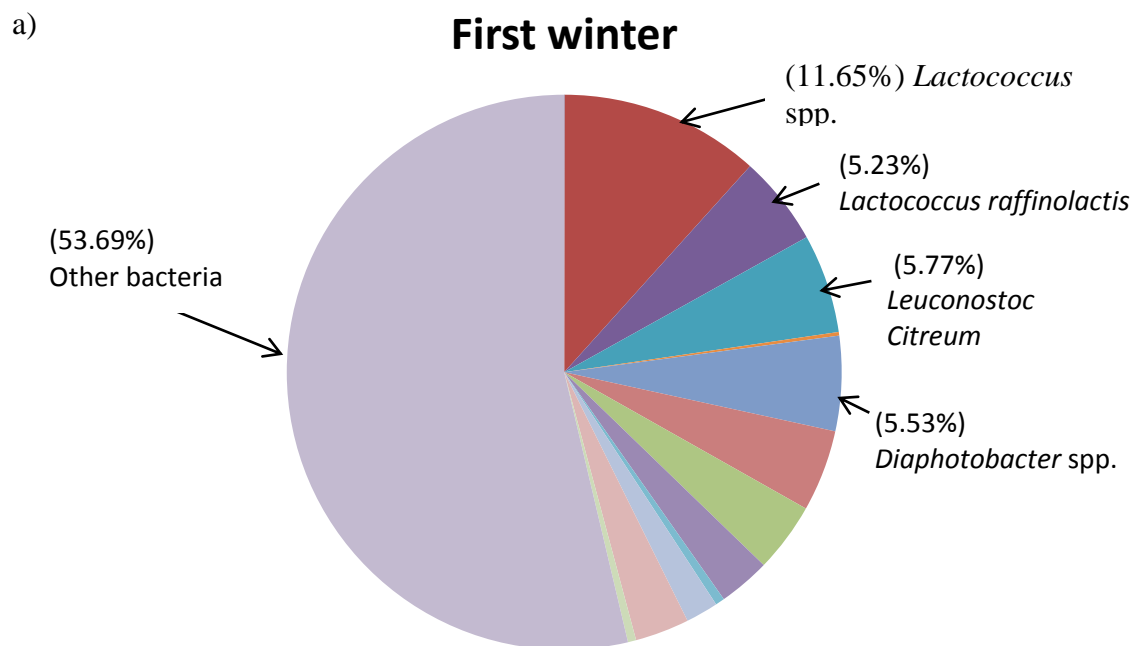
The most abundant microbes in first winter identified at species level, for both diet groups were *Lactococcus* spp., *Lactococcus raffinolactis*, *Leuconostoc citreum* and *Diaphorobacter* spp.
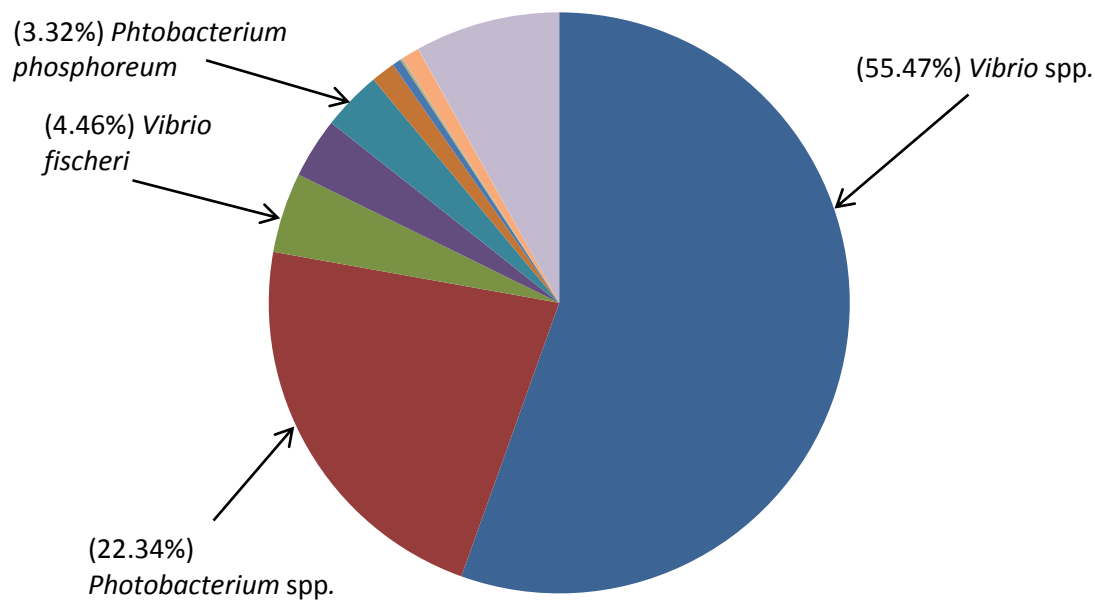
b)
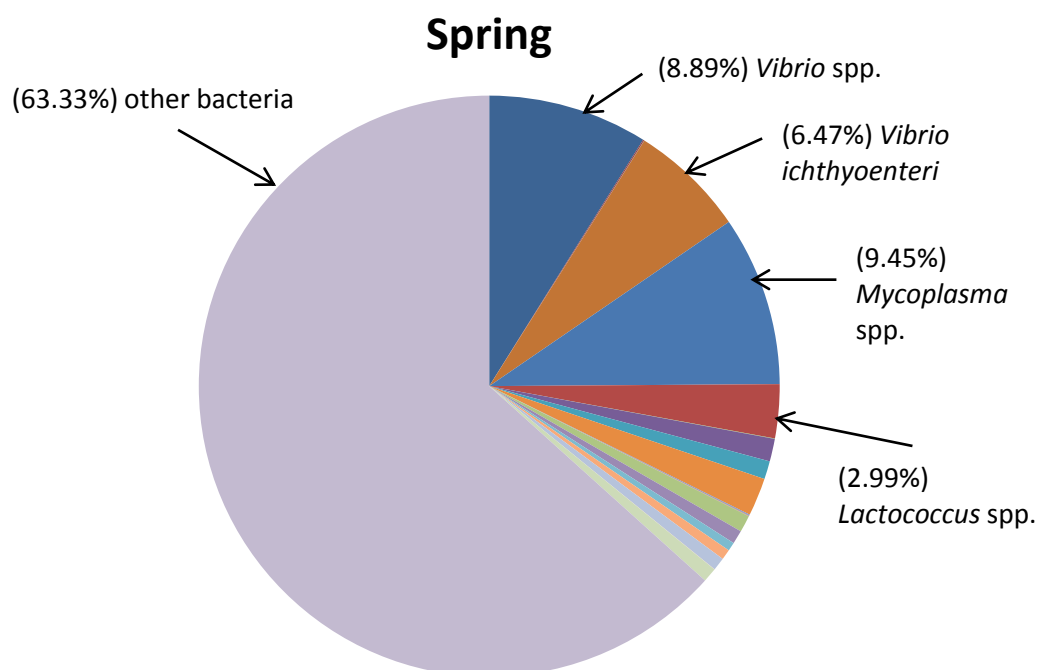
Spring

The most abundant microbes in spring identified at species level, for both diet groups were *Vibrio* spp., *Lactococcus* spp., *Mycoplasma* spp., and *Vibrio ichthyoenteri.*

c)

Summer

The most abundant microbes in spring identified at species level were *Vibrio* spp., *Photobacterium* spp., *P. phosphoreum* and *V. ichthyoenteri.*

d)

Autumn

(3.32%) *Phtobacterium phosphoreum*

(4.46%) *Vibrio fischeri*

(55.47%) *Vibrio* spp*.*

(22.34%) *Photobacterium* spp*.*

The most abundant microbes in autumn identified at species level were *Vibrio* spp.,
Photobacterium spp., *Vibrio fischeri* and *Photobacterium phosphoreum.*

e)

Second winter

(21.65%) *Vibrio* spp.

(5.87%) *P. phosphoreum*

(5.99%) *Photobacterium* spp.

(17.31%) *Pseudomonas* spp.

(29.20%) *Vibrio fischeri*
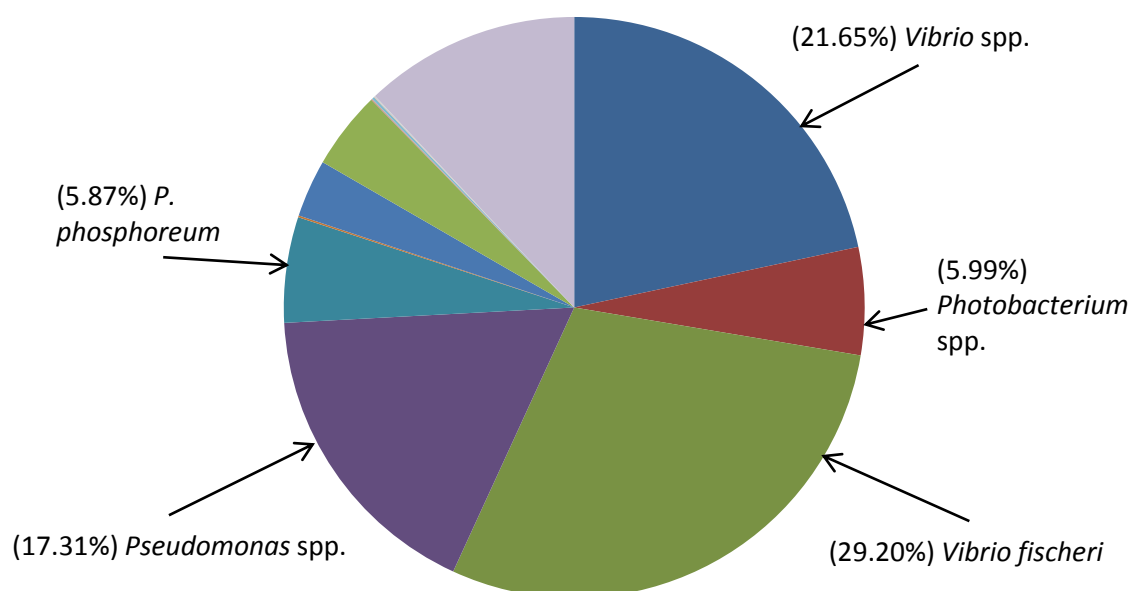
The most abundant microbes in autumn identified at species level were *Vibrio* spp.,
Pseudomonas spp., *Vibrio fischeri*, *Photobacterium* spp., and *Photobacterium phosphoreum.*