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Fishing for the right probiotic: Investigating the mechanism of mucosal-bacterial interactions at the interface of health and productivity in salmonid aquaculture

Luana Langlois, *The University of Western Ontario*

Supervisor: Reid, Gregor, *The University of Western Ontario*

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Abstract

Aquaculture is vital for the global food supply, but the high incidence of infectious diseases threatens the industry's productivity. The intestinal mucosa is a key port of entry for pathogens and provides an extensive interface for host-microbe interactions. Tight junctions are at the core of gut barrier function and the mucosal health of finfish. Disruption of these complexes gives rise to sepsis, which leads to systemic inflammation and death. The present study employs a combinatorial approach that integrates *in vitro* and *in vivo* analyses to gain actionable insights into the mechanism of microbial-mediated modulation of host health. The experiments outlined in chapters 2 and 3 examine the suitability of several candidate probiotics for promoting gut barrier function, immunity, and mitigating the deleterious effects of the highly virulent aquatic pathogen *Vibrio anguillarum*. These studies demonstrate the importance of investigating the mechanism underlying host-microbe and microbe-microbe interactions for maximizing salmonid health.

Keywords

Host-microbe interactions, aquaculture, salmonids, *Oncorhynchus*, probiotics, lactic acid bacteria, infectious diseases, immune modulation, gut barrier function, tight junctions.

Summary for lay audience

The aquaculture industry, which refers to the farming of aquatic species, is rapidly growing and its production has far surpassed that of capture hatcheries. Given that the human population will continue to increase, aquaculture must intensify its practices in the coming years to ensure the security of the global food supply. However, there are several interdependent factors that negatively impact the industry's ability to meet these growing demands. These include competition for natural resources, climate change, and infectious diseases. The latter has become a major limiting factor for further intensification of production, and despite great developments over the past three decades, viral and bacterial infections continue to cause multibillion-dollar losses for the industry every year. Microbes are ubiquitous both within and around fish. In addition to its role in digestion, the intestinal mucosa is home to the richest and most abundant community of resident microbes that can interact with the host cells and modulate health. This interface is also a target site for the onset of infection, but supplementation of probiotic bacteria has reportedly improved disease resistance and overall physiology in several fish species. In this thesis, the concept of using beneficial microbes for the improvement of host health by interacting with the intestinal epithelium was explored with the focus on salmonid species, which are the most economically important family of finfish. By using a cell-based laboratory model of the salmonid intestine, it was found that the candidate fish probiotics could protect the cells from the highly virulent fish pathogen *Vibrio anguillarum*, but the beneficial microbes had no effect on the expression of key gut barrier and immune molecules. To investigate these concepts in live animals, a disease trial was carried out at a fish farm in British Columbia. Chinook salmon were fed with

diets containing different probiotic strains and were then infected with the pathogen *V. anguillarum*. It was found that probiotic supplementation had no effect on the survival, growth, or gene expression of immune or gut barrier molecules in the intestinal tissue. This project established the use fish cell lines for the investigation of host-microbe interactions and represents a steppingstone to guide future researchers on the study of more suitable candidate fish probiotic strains.

Co-authorship statement

The experiments and data analyses herein presented were primarily conceived, executed, and analyzed by Luana Langlois with supervision and guidance from Gregor Reid. The manuscripts presented were written primarily by Luana Langlois with input from the co-authors.

Chapter 1: General introduction

Luana Langlois conceptualized the review, drafted the manuscript, and generated the figures, under the supervision of Gregor Reid. Luana Langlois and Nadeem Akhtar conducted the literature review and Nadeem Akhtar wrote some sections of the manuscript. Luana Langlois, Nadeem Akhtar, Kam Tam, Brian Dixon, and Gregor Reid were all involved in reviewing and revising the article.

Chapter 2: Establishment of an *in vitro* model of the salmonid gut for the investigation of host-microbe interactions

Luana Langlois and Gregor Reid conceptualized the experiments. Luana Langlois performed the experiments, collected and analyzed data, generated figures, and drafted the manuscript. Eric Patterson and Gediminas Cepisnkas provided technical support and expertise with the transepithelial electrical resistance (TEER) assay. Brian Dixon provided technical expertise with the maintenance of the salmonid intestinal cell line RTgutGC. All authors read, revised, and approved the final manuscript.

Chapter 3: Chinook salmon field study: Investigating the effects of probiotic supplementation in the context of a pathogenic infection with *Vibrio anguillarum*

Manuel Davila, Luana Langlois, Maureen Latimer, Brian Dixon, and Gregor Reid conceptualized the experiments. Technical support with animal husbandry was provided by John, Ann, Earl, and George Heath. Luana Langlois, Maureen Latimer, Gillian McDonald and Manuel Davila performed the experiment and collected data. Luana Langlois analyzed the growth and survival data. Gillian McDonald, Maureen Latimer, and Manuel Davila dissected hindgut tissues and Luana Langlois performed RNA extractions and gene expression analyses.

Epigraph

'Oh, the depth of the riches both of the wisdom and knowledge of God!

How unsearchable are His judgments and His ways past finding out!

“For who has known the mind of the LORD?

Or who has become His counselor?”

“Or who has first given to Him, and it shall be repaid to Him?”

For *of* [Christ] and *through* [Christ] and *to* [Christ] are all things, to whom be glory forever. Amen.'

Romans 11:33-36 NKJV

Dedication

For Mom, Dad, Brother, and Matt.

I love you more than words can say.

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List of abbreviations

LT	Long-term
ST	Short-term
IP	Intraperitoneal
ID	Intradermal
RT	Rainbow trout
SOD	Superoxide dismutase
HK	Head kidney
dpi	Days post-infection
GI	Gastrointestinal
FAO	Food and Agriculture Organization
MRS	De Man, Rogosa, and Sharpe (media)
LAB	Lactic acid bacteria
FW	Freshwater
SW	Saltwater
GF	Germ-free
GALT	Gut-associated lymphoid tissue
MALT	Mucosal-associated lymphoid tissue
CFU	Colony-forming units
RT-qPCR	Real-time quantitative polymerase chain reaction
mRNA	Messenger ribonucleic acid
cDNA	Complementary deoxyribonucleic acid
TJ	Tight junction
ZO-1	Zonula occludens 1
SCFA	Short-chain fatty acid
RTgutGC	Rainbow trout intestinal epithelial cell line
MAMP-PRR	Microorganism-associated microbial pattern-pattern recognition receptor
QS	Quorum sensing
QQ	Quorum quenching
AHLs	Acylated homoserine lactones
NGS	Next-generation sequencing

ATCC	American Type Culture Collection
BHI	Brain heart infusion (media)
L-15	Leibovitz's 15 (tissue culture media)
TEER	Transepithelial electrical resistance
YIAL	Yellow Island Aquaculture Ltd.
O_2	Oxygen
CO_2	Carbon dioxide
$CaCl_2$	Calcium chloride
NaCl	Sodium chloride
PCR	Polymerase chain reaction

Chapter 1

1. General introduction

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Langlois L, Akhtar N, Tam CK, Dixon B, Reid G. Fishing for the right probiotic: Host-microbe interactions at the interface of effective aquaculture strategies. 2021. FEMS Microbiology Reviews.

1.1 Abstract

Effective aquaculture management strategies are paramount to global food security. Growing demands stimulate the intensification of production and create the need for practices that are both economically viable and environmentally sustainable. Importantly, pathogenic microbes continue to be detrimental to fish growth and survival. In terms of host health, the intestinal mucosa and its associated consortium of microbes have a critical role in modulating fitness and present an attractive opportunity to promote health at this interface. In light of this, the administration of probiotic microorganisms is being considered as a means to restore and sustain health in fish. Current evidence suggests that certain probiotic strains might be able to augment immunity, enhance growth rate, and protect against infection in salmonids, the most economically important family of farmed finfish. This review affirms the relevance of host-microbe interactions in salmonids in light of emerging evidence, with an emphasis on intestinal health. In

addition, the current understanding of the mode of action of probiotics in salmonid fish is discussed, along with delivery systems that can effectively carry the living microbes.

1.2 Introduction

Aquaculture refers to the farming of aquatic species, including finfish, shellfish, crustaceans, and plants, primarily for human consumption. This is the fastest-growing sector in the livestock industry and is responsible for the production of over 50% of the fish consumed worldwide (FAO 2020). A conservative estimate by the United Nations' Food and Agriculture Organization (FAO) is that an additional 40 million tonnes of fish protein will be needed to feed the human population by 2030 (FAO 2020). As such, aquaculture production must be intensified to meet growing global demands and prevent irreversible losses in biodiversity due to overfishing of wild populations. It is, therefore, essential that the practices employed are environmentally safe and sustainable, while remaining economically viable.

Salmonids, such as trout and salmon species, represent the largest single fish commodity by value in the aquaculture world trade (FAO 2020). The salmonid market is currently dominated by farming of Atlantic salmon (*Salmo salar*), followed by rainbow trout (*Oncorhynchus mykiss*). Other members of the Salmonidae family include other trout and char (*Salvelinus* sp.) species, as well as Pacific species, such as Chinook salmon (*Oncorhynchus tshawytscha*) (FAO 2020). Salmonids attract high market values because they are a stable source of quality fish protein, as well as a versatile raw material that can be processed into a wide range of food products (Merrifield *et al.* 2010). These features have amplified the interest in commercial farming, which greatly increased global production of these species in the last 20 years (FAO 2020).

To date, scientific research has largely focused on the genetics of elite broodstock management, nutrition, and the health of salmonids. Challenges remain, especially as they pertain to infectious diseases that afflict these fish. The high incidence of infectious diseases creates a substantial financial burden and instability for the farmers. With losses that surpass US$6 billion annually worldwide, aquaculturists are left with limited options to prevent and combat outbreaks (Brummett et al., 2014). Current strategies consist primarily of prophylactic antibiotic administration; however, their use is highly discouraged due to the rise in multidrug-resistant bacterial strains (Verschuere et al., 2000; Watts et al., 2017; Santos and Ramos, 2018).

In addition, antibiotics disrupt the microbiota of fish, and this in turn can increase the susceptibility to secondary infections (He *et al.* 2017). For example, administration of oxytetracycline has been shown to cause dysbiosis in the intestinal microbiota of juvenile Atlantic salmon (Navarrete *et al.* 2008). Similarly, in zebrafish, sub-therapeutic doses of olaquindox, an antibiotic growth promoter commonly used in aquaculture, led to a trend of higher mortality after *Aeromonas hydrophila* challenge. This phenomenon was associated with a profound shift in the microbiota composition of the antibiotic-treated group from a Fusobacteria- to a Proteobacteria-dominated community, which suggests that antibiotic-induced disruptions in the gut microbiota can increase the susceptibility to secondary infections (He *et al.* 2017).

Vaccination is also widely used, albeit with limited efficacy. The under-characterization of finfish immunology makes vaccine design challenging; the immature adaptive immune response in juvenile finfish makes them unsuitable for vaccination, leading to high mortality rates due to infections; and vast host genetic variability

contributes to poor protection between fish families (Dixon 2012; Pérez-Sánchez, Mora-Sánchez and Balcázar 2018; Figueroa *et al.* 2020). These sub-optimal outcomes have fuelled an interest in alternative eco-friendly strategies, such as the use of microbial-based therapeutics, that can mitigate losses due to infectious diseases and improve the value chain of fish production.

The intestinal mucosa and microbiota are critical to host health, locally and at distant sites. These findings are also relevant to fish species, given that nutrient absorption, immunity, and prevention of infection also occur at this interface (Merrifield et al., 2010; Jutfelt 2011). Likewise, high-quality animal nutrition is vital for growth, immune competence, disease resistance, and stress mitigation (Ringø *et al.* 2016). Approximately half of the costs associated with aquaculture are directly related to feed and nutrition. Thus, strategies that maximize feed conversion rates and prevent additional costs related to infectious disease outbreaks have become an industry priority (FAO 2018).

There is a growing trend towards the use of dietary supplements that can promote health and limit pathogens at the gastrointestinal interface. Given the importance of microbes in the gut, the concept of supplementing beneficial ones (probiotics) is being considered. Probiotics are 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host' (Hill *et al.* 2014). In fish, attempts at using probiotics have been reported to promote growth, improve feed conversion rates, enhance immune function and disease resistance, and counter pathogens (Gram *et al.* 1999; Wang, Li and Lin 2008; Zorriehzahra *et al.* 2016).

The purpose of this review is to discuss the impact of host-microbe interactions in salmonids, particularly those associated with intestinal health. This will also include an overview of the different mechanisms of action of probiotics in salmonids, and a survey of vehicles for their delivery in aquaculture systems. Although several reviews have addressed some of the aspects herein discussed, to our knowledge, none have endeavoured to integrate the host-microbe interactions specifically in farmed salmonids, as well as key knowledge gaps to be addressed in the field. The aquaculture industry stands at a new frontier of sustainable intensification of production and an amplified interest in alternative rearing systems, such as Norway's land fish farms (Gibson 2021). This article is particularly timely to inform research and development of salmonid production and contribute to the optimization of aquaculture strategies through microbial-mediated modulation of host health.

1.1.1 Salmonid intestinal microbiome

The microbial ecosystems inhabiting all mucosal surfaces in fish have a key role in the modulation of host fitness. Studies have shown that microbial counterparts vastly outnumber their host cells, and the metabolic capacity of this community facilitates processes vital for host health and homeostasis (Qin *et al.* 2010). Recent efforts have examined the taxonomical composition of the intestinal microbiota of some fish species, its functional stability over time, and the response to physiological stimuli, such as stressors, infectious diseases, and nutrition (Wang *et al.* 2018a).

Using next-generation sequencing (NGS) technologies and high-throughput analyses, the composition and function of the microbiota can be identified. Early efforts have revealed the presence of a strikingly similar 'core microbiota' comprised mainly of

γ-Proteobacteria and Fusobacteria in zebrafish shared between laboratory-reared and wild-caught fish (Roeselers *et al.* 2011). This suggests that laboratory studies could be relevant to fish in the wild in terms of their microbiome, and that host selective pressures might play a central role in determining the composition of this microbial community in fish. Although the gut microbiota of Atlantic salmon along disparate sampling sites share 80% of their composition, there are still substantial differences between the intestinal mucosa and digesta, which might suggest that spatial microbe groupings have evolved to exert different functions due to their proximity with the epithelial interface (Gajardo et al., 2016). The phyla Proteobacteria and Firmicutes were found to be present in highest abundance in the gut, primarily represented by members of the *Pseudomonas*, *Acinetobacter*, *Microbacterium*, *Janthinobacterium*, and *Burkholderia* genera (Gajardo et al., 2016).

More recently, the microbial composition in the water, feces, and feed were compared for Chinook salmon in a seawater recirculation aquaculture system (Steiner *et al.* 2021). The study found overlaps between microbes in all three ecosystems, with the feces having the highest microbial abundance and richness. The core microbiota in the feces was comprised mainly of the phyla *Firmicutes*, *Proteobacteria*, *Bacteriodetes*, and *Actinobacteria* which is in line with other reports in salmonids (Gajardo et al., 2016; Al-Hisnawi et al., 2019). However, without distinguishing 'healthy' versus 'dysbiosis' states, it will be difficult to develop tools to rapidly diagnose and mitigate the uncontrolled expansion of pathobionts in the fish gut.

Lactic acid bacteria (LAB), known to be associated with health in humans and other species (Trinder et al., 2015; Puebla-Barragan and Reid, 2019), are also common

residents of the salmonid gut (Gajardo et al., 2016; Al-Hisnawi et al., 2019). Interestingly, an increased abundance of LAB, such as *Leuconostoc* and *Weissella,* in the distal gut of salmon is associated with plant-based diets and is, at least in part, affected by seasonal changes (Zarkasi et al., 2014; Gajardo et al., 2016). In winter, LAB are more abundant, whereas members of the Vibrionaceae and Pseudomonaceae families, which contain pathogens able to cause opportunistic infections, increase in abundance in warmer months (Hatje et al., 2014; Zarkasi et al., 2014, 2016; Gajardo et al., 2016). Temperature has a fundamental effect on bacterial growth as well as host physiology. Therefore, as the water temperature changes, some taxa lose competitiveness and their depletion allows others to take their place (Zarkasi *et al.* 2014). This provides an opportunity to test the concept of seasonal supplementation of probiotic organisms that counter these pathogens by administering them just prior to summer.

As anadromous fish, salmonids experience significant habitat transitions, which is reflected by considerable changes in the composition of the intestinal microbiota. The intestine of saltwater (SW) Atlantic salmon has a greater abundance of Firmicutes, as well as increased bacterial load, higher alpha diversity, and lower beta diversity compared to freshwater (FW) fish (Rudi *et al.* 2018). In spite of these differences, several reports point towards the presence of a 'core' gut microbiota shared among different conditions, such as FW to SW transition, diets, rearing densities, geographic location, and wild *vs.* captive-reared fish (Sullam *et al.* 2012; Wong *et al.* 2013; Zarkasi *et al.* 2014, 2016; Gajardo *et al.* 2016; Llewellyn *et al.* 2016; Rudi *et al.* 2018; Steiner *et al.* 2021). These core microbe assemblages are highly adapted to the salmonid intestinal ecosystem and occupy ecological niches in a symbiotic relationship with their hosts. The host

provides shelter and nutrients, and the microbes facilitate key metabolic processes and help reduce infection. The remarkable adaptability of this core microbiota points to a functional niche that these microbes have evolved to fulfill by being in close association with the host. The administration of antibiotics, whether prophylactically or as a treatment, disrupts this core community causing a significant rearrangement of the ecological composition and function. Ironically, a consequence is that nutritional and ecological niches harbouring beneficial microbes are vacated due to antibiotics and invariably filled by pathobionts (Gatesoupe 2008).

The high-resolution characterization of the salmonid gut microbiota and the mapping of factors that affect this community provide important insights into the relationships between host and microbe. However, the functional significance of these changes for host health remains largely unclear. As such, multi-omics studies are needed to identify which microbial associations equate with health-promoting metabolic function in fish and how detrimental associations can be manipulated (Uengwetwanit *et al.* 2020). Furthermore, shotgun deep-sequencing and metabolomic analyses can provide a more comprehensive framework of the taxonomy and metabolic potential of the salmonid microbiome, thus paving the way for actionable insights derived from such analyses.

1.1.2 Intestinal mucosa at the interface of fish health

The intestinal mucosa was once regarded simply as a digestive organ. In fish, it has been proposed that differences in immune competence are related to the ability to prevent pathogen attachment and invasion at mucosal tissues (Palaksha et al., 2008; Rajan et al., 2011; Gao et al., 2016). It is estimated that the distal intestine harbours between 10^7-10^8 CFU/g of intestinal content, which constitutes the most prominent

microbial community in terms of richness and abundance (Austin 2006; Gómez and Balcázar 2008).

The continual exposure to commensal and transient microbes at mucosal surfaces creates the need for mechanisms that provide broad-range protection for the host. In the gut, a layer of mucus offers the first line of defence. It contains several antimicrobial and protective compounds, including antibodies, complement components, enzymes, antimicrobial peptides, and mucins (Zou et al., 2007; Brunner et al., 2020). The gut-associated lymphoid tissue (GALT) has additional mechanisms which include diffusely organized lymphoid cells, granulocytes, macrophages, and mucus IgM and IgT antibodies to distinguish between commensal microbes and provide broad-range protection against potential pathogens (Zhang et al., 2010; Lazado and Caipang, 2014; Salinas and Parra, 2015). The mucosal tolerance to microbes in the gastrointestinal tract promotes homeostasis in the commensal community while simultaneously limiting the expansion of opportunistic pathogens.

The intimate relationship between host and microbe begins as soon as the eggs are laid (Yoshimizu, Kimura and Sakai 1980). During development, the resident microbes promote epithelial cell growth and differentiation, as evidenced by studies in gnotobiotic zebrafish (*Danio rerio*) embryos. Germ-free (GF) zebrafish were found to have immature gastrointestinal tracts and a significantly lower population of epithelial cells actively proliferating compared to the conventional-reared embryos (Rawls et al., 2004; Bates et al., 2006). Additionally, several genes related to cell proliferation, nutrient metabolism, and innate immunity were differentially expressed in GF *versus* conventional-reared embryos (Rawls, Samuel and Gordon 2004). These findings highlight the profound

codependence between host and microbiota for proper gastrointestinal development. A more recent study accordingly found that gnotobiotic rainbow trout larvae had fewer Goblet cells compared to their conventionally-reared counterparts (Perez-Pascual *et al.* 2021). In rainbow trout and Coho salmon (*Oncorhynchus kisutch*), the core phyla of microbes present throughout their life cycle stably colonize the gastrointestinal tract upon first feeding (Romero and Navarrete, 2006; Ingerslev et al., 2014). As such, these resident microbes help stimulate immune cells and establish mucosal tolerance through the presentation of antigens by the microbiota, which prime the immune system and promote maturation of the GALT (Gomez, Sunyer and Salinas 2013).

One could rightly ask if these core microbiota assemblages have evolved over time to optimally prime the host's gastrointestinal and immune functions, why do they not protect the fish from disease and poor nutritional uptake? The answer is not known, but the presumption is that external anthropogenic factors such as pollutants, pathogens, stress, and artificial food sources alter the microbiota composition and metabolic output, thus leading to diminished health.

1.1.3 Infectious diseases in aquaculture and the threat posed by Vibrio anguillarum

As is the case in other forms of farming, aquaculture animals are reared in settings that differ substantially from those in the wild. The combination of several factors, such as chemical stress (e.g. water quality, diet), biological stress (e.g. high rearing densities and increased abundance of microorganisms), and physical stress (e.g. temperature fluctuations) creates a formidable environment for opportunistic pathogenic infections (Frans *et al.* 2011). Accordingly, the shift from extensive (low stocking densities, no

exogenous feeding) to more intensive (higher stocking densities and artificial feeding) forms of aquaculture has been associated with a dramatic increase in the incidence and severity of disease outbreaks, which leads to devastating economic losses (Bayliss *et al.* 2017).

The following bacterial pathogens particularly affect salmonids: *Aeromonas salmonicida* (furunculosis), *Vibrio anguillarum* (vibriosis), *Vibrio salmonicida* (cold water vibriosis), *Piscirickettsia salmonis* (piscirickettsiosis), *Yersinia ruckeri* (enteric red mouth disease/yersiniosis), *Renibacterium salmoninarum* (bacterial kidney disease), *Flavobacterium psychrophilum* (bacterial cold water disease), *Lactococcus garviae* (lactococcosis), and *Moritella viscosa* (winter ulcer; Merrifield et al., 2010). Although these pathogens differ in their disease presentation, their ability to translocate the intestinal epithelium is a key route of entry to the blood (Ringø et al., 2003; Jutfelt et al., 2006; **Figure 1-2**). Thus, the survival advantage provided by probiotic supplementation has been attributed, at least in part, to enhancement of immune and barrier function (Ringø et al., 2007a, 2007b; Salinas et al., 2008; Nayak, 2010; **Table 1-1**). Notably, evidence also suggests an important role for microbe-microbe interactions in probiotic strains reducing pathogen adherence to mucosal surfaces, limiting growth through nutrient exclusion, antagonising the pathogens through the secretion of antimicrobial molecules, or lowering the local pH (Ringø et al., 2020; **Figure 1-1**).

The pathogen *Vibrio anguillarum* is particularly concerning to the aquaculture industry due to its virulence, widespread prevalence, and wide range of targets (Frans *et al.* 2011). This species is ubiquitously found in saltwater, freshwater, and brackish water worldwide and infects over 50 fish species of economic importance to the aquaculture

industry, including finfish and shellfish species (Frans *et al.* 2011). The pathogen is a Gram-negative, non-spore-forming, comma-shaped rod bacterium (Toranzo, Magariños and Romalde 2005; Frans *et al.* 2011). The bacterium bas a 4.2Mb bipartite genome with a 43-46% GC content and a 65-67Kb virulence plasmid, pJM1 and other newly-identified pJM1-like plasmids (Naka and Crosa 2011; Akter *et al.* 2020).

The pJM1 plasmid is an essential component in *V. anguillarum*'s virulence and pathogenicity, as pMJ1-less O1 strains were more sensitive to lysozyme, were unable to colonize the skin, but could still colonize the gut with limited motility and could not transport the ferrin-anguibactin complex into the cell (Weber, Chen and Milton 2010). This organism's arsenal of virulence factors includes a flagellum for chemotaxis and motility (Milton *et al.* 1996; O'Toole, Milton and Wolf-Watz 1996), proteases (Norqvist, Norrman and Wolf-Watz 1990), hemolysin activity (Hirono, Masuda and Aoki 1996), and an iron-sequestering mechanism (Crosa *et al.* 1985). There are 23 known O serotypes (O1–O23), of which only serotypes O1, O2, and O3 (the latter to a lesser extent) have been associated with disease with a distinct pathogenicity and host specificity in fish species (Pedersen *et al.* 1999).

Vibriosis is characterized by a highly fatal haemorrhagic septicaemia, and clinical signs include weight loss, lethargy, redness on the skin, abdominal distension, liquefaction of organs, exophthalmia, and haemorrhaging in the gills and fins (Toranzo, Magariños and Romalde 2005; Frans *et al.* 2011). High bacterial concentrations are found in the blood and haematopoietic tissues during infection (Frans *et al.* 2011). Importantly, in acute epizootic infection, fish may succumb to the disease prior to demonstrating

classical symptoms due to rapid disease transmission, which directly translates to substantial economic losses to the farmers (Frans *et al.* 2011).

Regarding the mode of entry of the pathogen, evidence suggests that *V. anguillarum* infects the host through the skin and gills, as well as through ingestion of contaminated food and water. It then localizes, initially to the distal gut, prior to entering the bloodstream by translocating across the epithelial barrier (Grisez *et al.* 1996; Olsson *et al.* 1996, 1998; O'Toole *et al.* 1999) (**Figure 1-2**). Skin colonization with the pathogen may also play a role during infection, but it is possible that the plethora of antimicrobial peptides keep the pathogen at bay (Weber, Chen and Milton 2010). Exposure to intestinal mucus supports rapid growth of *V. anguillarum* and the bacterium becomes particularly virulent, as several of its virulence factors are specifically induced by contact with intestinal mucus (Garcia *et al.* 1997; Olsson *et al.* 1998; Denkin and Nelson 1999; Li *et al.* 2015). Indeed, a mechanistic analysis of an infection with *V. anguillarum* in turbot (*Scophthalmus maximus*) elicited an enrichment in pathogen adherence and attachment with a simultaneous downregulation of intracellular pathogen recognition molecules, which suggests immune evasion through the impairment of several innate immune pathways in the host (Gao *et al.* 2016). Leukocyte respiratory burst activity and apoptotic cascades were also downregulated in sea bass (*Dicentrarchus labrax* L.; Sepulcre *et al.* 2007). These findings further support the hypothesis that the intestine is indeed a major portal of pathogen entry into the bloodstream.

Prevention and containment strategies consist of prophylactic measures to promote the health and welfare of the animals, such as lower stock densities, high-quality nutrition, and maintenance of optimal temperature. Antibiotic use, although still

employed in some cases, is increasingly being strictly regulated against as these compounds enrich for resistant organisms and disrupt the indigenous microbiota (He *et al.* 2017). Immunization has also been investigated as a preventative strategy, albeit insufficiently efficacious depending on the fish species as well as host genetic variability that can impact the degree of protection provided by the vaccine (Dixon 2012; Figueroa *et al.* 2020). Furthermore, commercial vaccines mainly protect fish from outbreaks caused by serotypes O1 and O2, but serotype O3 cannot be completely prevented (Mikkelsen *et al.* 2007).

More recently, phage therapy has been explored as an emerging disease control strategy. The use of phages against *V. anguillarum* infection has been described with some degree of success in zebrafish and Atlantic salmon (Higuera *et al.* 2013; Silva *et al.* 2014). However, further examination of phage-resistant strains has revealed that the *V. anguillarum* phage isolated, CHOED, can exert a selective pressure to drive the proliferation of resistant strains with enhanced virulence phenotypes (León *et al.* 2019). As such, unless novel containment strategies are developed, vibriosis will continue to be rampant in aquaculture settings.

1.3 Probiotic-mediated modulation of host health

Collectively, there has been an abundance of scientific literature underpinning the benefits of probiotic supplementation in mammals. Despite the incorrect use of the term ‘probiotic’ by too many authors and companies who have not characterized the strain, its properties, and proven a health benefit, there are reports in several agriculture-relevant animals, such as cattle (McGilliard and Stallings 1998; Zhao *et al.* 1998), poultry (Morishita *et al.* 1997), pigs (Hossain, Sadekuzzaman and Ha 2017), and honeybees

(Daisley et al., 2019; Chmiel et al., 2020) in addition to humans showing the breadth of applications for probiotic microorganisms. In fish, certain probiotic strains have been reported to improve immunity, enhance growth rate, and protect against infection (Nikoskelainen et al., 2003; Balcázar et al., 2007a; Vendrell et al., 2007).

There appears to be primarily two modes of action, including **direct** modulation of host physiology at the mucosal interface, and **indirect** alteration of the structure and function of the microbial community in the gut, which limits the expansion of pathogens and promotes homeostasis in this community (Iman et al., 2013; Gatesoupe, 2016). Here, the latest findings are reviewed regarding the mode of action of probiotic microorganisms in salmonids, with an emphasis on intestinal health, in the hope of identifying strategies to leverage microbial-mediated interventions that contribute to maximizing productivity in the industry.

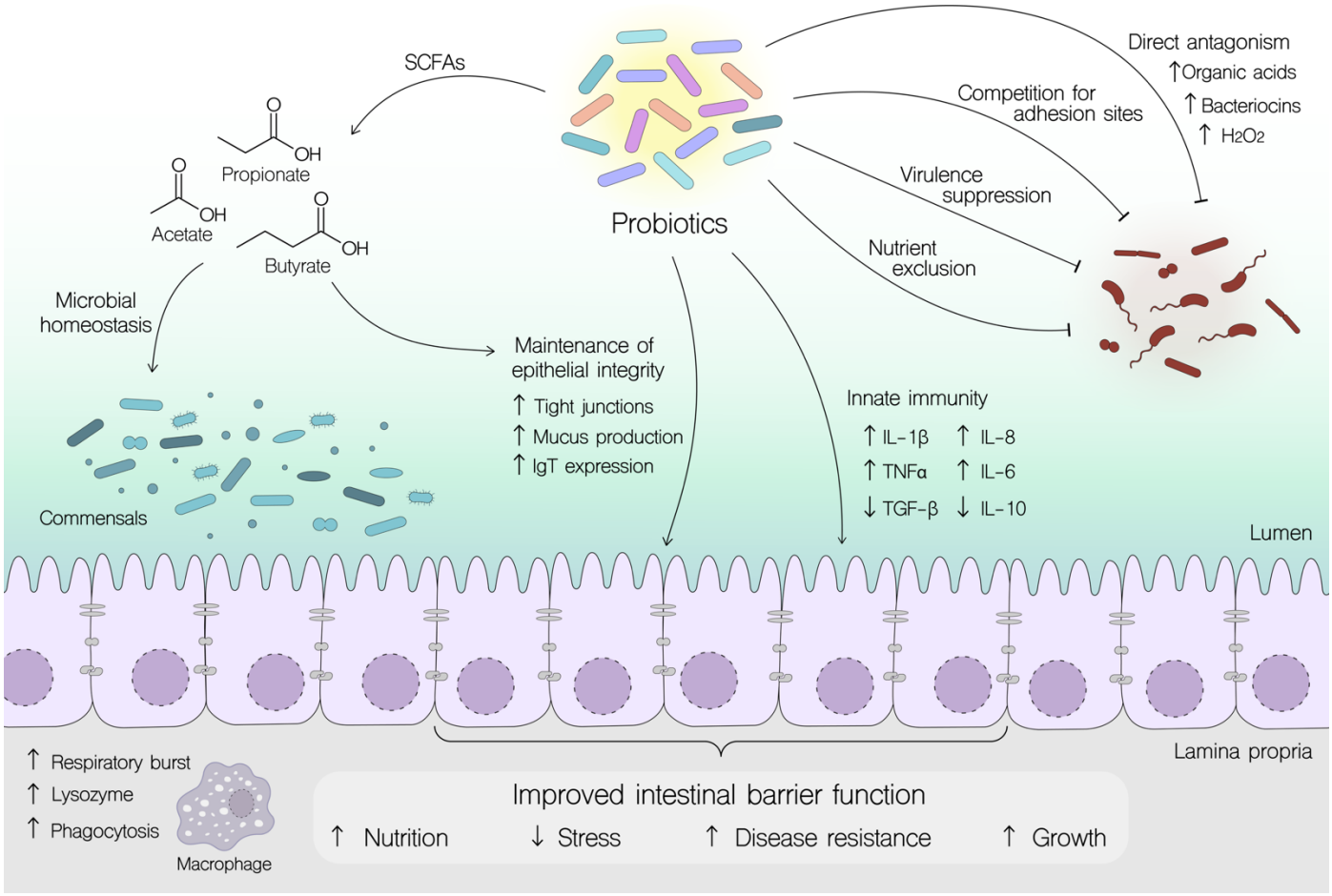

Figure 1-1. Schematic of the concerted mechanism of action of probiotics in salmonid fish. Probiotic microorganisms are believed to promote host fitness primarily *via* the direct modulation of innate immunity and through the maintenance of epithelial integrity. Indirect mechanisms include the production of metabolites, such as SCFAs, that promote homeostasis in the gut commensal community, and the inhibition of pathogen growth and expression of virulence factors. The harmonized effect is the improvement in intestinal barrier function, which in turn has substantial physiological benefits for the host.

1.2.1 Intestinal barrier function in salmonids

At the core of the intestinal mucosa is a single layer of epithelial cells and their intercellular junctions (comprised of tight junctions, desmosomes, adherens' junctions,

and gap junctions). This complex network exerts a dual function of 'fence' and 'gate', in which the 'fence' function refers to the preclusion of most of the microbes and metabolites to the lamina propria and the gut-associated immune system while simultaneously allowing for the absorption of nutrients, which refers to the 'gate' function (Clayburgh, Shen and Turner 2004; Rajasekaran, Beyenbach and Rajasekaran 2008; Suzuki 2013; Wells *et al.* 2017).

Low-grade inflammation resulting from a faulty intestinal barrier is being increasingly recognized as the basis for a myriad of chronic and autoimmune conditions in humans (Ukena et al., 2007; Bron et al., 2017). Importantly, a 'leaky' gut barrier may lead to deficits in nutrient absorption and impaired immunity due to the continuous stimulation by the passage of antigens from the intestinal lumen to the lamina propria and portal blood (Kosińska and Andlauer 2013). As such, the gut barrier plays a critical role in organismal health, and damage to the intestinal integrity can have significant systemic consequences.

Probiotic strains can act at this interface and upregulate the expression of tight junction molecules, such as claudins, occludins, and zonula occludens 1 (ZO-1). This activity enhances intestinal barrier integrity against chemical- or pathogen-induced insults to the epithelium (Ringø et al., 2007a; Ukena et al., 2007; Patel et al., 2012; Vasanth et al., 2015; Wang et al., 2018b). The precise mechanism of action remains to be elucidated, but evidence suggests that microbial metabolites, such as short-chain fatty acids (SCFAs), can promote intestinal integrity (Mariadason, Barkla and Gibson 1997). These compounds are a product of anaerobic fermentation of carbohydrates in the gut and are comprised primarily by propionate, butyrate, and acetate (Ganapathy *et al.* 2013). This

class of metabolites is the most important microbial end-product both quantitatively and metabolically, and displays pleiotropic links with the maintenance of community homeostasis and host health (Louis, Hold and Flint 2014; Rivière *et al.* 2016). Notably, butyrate has been shown to promote epithelial barrier function in a human colon carcinoma (Caco-2) epithelial cell model by suppressing the expression of proteins associated with gut permeability, facilitating assembly of TJ complexes, and modulating the epigenetic landscape in host cells (Peng et al., 2009; Chang et al., 2014; Kelly et al., 2015; Zheng et al., 2017). In salmonids, dietary butyrate has been shown to upregulate the expression of tight junction molecules and innate immune parameters *in vivo* (Hoseinifar, Sun and Caipang 2017; Mirghaed *et al.* 2019). These studies are beginning to unravel the complex relationship between host and microbe and how microbial metabolites can promote intestinal integrity, although more research is warranted to identify strains that produce butyrate *in situ* in the salmonid gut.

The *in vivo* evidence accumulated to date substantiates the concept of the intestinal barrier as a key factor in host health. Therefore, if probiotic strains can improve barrier function in salmonids, premature deaths and morbidity could be reduced and nutrient uptake increased, resulting in a higher yield of commercial fish (**Figure 1-1**).

1.2.2 Growth and nutrition

Given that the quality and quantity of fish protein is central to aquaculture productivity, growth promotion is arguably one of the most appealing outcomes of probiotic supplementation for the industry. Indeed, there are some data suggesting that supplementation of beneficial microbes positively affects growth parameters in several salmonid species. For example, a report investigating the differences between the gut

microbiota composition of slow- and fast-growing rainbow trout indicated that the fast growers had a higher proportion of Firmicutes, whereas slow growers had a community dominated by members of the Actinobacteria phylum, which is known to include pathobionts in fish (Chapagain *et al.* 2019). These findings substantiate the idea that gut microbes play a central role in modulating nutrient absorption, metabolism, and immunity. Identification of specific members of the community responsible for the growth advantage could lead to new probiotic products.

In Atlantic salmon, rainbow trout, and Caspian trout (*Salmo trutta caspius*), diets containing *Lacticaseibacillus* (formerly *Lactobacillus rhamnosus* GG; Zheng et al., 2020) *Lactiplantibacillus plantarum* CLFP 238 and *Leuconostoc mesenteroides* CLFP 196 plus *L. plantarum*, led to improved specific growth and feed conversion ratios (Nikoskelainen et al., 2001; Vendrell et al., 2007; Ramos et al., 2015). One of the mechanisms through which these probiotics strains achieve physiological changes is presumed to be modulating the expression of tight junction molecules at the intestinal mucosal surface, though further studies are warranted (**Figure 1-1**). The modulation of the inflammatory and stress responses, as well as the microbial metabolism of otherwise indigestible nutrients, may also play an important role in fish growth and nutrient assimilation.

Central to nutrition is the quality of the diet that the fish receive. Salmonids thrive on a diet rich in fishmeal (FM) and fish oil. However, given the intensification of salmonid aquaculture, producers are shifting to less costly sources of protein, such as soybean or insect meal (Rana, Siriwardena and Hasan 2009). An unintended consequence of the use of plant-derived proteins, particularly saponins, is that they can cause enteropathy in salmonids, as evidenced by considerable damage to the intestinal

epithelium, the upregulation of proinflammatory cytokines, as well as appreciable alterations in the gut microbiota, indicative of dysbiosis (Dimitroglou et al., 2009; Marjara et al., 2012; Green et al., 2013; Krogdahl et al., 2015).

The deleterious effects induced by antinutrients might be mitigated by probiotic administration. In rainbow trout, supplementation of a multi-strain commercial product containing strains of *Saccharomyces cerevisiae*, *Enterococcus faecium*, *L. acidophilus*, *L. casei*, *L. plantarum*, and *L. brevis*, in conjunction with soybean meal, rescued the deficits in growth observed in the soybean-only diet. Furthermore, the fish fed with this product and soybean meal starter diets exhibited higher digestibility and growth during grow-out phases compared to starter diets devoid of the microbes (Sealey *et al.* 2009). These findings suggest that certain microbes can promote intestinal health and prevent injury induced by antinutrients. Notably, however, these effects were not observed long-term following cessation of supplementation, presumably because the strains did not colonize the intestinal tract (Sealey *et al.* 2009). Similar studies are warranted to identify strains of beneficial microbes that support digestion and protect intestinal health from antinutrients. In addition, research is needed to determine the optimal inclusion ratio of plant-derived proteins in fish diets, which can help reduce the industry's dependence on marine harvest to produce fish feeds.

Recently, Rimoldi *et al.* (2021) investigated the effect of an insect meal (IM) diet on the gut microbiota of rainbow trout and found that a 15% inclusion ratio preferentially enriched Firmicutes, primarily represented by *Bacillus* and *Lactobacillus* genera, while decreasing the abundance of *Aeromonas,* compared to a conventional FM diet. An analysis of the metagenome revealed functional differences between treatments in the IM

diet, indicating an enhancement of sugar and starch metabolism pathways; whereas the FM diet enriched for peptidoglycan synthesis pathways. These data suggest that alternative protein sources can directly modulate the gut microbiota through nutrient availability, and thereby modify the function and metabolism to enhance digestion and nutrient assimilation, thus benefiting the host. Increased attention should be given to the effects of alternative diets and probiotic feed additives to preferentially enrich for key microbes and pathways that can maximize host health.

A number of prebiotic compounds have been used to stimulate growth of beneficial microbes in the gut. Prebiotics are defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (Gibson *et al.* 2017), and synbiotics as "a mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit on the host" (Swanson *et al.* 2020). The latter includes complementary synbiotics and synergistic synbiotics where the substrate is selectively utilized by the co-administered microorganisms. A product containing a plant- or insect- derived compound and probiotic could well be an option worth considering to support a healthy gut microbiota and the expansion of beneficial strains, thus leading to improved fish health.

1.2.3 Immune performance

Immune modulation is among the most widely studied effects of probiotic supplementation in aquatic and terrestrial organisms. In fish, probiotic supplementation augments the innate immune response through the modulation of both cellular and humoral parameters, thereby promoting more efficient pathogen clearance and improving disease resistance (Nayak, 2010; Table 1). Several reviews address this topic specifically

(Nayak, 2010; Hoseinifar et al., 2018; Ringø et al., 2018). For instance, the administration of LAB (*Lc. lactis* subsp. *lactis* CLFP 100, *L. sakei* CLFP 202, and *Leuc. mesenteroidetes* CLFP 196) to brown trout (*Salmo trutta*) led to an upregulation of non-specific humoral responses, such as lysozyme and alternative complement activities (ACH50), as well as plasma immunoglobulin levels (Balcázar *et al.* 2007a). This outcome was also observed in rainbow trout fed with *Lacticaseibacillus rhamnosus* JCM 1136. Interestingly, the authors noted an upregulation of non-specific humoral immunity when the probiotic was administered in live spray or freeze-dried forms, but not when they were heat-killed. This highlights the importance of metabolically active beneficial microbes during gastrointestinal transit (Panigrahi *et al.* 2004). The same group found that administration of a microbial mix (containing three strains of *Lacticaseibacillus rhamnosus*, *Enterococcus faecium*, and *Bacillus subtilis*) increased superoxide anion production in the head kidney leukocytes and ACH50 in the serum, along with an upregulation of *il1β*, *tnf1*, *tnf2*, and *tgfβ* in the spleen and head kidney (Panigrahi *et al.* 2007).

Additionally, injection of a candidate probiotic strain of *Enterobacter* spp. led to reduced mortality upon infection with *Flavobacterium psychrophilum*, potentially mediated by cross-reactive antibodies generated in response to the microbial supplement, also in rainbow trout (LaPatra, Fehringer and Cain 2014). Unfortunately, it is not clear why that strain was chosen, as it is not a species customarily used in probiotic applications. Another species more commonly regarded as a human pathogen, *E. faecalis,* was shown to increase mucus production in rainbow trout and *Pediococcus acidilactici* MA18/5M upregulated IgT expression in the gut, which is a mucosal antibody known to

promote homeostasis in the gut microbiota (Rodriguez-Estrada et al., 2013; Al-Hisnawi et al., 2019). Further, dietary supplementation of *P. acidilactici* MA18/5M locally stimulated an increase in the levels of *il1* and *il8* while decreasing *il10* expression in the distal intestine, and simultaneously preserving the intestinal epithelium from detrimental injury (Al-Hisnawi *et al.* 2019). Further studies with *P. acidilactici* MA18/5M showed induction of an innate antiviral response in Atlantic salmon by upregulating the expression of toll-like receptor 3, interferon-alpha, and other molecules upstream of the cascade of the primary antiviral response (Abid *et al.* 2013; Jaramillo-Torres *et al.* 2019).

Administration of rainbow trout with a yeast supplement containing strains of *Saccharomyces cerevisiae* and *Cyberlindnera jardinii* upregulated the expression of molecules associated with toll-like receptor 2 signalling, effector cytokines, and transcription factors upstream of the T cell regulatory response in the rainbow trout distal intestine. Supplementation induced the expression of proinflammatory molecules such as *tnfα* and *il1β*, as well as anti-inflammatory cytokines such as *il10* and *tgfβ*, indicative of a balance between immune stimulation and mucosal tolerance presumably mediated by glycans, glycolipids, and glycoproteins in the fungal cell wall. In addition, supplemented fish had significant increase in Goblet cell density, which suggests that the yeast can stimulate increased antigen uptake and immune surveillance (Rawling *et al.* 2021). Such studies investigating the molecular mucosal response to beneficial microbes are beginning to unravel the mechanism underlying cellular and humoral immune stimulation.

In general, the evidence from human studies suggests that probiotic strains elicit a regulatory response that leads to immune homeostasis (Bron, Van Baarlen and

Kleerebezem 2012). This is achieved through microorganism-associated microbial pattern-pattern recognition receptor (MAMP-PRR) interactions that activate the nuclear factor kappa beta (NFκB) pathway (Rescigno 2010). This, in turn, activates GALT cells that secrete cytokines, such as tumour growth factor beta (TGFβ) and interleukin (IL) 10, which modulate the activation and differentiation of T cells, while downregulating proinflammatory cytokines (Lorea Baroja et al., 2007; Bron et al., 2012). In contrast, probiotic supplementation in salmonids elicits a proinflammatory response, typically characterized by the upregulation of IL-1β, IL-6, IL-8, and tumour necrosis factor alpha (TNF-α) in the gut (Kim and Austin 2006a, 2006b; Panigrahi, Viswanath and Satoh 2011; Ringø *et al.* 2018). Although some LAB strains induce an upregulation of IL-10 in other finfish (Beck *et al.* 2016; Maji *et al.* 2017). This inflammatory response in salmonids is directly correlated with a survival advantage against pathogenic challenges (Table 1), implying an upregulation of defence mechanisms. These observations indicate that selection of probiotic strains for salmonids requires careful experimentation not based upon warm-blooded hosts. Strain-specific responses may also be linked to the biochemical variability and complexity of bacterial cell wall components that elicit differential responses in the host through pattern recognition receptor (PRR) interactions (Bron, Van Baarlen and Kleerebezem 2012).

Another consideration is when to administer probiotic strains. When finfish transition from the cold winter to the hot summer temperatures, their immune system is part of the coping mechanism. The idea of administering probiotic bacteria or yeast prior to summer when pathogen loads are higher, could prove to be an effective strategy to prime the innate immune response without inducing excessive inflammation in the tissue.

But how the probiotic organisms' function inside fish swimming in cold versus warm water needs to be investigated.

Studies employing transcriptomic approaches to survey the piscine response to probiotic supplementation at the molecular level are beginning to unravel the mechanistic links between host and microbe. For instance, supplementation of rainbow trout with a strain of *S. cerevisiae* led to an upregulation of innate immune pathways in conjunction with a mitigation of the stress response associated with high-density rearing systems (Gonçalves, Valenzuela-Muñoz and Gallardo-Escárate 2017). This suggests that this *S. cerevisiae* strain is able to overcome stress-induced immunosuppression and locally stimulate the immune system to prime the organism for a more effective clearance of pathogens. As such, future studies employing high-throughput methods to understand the strain-specific molecular cascade of immunomodulation would do well to pave the path towards enabling the leveraging of these properties for sustainable maximization of aquaculture production.

Recent advances made with *in vitro* systems, such as the rainbow trout intestinal epithelial cell line RTgutGC and gut-on-chip models, offer opportunities to investigate host-microbe interactions at the molecular level (Kawano et al., 2011; Drieschner et al., 2019; Pumputis et al., 2019; Wang et al., 2019). A gnotobiotic rainbow trout model may also help with mechanistic investigation of these phenomena (Perez-Pascual *et al.* 2021). It is hoped that such studies will aid in understanding probiotic effector molecules and cognate host signaling pathways underlying the functional modulation of cellular physiology in salmonids. Such models might provide a platform for rapid screening of functional supplements. Though the translation of findings in the lab to the complex

mucosal system in the field is not trivial, the knowledge gained represents a starting point to address this key knowledge gap in the field.

Table 1-1. Summary of studies investigating the effects of probiotic supplementation in the context of a disease challenge in salmonids.

Host species	Pathogen	Pathogen dose	Strain(s) designated as 'probiotic'	Duration of supp.	Dose (CFU/g feed)	Main findings	Ref.
Rainbow trout (*Onco-rhynchus mykiss*)	*Vibrio anguillarum* (90-11-287, serotype O1)	10^5-10^6 CFU/ml, bath immersion	*Pseudomonas fluorescens* AH2	5 days prior to infection (LT) and during infection (ST)	LT: 10^5 CFU/ml ST: 10^7 CFU/ml, bath immersion	• Inhibition of *V. anguillarum in vitro* • ↑ survival	(Gram *et al.* 1999)
	Aeromonas salmonicida SN1	$1.8x10^7$ CFU/mL, bath immersion	*Lacticaseibacillus rhamnosus* GG (ATCC 53103)	15 days	10^9 and 10^{12}	• ↑ survival • No difference in SGR	(Nikoskelainen *et al.* 2001)
	Yersinia ruckeri O1	$1.8x10^6$ CFU/mL, 0.1mL IP injected	*Bacillus subtilis* and *B. licheniformis*	16 weeks	$4x10^4$	• ↑ survival • No difference in growth parameters • No difference in haematocrit, plasma protein, or lymphocyte counts	(Raida *et al.* 2003)
	Lactococcus garviae 29-99 and *Strepto-coccus iniae* 00-318	$2x10^7$ CFU/ml, 0.1mL IP injected	*Aeromonas sobria* GC2 (indigenous isolate)	14 days	10^8	• ↑ survival • ↓ morbidity • Dosage optimum • Live candidate probiotic cells necessary for benefits • ↑ leukocyte count, phagocytic activity, and respiratory burst	(Brunt and Austin 2005)
	Aeromonas salmonicida Hooke and *Yersinia ruckeri* T1	*A.s.*: $2.4x10^7$ cells/ml *Y.r.*: $1.6x10^7$ cells/ml, 0.1mL IP injected	*Carnobacterium maltaromaticum* B26 and *Carnobacterium divergens* B33 (indigenous isolates)	14 days	10^7	• Viable in feed for up to 3 weeks • ↑ phagocytic activity, SOD (B33 only), and lysozyme activity • No difference in anti-protease activity	(Kim and Austin 2006b)

	Aeromonas salmonicida ABE1	IP: 10^2 CFU/mL IM: 10^8 CFU/mL	*Bacillus subtilis* AB1	14 days	10^7	• ↑ survival • Dosage optimum • ↑ erythrocyte count, phagocytic activity, respiratory burst, lysozyme and protease activity	(Newaj-Fyzul *et al.* 2007)
	Aeromonas salmonicida CLFP 501	$1.7x10^6$ CFU/mL; 0.1mL IP injected	*Lactococcus lactis* ssp. *lactis* CLFP 100, *Leuconostoc mesenteroi-des* CLFP 196, and *Latilacto-bacillus sakei* CLFP 202 (indigenous isolates)	14 days prior to infection	10^6	• ↑ survival • ↑ phagocytic activity of HK leukocytes • ↑ SOD production, phagocytosis, and complement activity (ACH50) after 2 weeks	(Balcázar *et al.* 2007c)
	Aeromonas salmonicida, Lactococcus garvieae 29-99*, Streptococcus iniae* 00-318*, Vibrio anguillarum* VIB1*, Vibrio ordalii* VIB2 *and Yersinia ruckeri* Pri10	~$2.5x10^7$ CFU/mL, 0.1mL IP injected	*Bacillus sp.* JB-1 and *Aeromonas sobria* GC2	2 weeks	$2x10^8$	• *In vitro* inhibitory activity to at least 1 pathogenic strain • ↓ morbidity • ↑ respiratory burst, macrophage, and protease activities • Dosage optimum	(Brunt, Newaj-Fyzul and Austin 2007)
	Lactococcus garviae CLFP LG1	3.4 x 10^3 CFU/mL, 0.1mL IP injected	*Leuconostoc mesenteroi-des* CLFP 196, *Lactiplanti-bacillus plantarum* CLFP 238	4 weeks	10^7	• ↑ survival • Supplemented strains recoverable from intestine	(Vendrell *et al.* 2007)
	Aeromonas bestiarum and *Ichthyophthirius multifilii*	$1x10^5$ CFU/mL, 0.1mL ID injected	*Aeromonas sobria* GC2 or *Brochothrix thermos-phacta* BA211	2 weeks	10^8 (CG2) or 10^{10} (BA211)	• ↑ survival • ↑ phagocytic activity (GC2) • ↑ respiratory burst activity (BA211)	(Pieters *et al.* 2008)
	Vibrio anguillarum	$3x10^5$ CFU/mL, 0.1mL IP injected	*Kocuria* SM1 (indigenous isolate)	Up to 4 weeks	10^8	• ↑ survival • ↑ phagocytic activity in HK, total serum antiprotease, and lysozyme activity	(Sharifuzzaman and Austin 2009)
	Vibrio anguillarum	*V.a.*: $3x10^5$ CFU/mL	*Kocuria* SM1 (indigenous isolate)	2 weeks	10^8	• ↑ survival	(Sharifuzzaman and

	and *Vibrio ordalii*	*V.o.*: $5x10^4$ CFU/mL, 0.1mL IP injected				• ↑ cellular and humoral immune parameters • ↑ in leukocytes, erythrocytes, globulin, albumin • ↑ respiratory burst, complement, and lysozyme activities	Austin 2010a)
	Vibrio anguillarum	$3x10^5$ CFU/mL, 0.1mL IP injected	*Kocuria* SM1 (indigenous isolate)	2 weeks; trial up to 5 weeks following cessation of supplementation	10^8	• ↑ survival • ↑ serum lysozyme and respiratory burst activities of blood • ↑ serum peroxidase and bacterial killing activities	(Sharifuzzaman and Austin 2010b)
	Streptococcus iniae ATCC 29178	$1.2x10^6$ cells/mL, 0.1mL IP injected	*Enterococcus casseliflavus* NC0209951 (indigenous isolate)	8 weeks	T1. 10^7 T2. 10^8 T3. 10^9	• ↑ growth • ↑ survival • ↑ neutrophil count at week 4 • ↑ serum IgM and C3 levels at week 4 and 8 in all diet groups; ↑ in total serum protein and albumin in T3 at week 8 • ↑ respiratory burst activity in T2 and T3 at week 8 • No change in intestinal morphology • In vitro inhibition of the pathogen • Dose-response relationship	(Safari *et al.* 2016)
	Vagococcus salmoninarum and *Lactococcus garvieae*	*V. s.*: $1.8x10^8$ CFU/fish *L.g.*: $1.2x10^7$ CFU/fish, 0.1mL IP injected	Fish isolates: *Lactobacillus lactis* spp. *Pediococcus acidilactici Latilactobacillus sakei*	3 weeks	10^8	• In vitro inhibition of the pathogen • ↑ survival	(Didinen *et al.* 2018)
	Vaccine against *Yersinia ruckeri* KC291153	Immersion vaccine	*Lactiplantibacillus plantarum* 426951 (indigenous isolate)	72 days	$2x10^7$	• ↑ serum total protein and complement activity in supplemented + vaccine group • ↑ lysozyme activity and growth performance in supplemented + vaccine group	(Soltani *et al.* 2019)

Rainbow trout (*Onco-rhynchus mykiss*) and Atlantic salmon (*Salmo salar*)	*A. salmonicida* Hooke *V. anguillarum* V72 *V. ordalii* V453 *Y. ruckeri* PR110	$5x10^7$ CFU/ml, 0.1mL IP injected	*Carno-bacterium* sp. K1 (indigenous isolate)	4 weeks	$5x10^7$	• ↑ survival after 14 days • Transient strains (not recoverable after cessation of supplementation)	(Robertson *et al.* 2000)
Brook Charr (Salveli-nus fontina-lis)	*Aeromonas salmonicida* subsp. *salmonicida*	Only *in vitro* studies	*Pediococcus acidilactici* MA 18/5M, *Peudomonas fluorescens* ML11A, *Aeromonas sobria* TM18	16 weeks	$2x10^5$ CFU/mL tank water, bath immers.	• In vitro inhibition of the pathogen • ↑ Fulton index (growth) with *Pedicoccus* supplementation • Modulation of innate immune response	(Gauthier *et al.* 2019)
Brown trout (*Salmo trutta*)	*Aeromonas salmonicida* NCIMB1102	Infection induced by heat stress (opportun istic pathogen)	*Lactococcus lactis* CLFP 100 or *Leuc. mesenteroi-des* CLFP 196	4 weeks	10^6	• ↑ survival • Presence of both strains in the fish intestine • ↑ phagocytic activity in HK leukocytes • ↓ pathogen load	(Balcázar *et al.* 2009)

LT = long-term; ST = short-term; IP = intraperitoneal; IM = intramuscular; ID = intradermal; SOD = superoxide dismutase; RT = rainbow trout; HK = head kidney; dpi = days post-infection; GI = gastrointestinal.

1.2.4 Pathogen inhibition

Microbe-microbe interactions play a vital role in the modulation of host fitness. Evidence suggests an important role for probiotic-mediated reduction in pathogen adherence to mucosal surfaces, limiting growth through nutrient exclusion, direct antagonism through the secretion of antimicrobial molecules, or lowering the local pH (Ringø et al., 2020; **Figure 1-1**). LAB isolated from healthy rainbow trout have been shown to exhibit strain-specific inhibitory abilities against a panel of aquatic pathogens. All isolates (namely *Lactococcus lactis* subsp. *lactis* CLFP 100, *Lc. lactis* subsp. *cremoris* CLFP 102, *L. curvatus* CLFP 150, *Leuc. mesenteroidetes* CLFP 196, and *L. sakei* CLFP 202) preferentially adhered to intestinal mucus and caused a substantial

reduction in the adherence of *A. salmonicida*, *C. piscicola*, *Lc. garviae*, and *Y. ruckeri* to intestinal mucus (Balcázar *et al.* 2007c). Interestingly, however, *Leuc. mesenteroidetes* CLFP 196 induced a slight increase in the adherence of *A. salmonicida* to intestinal mucus, from which the authors substantiate the argument that the investigation of strain-specific effects is needed to assess microbe-microbe interactions (Balcázar *et al.* 2007c). A cautionary note should follow that the *in vitro* adhesion experiments may not be indicative of what happens *in vivo*. Inhibition of pathogen growth has been reported in several studies, some of which are summarized in Table 1.

Box 1. Disease challenge methods

Given that commensals, pathogens, and probiotic strains primarily act locally in the gastrointestinal tract, the gut of fish has been considered the battleground for these microbe-microbe interactions (Ringø et al. 2003). As such, disease challenge methods that employ intraperitoneal infections circumvent the potential inhibition of these pathogens by probiotics in the gut. Thus, the beneficial effects of probiotics in fish against pathogens could be more powerful than such experimental evidence currently indicates (Merrifield *et al.* 2010). Future studies must consider employing challenge methods that aim to carefully mimic the real-life setting so as to obtain biologically relevant insights and take into account the microbe-microbe interactions that might be happening on mucosal surfaces, especially in the gut.

In an effort to better understand microbial dynamics in the context of the intestinal mucosa, *ex vivo* approaches have been employed. For instance, *P. acidilactici* MA18/5M outcompeted *V. anguillarum* in the rainbow trout gut and the probiotic was also able to

populate the anterior intestine temporarily (Harper *et al.* 2011). Strains of *L. delbrueckii* subsp. *lactis* and *Carnobacterium divergens* Lab01 have been found to exert a protective effect against insults induced by *A. salmonicida* subsp. *salmonicida* on the intestine of Atlantic salmon *ex vivo* (Salinas et al., 2008; Hartviksen et al., 2015). Further, *C. divergens* was able to decrease the adherence of *A. salmonicida* to the intestinal mucosa (Hartviksen *et al.* 2015). These approaches are supportive of an *in vivo* effect, but mechanistic studies are needed to prove how they achieve this.

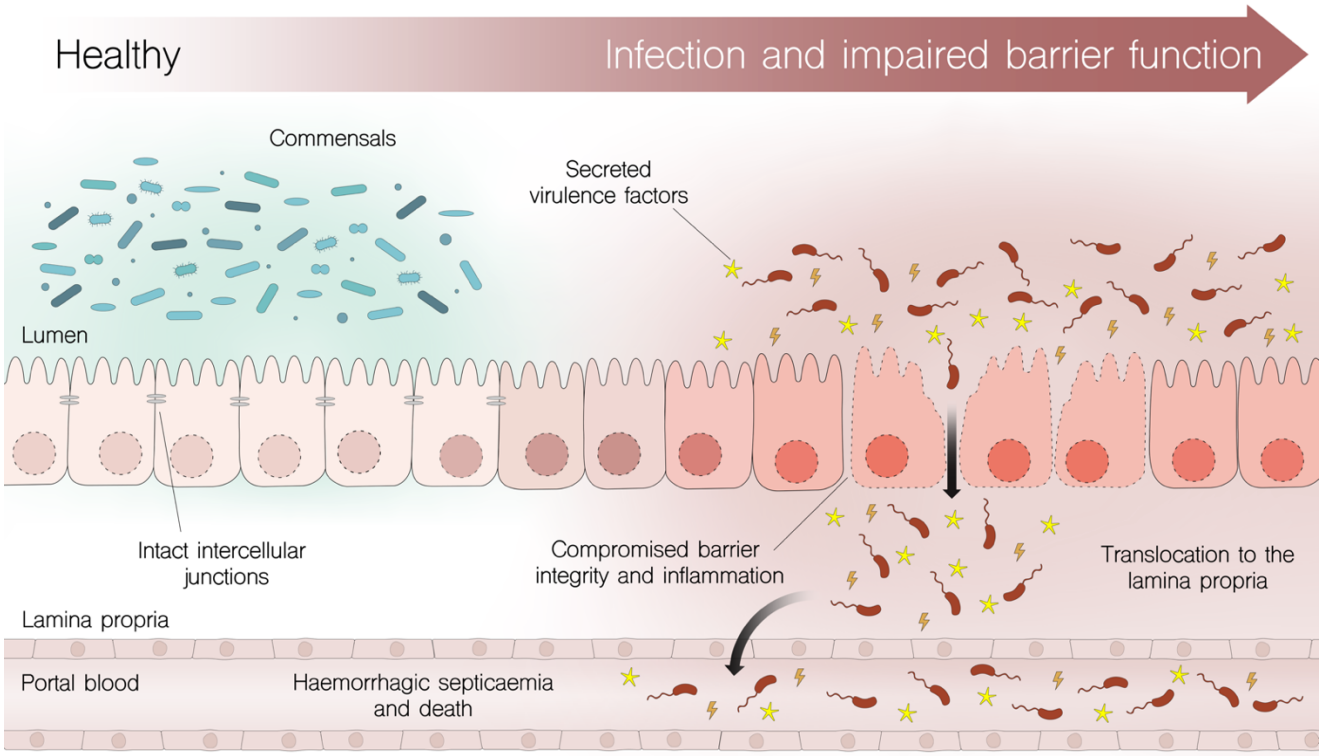

Figure 1-2. The salmonid intestinal mucosa as a portal of entry of pathogens into the bloodstream. The fish intestinal microbiota exists on a spectrum, in which the healthy state is characterized by homeostasis in the community and minimal damage to intercellular tight junctions. When dysregulation occurs, pathogens proliferate in the lumen and secrete virulence factors, which degrade the epithelial barrier and cause inflammation. Pathogens can then translocate to the lamina propria and enter into the circulation, thus causing haemorrhagic septicaemia and death.

1.2.5 Bacteriocin production

Beneficial microbes can directly antagonize the growth of bacterial pathogens through the secretion of bacteriocins, which are essentially peptides that exhibit antimicrobial activity against specific targets (Dobson *et al.* 2012). In the complex milieu of the gastrointestinal tract, bacteriocins play diverse roles that include microbial signaling, colonization, as well as killing organisms (Fajardo and Martínez, 2008; Dobson et al., 2012). Several studies have investigated salmonid-derived isolates that produce bacteriocins and aid in biopreservation of fish products (Gatesoupe, 2007; Desriac et al., 2010). Three strains of *Carnobacterium* spp. isolated from cold-smoked Atlantic salmon appear to have bactericidal properties against *Listeria monocytogenes* (Nilsson et al., 2002; Brillet et al., 2004). Sahnouni et al. (2016) isolated four strains of fish gut-associated LAB (*Loigolactobacillus coryniformis* L.11, *Limosilactobacillus fermentum* L.03, and *Carnobacterium* spp. Cb04 and Cb10) and demonstrated the bacteriocin-mediated inhibition of a range of pathogenic microorganisms, including *Vibrio*, *Aeromonas*, *Salmonella*, and *Escherichia* spp. Nevertheless, *in vivo* evidence reporting bacteriocin-mediated pathogen inhibition remains scarce (Gatesoupe 2007). As such, while bacteriocins appear to be a factor in modulating microbiota homeostasis in the gut, other components, such as organic acids and hydrogen peroxide, may play a more prominent role (Vázquez et al., 2005; Tomé et al., 2006).

1.2.6 Suppression of virulence expression

Bacteria use quorum sensing (QS) to communicate with each other through the secretion of autoinducer molecules in a cell density-dependent manner (Suga and Smith, 2003; Defoirdt, 2014). This also plays an important role in the regulation of microbial

phenotypes, including biofilm formation, virulence expression, swarming, and bioluminescence (Waters and Bassler 2005). Acylated homoserine lactones (AHLs) are key molecules involved in QS. These are produced by several aquaculture-relevant pathogens, such as *A. salmonicida*, *A. hydrophila*, *V. anguillarum*, *V. harveyi*, *V. salmonicida*, and *Y. ruckeri* (Kastbjerg et al., 2007; Defoirdt, 2014). Since bacterial pathogenesis is to a degree regulated by QS, an anti-infective strategy could be devised to employ quorum quenching (QQ), which disrupts the bacterial communication. Indeed, several fish-associated *Bacillus* strains have been investigated for their QQ properties against aquatic pathogens (Kuebutornye *et al.* 2020). For example, *Bacillus cereus* BP-MBRG/1b and *Bacillus* sp. QSI-1 have been shown to degrade AHL molecules of *A. hydrophila*, thus disrupting QS and preventing infection in prawns (*Macrobrachium rosenbergii*), goldfish (*Carassius auratus*) and zebrafish, respectively (Chu et al., 2014; Zhou et al., 2016; Wee et al., 2018). Delshad and colleagues (2018) identified five rainbow trout-associated microbes that exhibit QQ properties against *Y. ruckeri,* the etiological agent of enteric red mouth disease. While pathogen growth was unaffected, biofilm formation, motility, and AHL production were significantly inhibited *in vitro*. Furthermore, the strains conferred a survival advantage of up to 50% in a disease challenge, albeit differences were substantial between QQ strains (Delshad *et al.* 2018). These studies highlight the use of candidate probiotic strains for disease control in aquaculture through the modulation of pathogen virulence.

Box 2. Host-associated beneficial microbes

The selection of probiotic strains has tended to be based on species already characterized. Since most work has been done on human strains, this has led to these same species being applied to fish. Although the origin of species need not detract from its successful application to a different host, autochthonous strains from fish are worthy of exploration as probiotic candidates. Several isolates derived from aquatic animals have been shown to inhibit pathogens, promote growth, and modulate mucosal immunity in the host (Van Doan et al., 2020; **Table 1-1**). Normally, probiotic strains do not colonize the host gut. It would be interesting to determine whether autochthonous strains from fish can colonize the intestine and be better choices for aquaculture applications. The indigenous microbes will likely have coevolved with aquatic pathogens, making them more adaptable to preventing infection. Concerns remain, however, regarding the safety and market-readiness of these emerging beneficial microbes. Thus, all commercial probiotic strains must be proven to be safe.

1.4 Methods of probiotic encapsulation and delivery

To achieve health benefits in humans, it is generally believed that a minimum of 10^8-10^9 viable probiotic cells are required to reach the intestine (Hou *et al.* 2003; Oliveira *et al.* 2009). Another report suggested that an efficient probiotic formulation should contain a minimum viable cells number of 10^6 CFU/g by the end of shelf-life (Neffe-Skocińska *et al.* 2018). However, the number per se is not the issue. The evidence of benefit must come from tests in the host. Thus, if less or more viable organisms are needed, then that is what should be delivered. To act as a substitute for prophylactic

antibiotics in aquaculture and promote salmonid health, 10^6-10^8 probiotic CFU/ml per tank or CFU/g have been used (Table 1). The main challenge is to maintain this viability at time of use, which requires stringent documentation during their industrial production and along the storage and handling stages.

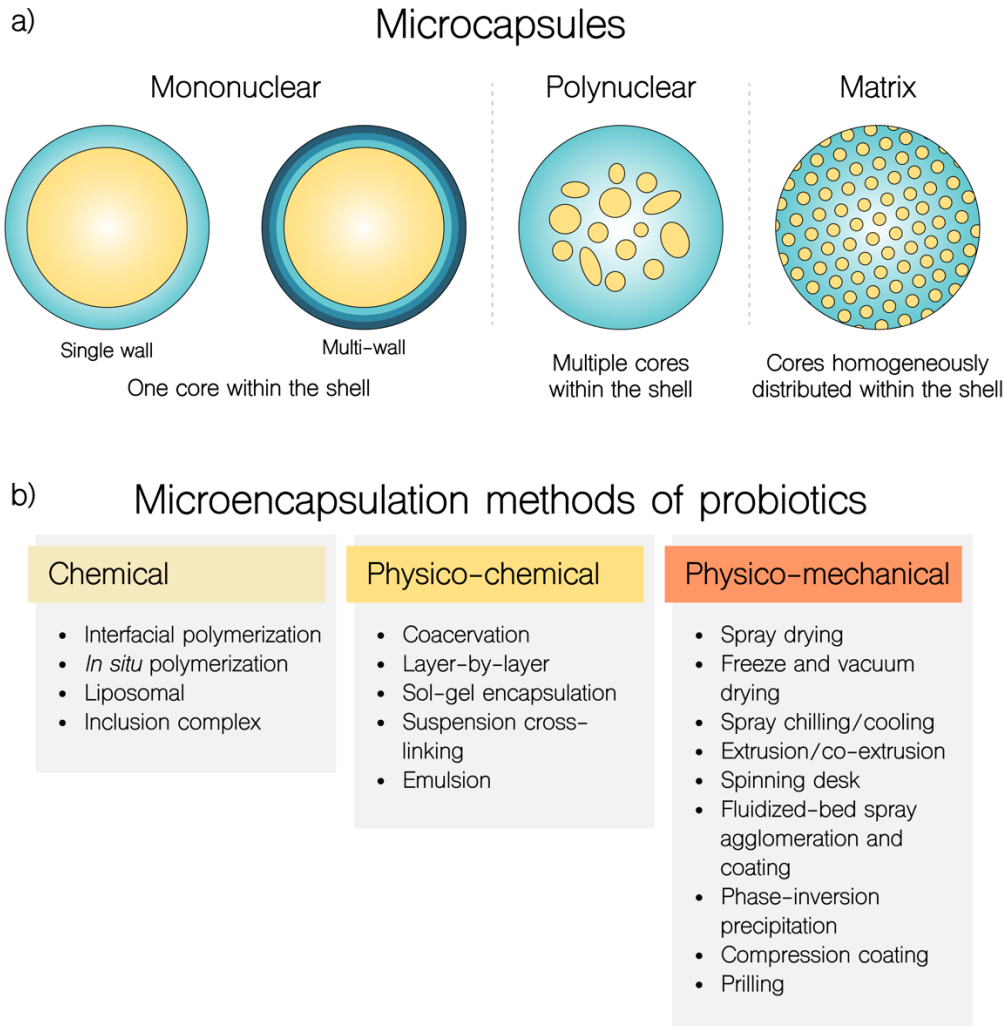

Figure 1-3. Delivery vehicles of live microorganisms in aquaculture systems. Types of microcapsules **(A)** and commonly used microencapsulation methods for probiotics **(B)**.

1.4.1 Probiotic delivery methods

The route of probiotic administration for aquatic animals has included application directly into rearing water, supplementation of live food (Artemia and rotifers), inclusion in pelleted feed, and use of oral gavage (Masoomi Dezfooli *et al.* 2019). It is important to note, however, that probiotic delivery to the fish through water might reach untargeted aquatic animals, which may raise safety concerns since the effect on these animals is unknown. Having stated that, a detrimental disturbance of the aquatic body is unlikely given the lack of pathogenic attributes of probiotic strains and the considerable inoculum that would be needed.

Oral gavage and injection routes are labour-intensive and time-consuming processes, which incur additional costs and stress to the animals. The use of probiotic-coated pelleted feed would enable dosage control of the viable number of administered organisms, thus preventing loss and untargeted delivery. As such, coated pelleted feeds are a more suitable and economical probiotic delivery method to reach the fish intestine. Importantly, incorporating the organisms in the feed has to be achieved without compromising their viability in the nutrient formula and during exposure to the water. Further research is required to optimize this process and monitor the enclosure to make sure the less aggressive feeders still acquire the probiotic strains in adequate amounts.

1.4.2 Effect of acid, bile, and storage conditions on probiotics

One criterion for selecting a probiotic is the ability of the organism to survive in the low gastric pH (1.0-3.5) and intestinal bile salt environment (Merrifield *et al.* 2010). The gastric pH of rainbow trout before feeding is ~2.7 (Bucking and Wood 2009), which is low enough to kill bacterial strains unless they have resistance traits or are in a delivery

vehicle or coating that protects them. Many strains of *Lactobacillus acidophilus* and *Bifidobacterium* spp. are unable to survive at low pH and bile salt-rich environment (Shah *et al.* 1995). Even yoghurt-producing bacteria *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* often fail to reach the small intestine with robust viability (Desai 2008). Similarly, proper storage conditions of probiotics in dry and cool environments are required to ensure stability of the viable count. These issues have led to some spore-forming bacteria being used as probiotics, as they can retain stability in a range of environmental conditions. However, verification that the strains come out of their spore form and provide a benefit needs more documentation.

1.4.3 Encapsulation strategies and delivery systems

Encapsulation is a strategy that uses a shell or coating material to protect and preserve the active ingredients (probiotics, in this case). The size and shape of the encapsulated ingredient may vary depending on the coating material and technique used (**Figure 1-3 A**). The concept is to protect the organisms during storage and passage through the stomach and ensure controlled release of the strains in the small intestine or colon (Berkland et al., 2004; Anal and Singh, 2007; Champagne and Fustier, 2007; Wagdare et al., 2010). The use of capsules for fish presents its own challenges, such as the small size of young salmon, exposure to lake or sea water, and the rate at which the organisms need to be released inside the host. The controlled release of probiotics based on pH or other physiological conditions of the intestinal segments may provide an opportunity for the encapsulation material to site-specific release of the contents in the gastrointestinal tract.

There are a variety of encapsulation techniques available, which are generally prepared by three methods *viz*. chemical, physico-chemical, and physico-mechanical techniques (**Figure 1-3 B**). Often, these methods are combined. Among these, spray-drying is the most common, economical, and effective technique for production of food ingredients, however it has not been widely used for the encapsulation of probiotics (Chavarri et al., 2015). Freeze-drying is the preferred and least harmful drying method for probiotics, but it is more costly relative to spray-drying (Chávez and Ledeboer 2007). Innovative approaches that strive to maintain the viability of live active ingredients while remaining economically feasible are of interest to the aquaculture industry.

1.4.4 Encapsulating materials for probiotics

Biomaterials are often used in capsule formation as they are biocompatible, widely available, and have minimal to no effect on probiotic viability and host health (Solanki *et al.* 2013). Biomaterials are defined as "natural or engineered synthetic macromolecules, which can be used to establish interaction with biological systems for therapeutic or diagnostic purposes". The chemical structure and the conformation of the monomer units provide specific functionality, such as cross-linking to form gels (Renard and Reddy 2007). Over the past few decades, biopolymer alginate has been used extensively for the encapsulation of probiotics (reviewed by Shori, 2017). Generally, calcium alginate is suitable for encapsulation due to its simplicity, non-toxicity, biocompatibility and low cost (Krasaekoopt, Bhandari and Deeth 2003). However, alginate beads are sensitive to the acidic environment, an obvious flaw when placed into gastric pH (Mortazavian *et al.* 2008). Furthermore, the impact of the alginate on the intestinal microbiome remains to be shown. Hydrogels, such as alginate-chitosan,

alginate-whey protein, alginate-pea protein, and alginate-gelatin are also used to encapsulate probiotic strains (Kwiecien and Kwiecien, 2018).

Other biomaterials such as κ-carrageenan, gelatin, chitosan, whey proteins, cellulose acetate phthalate, locust bean gum, and starches have also been explored (Anal and Singh 2007; Shori 2017). In one study, strains of *L. rhamnosus, B. longum,* and *L. acidophilus* entrapped in κ-carrageenan microcapsules showed higher acid and bile tolerance (Ding and Shah 2009). K-carrageenan-based hydrogels have been successfully used as delivery systems for lactobacilli*, Streptococcus,* and *Bifidobacterium* species (Kwiecień and Kwiecień 2018). Xanthan-alginate, xanthan-alginate-chitosan, xanthan-chitosan, xanthan–chitosan–xanthan, pectin, pectin–chitosan, pectin–whey protein, pectin–rice bran extract have also been used (Kwiecień and Kwiecień 2018). Chitosan has antimicrobial properties, hence it is not suitable for the encapsulation of probiotics (Sonia and Sharma 2011). Interestingly, a carboxymethyl cellulose-chitosan hybrid showed improved viability under simulated gastric conditions (Singh *et al.* 2017).

Among nanomaterials, cellulose nanocrystals (CNCs), starch nanoparticles, and silica nanoparticles, in combination with other materials, are being used for encapsulation of probiotics. Microcapsules consisting of alginate-CNC-lecithin showed greater viability of *L. rhamnosus* ATCC 9595 after passing through simulated gastric juice (Huq *et al.* 2017). Resistant starch, glucomannan, shellac, cellulose acetate phthalate (CAP), and κ-carrageenan are promising materials for encapsulation of probiotics when used with alginate (Cui *et al.* 2018). Probiotic strains can also be encapsulated using a colloidal system that comprises a dispersed phase in a liquid medium. There are different colloidal systems, such as oil-in-water (O/W), water-in-oil (W/O), water-in-water (W/W), and

water-in-oil-water (W/O/W) being investigated for the maintenance of strain viability (Vemmer and Patel, 2013; Picone et al., 2017; El Kadri et al., 2018; Singh et al., 2018).

The hydrophobic, hydrophilic, and amphiphilic nature of these nanomaterials determines how they are used to incorporate the organisms into the capsule. The nanocapsules are made using nanoparticles that are formed into particles that are actually micrometres in size in order to accommodate the microorganisms. The material is generally polymeric and selected based on diffusion, degradation, pH or temperature of the target site. The lack of availability of low-cost, generally regarded as safe (GRAS) nanomaterials that have minimal interference with probiotic strains, have limited their widespread application. To address these limitations, lipids are being considered as carrier for lipophilic pharmaceuticals, which are also described as solid lipid nano particles (SLNPs). The vehicle promotes the rapid absorption of the pharmaceuticals through lipolysis in the small intestine (Souto and Müller 2010). Further, nanostructured lipid carriers (NLCs) with lipids in the liquid form offer more versatility due to their broad physico-chemical properties compared to SLNs.

The delivery of probiotics using nano-gels and poly-electrolyte complexes have also shown promise. Nanogels are prepared using various methods of copolymerization of hydrophilic or water-soluble monomers in the presence of difunctional or multifunctional cross-linkers (Oh, Bencherif and Matyjaszewski 2009). Polyelectrolyte complex-mediated delivery involves electrostatic forces between biopolymers, which then contribute to the coating of probiotic-loaded microcapsules (Borges and Mano, 2014; Anselmo et al., 2016; Singh et al., 2017). Recently, Luan et al. (2018) developed a cellulose-based composite macrogel using cellulose fiber/cellulose nanofiber (CCNM)

for the delivery of probiotic strains to the intestine. Research is needed to delineate the effect of these substances on the host microbiota and to determine if all the nanoparticles are excreted.

Clearly, there is room for more research and development of methods to produce cost-effective, eco-friendly materials that deliver probiotic organisms to the fish gut to ensure that these strains confer measurable benefits on the host.

1.5 Hypothesis and objectives

Given that the intestinal mucosa plays a central role in host homeostasis and that host-microbe interactions can profoundly impact host health, it was hypothesized that microbial modulation of tight junctions and immune function may lead to improvements in survival and growth in Chinook salmon. To address this hypothesis, two complementary objectives were devised. First, an *in vitro* model of the salmonid gut was developed to study the mechanism of host-microbe interactions. The gold standard salmonid intestinal epithelial line, RTgutGC, was employed, and several coincubation experiments were performed to determine the molecular and physiological response of the cells to exposure with candidate fish probiotics and/or the marine pathogen, *V. anguillarum*. Second, to further validate the findings in the organism under study, an in vivo experiment was carried out in Chinook salmon, in which the animals were fed with the candidate beneficial microbes and then injected with live V. anguillarum. The purpose of the in vivo study was to determine the organismal response to the lactic acid bacteria and investigate whether the beneficial strains could mitigate the deleterious effects of the pathogenic infection. The goal of this research is to investigate the use of

candidate fish probiotics as an alternative strategy to reduce losses related to infectious diseases in the aquaculture sector.

1.6 Summary and prospects of fish probiotics

Host-microbe interactions at the intestinal mucosal interface display pleiotropic links with organismal health and are directly correlated with productivity in aquaculture animals. The rich microbial environment within and around salmonids cannot be overlooked as a key factor in the health and wellness of fish. Research over the past two decades, including novel modeling systems, has begun to unravel the key mechanisms and pathways through which probiotic microbes can be applied to this milieu and contribute health benefits. The key is to carefully select strains, study their properties, and administer them in an optimal formulation. Then, prove they confer a benefit in field trials complemented by high-resolution, high-throughput, and multi-omics analyses. To progress further, increased attention should be given to the metagenome and the functional state of the microbes to restore and maintain health. In order for probiotic strains to modulate the immune response effectively, studies are needed to better understand the direct interaction of bacterial cell wall components and metabolites with host receptors. The importance of delivery vehicles that ensure the viability and activity of the living therapeutics cannot be overstated. This is a critical area of research that has enormous potential for the aquaculture industry, particularly given the transient nature of the fish microbiome.

There is a degree of urgency to reduce losses in the aquaculture sector. The increasing demand of fish for human consumption is offset by an inability to control long-standing pathogens at a time when water pollution and competition for natural

resources are mounting. This is a juncture where bringing together expertise in microbiology, fish research, genetics, behavioural science, chemical engineering, and synthetic biology is essential to maximize production sustainably.

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Chapter 2

2. Investigation of host-microbe interactions in an *in vitro* model of the salmonid gut

2.1 Abstract

Host-microbe interactions are central to fish health and aquaculture's productivity. Given the immense burden of infectious diseases in the industry, novel microbial management strategies are imperative to ensure the sustainable intensification of production. The use of tissue culture models to investigate the mechanism underlying microbial modulation of fish health has not yet been explored in the context of probiotic microorganisms. The purpose of the present study was to assess the applicability of the salmonid intestinal epithelial cell line RTgutGC for the investigation of host-microbe interactions and to elucidate the mechanism through which the candidate probiotic strains modulate gut barrier function and protect from pathogenic organisms, such as *Vibrio anguillarum*. The expression of several key tight junction (TJ) and immune molecules was assessed, along with changes in transepithelial electrical resistance (TEER) given by the cell monolayer. While the candidate probiotic strains did not significantly upregulate tight junction molecules, pre-treatment with the microbes protected against pathogen-induced insults to the barrier. The expression of *occludin* was significantly induced by *V. anguillarum*, and this molecule might be implicated in the pathogenesis of this organism. Pre-treatment with lactic acid bacteria did not substantially alter the expression of TJs or immune molecules. The RTgutGC model provided a new means to identify candidate probiotic strains for salmonid aquaculture.

2.2 Introduction

Aquaculture is steadily growing and will need to intensify its production to meet growing global demands (FAO 2020). As such, greater attention has been given to factors that can help promote animal health in farmed fish, particularly salmonids, which constitute the most economically important family of finfish. Infectious diseases pose a huge threat to the industry (Groff and LaPatra 2000). The fish gut is thought to be a primary site for pathogen attachment, proliferation, and entry into the bloodstream (Olsson *et al.* 1996). The intestine is a key site for digestion and nutrient absorption, immune modulation, osmoregulation, and acting as a barrier against pathobionts (Dawood 2021). Unfortunately, pathogens can infect the host through damaging the intestinal lining. For example, *Vibrio anguillarum* is a common but deadly aquatic pathogen that afflicts farmed fish worldwide, causing substantial financial losses (Lafferty *et al.* 2015). This Gram-negative aquatic halophilic bacterium uses an arsenal of virulence factors to infect its host (Frans *et al.* 2011). The current proposed mechanism of infection suggests that the pathogen colonizes the skin and gills, but it is not until it reaches the intestine that it expresses its array of virulence factors that can cause injury to the epithelial lining (Denkin and Nelson 1999; Weber, Chen and Milton 2010). The damage caused thus opens the 'door' to bacterial translocation from the intestinal lumen to the lamina propria, and from there, to portal blood, therefore causing haemorrhagic septicaemia and death (Cisar and Fryer 1969). Although this is the proposed mode of entry based on the current understanding of *V. anguillarum*'s pathogenesis, mechanistic studies seeking to characterize the host response at the intestinal epithelial interface are lacking, as are studies on how non-pathogens might interfere with this process.

The current disease containment strategies employed by the industry are either inadequate (*ie*. antibiotics contribute to antimicrobial resistance; Watts *et al.* 2017) or insufficient (*ie*. vaccines offer limited protection; Dixon 2012; Figueroa *et al.* 2020). Given these constraints, interest has grown in the use of eco-friendly microbial therapeutics, such as probiotic supplementation, to mitigate and prevent infections in farmed fish (Ringø *et al.* 2020).

Several studies using different probiotic strains have reported health benefits in salmonids such as stress mitigation, reduction in mortality to pathogens, improvements in growth parameters, immune modulation, and homeostasis in the commensal microbiota (Merrifield *et al.* 2010; Fečkaninová *et al.* 2017; Gonçalves, Valenzuela-Muñoz and Gallardo-Escárate 2017; Al-Hisnawi *et al.* 2019; Gupta *et al.* 2019). The source of these microbes can differ, from human-derived strains to indigenous isolates from finfish species. The vast majority of candidate probiotics belong to lactic acid bacteria (LAB) genera, which encompass lactobacilli, as well as *Bacillus, Pediococcus*, and *Carnobacterium* spp (Ringø *et al.* 2018).

The selection of strains for use in salmonid aquaculture applications is often based on research in terrestrial host species, especially mammals. However, there are clearly substantial differences in the physiology of terrestrial and aquatic hosts, thus making generalizations challenging. An additional issue is the retention of strain viability from production to the field, and for successful delivery to the fish. Given the size of the losses and the increased demand for farmed fish, there is an urgent need to identify beneficial microorganisms that can effectively contribute to fish health.

The use of models can provide a high-throughput screening system to identify promising fish probiotic candidates. In humans, the Caco-2 colon carcinoma cell line is regarded as the gold standard intestinal epithelial cell model of the small intestine (Hubatsch, Ragnarsson and Artursson 2007). Recent reports on a salmonid intestinal epithelial cell line derived from rainbow trout (*Oncorhynchus mykiss*), RTgutGC, have established the usefulness of this cell line as a model for functional studies on fish feed development based on gut barrier function and immune competence (Kawano *et al.* 2011; Langan *et al.* 2017; Minghetti *et al.* 2017; Wang *et al.* 2019). Furthermore, by employing semipermeable membrane supports (Transwell®), it is possible to recapitulate the intestinal environment *in vitro* and conduct studies on the permeability and integrity of the cell monolayer (Hubatsch, Ragnarsson and Artursson 2007).

The objectives of the present study were to i) establish the RTgutGC model; ii) investigate the effect of candidate probiotic strains on barrier function and the expression of tight junction and immune molecules; iii) determine the extent to which *V. anguillarum* disrupts barrier function; and iv) assess whether probiotic strains can protect the epithelial monolayer integrity against pathogen-induced insults.

2.3 Materials and methods

2.3.1 Tissue culture maintenance

The rainbow trout intestinal epithelial cell line RTgutGC was generously provided by Dr. Brian Dixon's lab at the University of Waterloo. RTgutGC cells were cultured in Leibovitz's 15 media (HyClone, catalogue number: SH30525.01), supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS; Life Technologies, catalogue number: 26140079) and incubated in plates or flasks sealed with Parafilm (Bemis, ACAPM999) at 22ºC and atmospheric conditions. The medium was replaced every 3-4 days and cells were passaged when ≥80% confluent in a 1:2 to 1:4 subcultivation ratio, depending on the downstream experimental application. The RTgutGC cells in T75 flasks (Corning, catalogue number: 353136) were trypsinized by aspirating the medium with a glass Pasteur pipette (Fisher Scientific, catalogue number: 13-678-20A) connected to a vacuum line. Cells were washed with 4mL sterile PBS at room temperature, and residual buffer was aspirated in like manner. Four mL of trypsin (0.05% w/v; Thermo Fisher Scientific, catalogue number: 25200056) was then added and incubated for 10 minutes at room temperature on a flask vortex to facilitate detachment. Cells were monitored periodically using a Nikon inverted microscope to ensure detachment from the plastic. Upon detachment, 8mL of complete culture medium (L-15 + 10% FBS) was added to quench the trypsin protease activity. The suspension was then vigorously pipetted to break up clumps of cells, before transfer of the cells to a 15mL sterile conical tube from which a sample was taken for viable counting using a trypan blue (0.04%; Thermo Fisher Scientific, catalogue number: C10312) exclusion assay and the automated *Countess cell counter* (Invitrogen, catalogue number: C10281) prior to seeding into the cell culture

dishes. Medium renewal was performed 24-48h following trypsinization and seeding into new culture dishes to remove residual trypsin.

2.3.2 Bacterial strains and culture conditions

Bacterial strains were generously provided by Seed Health, Inc. and Lallemand Animal Nutrition, Inc. *Lacticaseibacillus rhamnosus* GG, *Limosilactobacillus rhamnosus* LR06, *Limosilactobacillus reuteri* LRE2, *Limosilactobacillus reuteri* 830, *Limosilactobacillus reuteri* RC-14, *Limosilactobacillus rhamnosus* GR-1, and *Pediococcus acidilactici* 18MA/5M were routinely cultured anaerobically at 37ºC in Mann, Rogosa, and Sharpe (MRS) medium (BD Difco, catalog number: DF0881-17-5). *Bifidobacterium breve* BR03 was routinely cultured anaerobically using a GasPak (BD Difco, catalogue number: 261205) at 37ºC in brain heart infusion-supplemented (BHI-S) culture medium (BD Difco, catalog number: DF0418-17-7), supplemented with yeast extract (5g/L), resazurin solution (25mg/100mL; add 4mL/L), L-cysteine•HCl (0.5g/L), and vitamin K1 (0.2mL/L). The *Leuconostoc mesenteroides* 8293 and *Leuconostoc mesenteroides* 1506 were routinely cultured anaerobically in MRS medium at room temperature (23ºC) for 48h.

Vibrio anguillarum serotype O1 was kindly provided by Dr. Brian Dixon's laboratory at the University of Waterloo. This strain was originally obtained from Pacific Biological Station (PBS) in Nanaimo, BC from Dr. Simon Jones, during an outbreak in winter Steelhead trout (*Oncorhynchus mykiss irideus*) in Little Campbell River, BC. The strain was routinely cultured in tryptic soy broth or agar (TSB/TSA; BD Difco, catalogue number: DF0370-17-3) supplemented with 2% (w/v) sodium chloride. Heat-killed *V. anguillarum* was prepared by transferring 1mL of a liquid culture of the bacterium (TSB

+ 2% NaCl (w/v) inoculated with a single colony and incubated for 24h aerobically at 23ºC in an orbital shaker at 250 rpm) to a 1.7mL tube, which was then centrifuged at 3000 rpm for 3 minutes. The supernatant was discarded and the bacterial pellet was resuspended in 1mL of sterile PBS and incubated at 100ºC for 30 minutes in a bead bath (Bennoit and Craig 2020). Then, 100µl of the heat-killed suspension was plated in TSA + 2% NaCl (w/v) to ensure sterility.

2.3.3 Coculture experiments

RTgutGC cells were cultured in 6- or 12-well plates (BD Falcon, catalogue number: 353046) for at least 3 weeks prior to the experiments to ensure that the cells established the brush border membrane and tight junction complexes. Frozen stocks of LAB and *V. anguillarum* were streaked onto agar plates of the appropriate medium and incubated for 24h. Single colonies were then re-streaked and incubated for another 24h. Fresh single colonies were used to inoculate 3mL of the appropriate growth medium and cultures were incubated for 48h. Assuming that the concentration per area of cells at confluency is approximately 1.3 x 10^5 cells/cm^2, the LAB were diluted to a final multiplicity of bacteria (MOB) of 1:100 gut cells to bacteria, while *V. anguillarum* was diluted to a final concentration of 2:1 MOB. Heat-killed *V. anguillarum* was diluted in like manner. The bacterial suspensions were mixed in the cell culture growth medium and the spent cell culture medium was aspirated using a sterile glass Pasteur pipette connected to a vacuum line. The bacteria were then added to the RTgutGC cells and incubated for various durations. Cells were then harvested at specific timepoints by aspirating the culture medium and adding 1 or 0.5 mL (for 6- or 12-well plates, respectively) of TRIzol reagent (Invitrogen; catalogue number: 15596018), and removed by vigorously pipetting

the RTgutGC cell lysate, which was then transferred to a 1.7mL tube and stored at 4ºC until further processing.

2.3.4 RNA extraction and cDNA synthesis

To prepare RNA for subsequent gene expression analyses, 0.3 volumes of chloroform per 1 volume of TRIzol were added to the RTgutGC cell lysates. Samples were vortexed for 15 seconds, incubated at room temperature for 10 minutes, then centrifuged at 16,000 rpm for 15 minutes at 4ºC. The aqueous layer was collected (being careful not to come near the layer interface) and transferred to a new 1.7mL tube. To improve RNA quality, 0.3 volumes of chloroform per 1 volume of the aqueous layer were again added, and this step was repeated. Samples were kept on ice henceforward.

To the new aqueous fractions, 0.7 volumes of 100% isopropanol per 1 volume of sample were added, vortexed briefly, and incubated at room temperature for 5 minutes. Samples were then centrifuged at 16,000 rpm for 15 minutes at 4ºC. The supernatant was discarded, and residual isopropanol was removed with a pipette (being careful to not disturb the RNA pellet). Then, 1mL of 70% ethanol in nuclease-free water (Invitrogen, catalogue number: 4387936) was added and samples were centrifuged at 16,000 rpm for 15 minutes at 4ºC. The ethanol was decanted, and this step was repeated to improve RNA quality and remove contaminants. The residual ethanol was carefully removed, and pellets were air-dried for 15 to 20 minutes. The RNA was then resuspended in 30µl of warm (56ºC) nuclease-free water and quantified using a NanoDrop spectrophotometer. The RNA concentration was consistently between 100-500ng/µl, depending on the size of well used for the experiments. The cDNA was synthesized from 1µg of the freshly isolated RNA using a High-Capacity cDNA Reverse Transcription Kit, following

manufacturer's instructions (Applied Biosystems, catalogue number: 4368814), for a total volume of 40µl per reaction. Remaining RNA was stored at -80ºC.

2.3.5 Real-time quantitative PCR analysis

Reverse-transcribed cDNA was diluted 10x and used in qPCR reactions with Power SYBR Green Kit (Thermo Fisher Scientific, catalogue number: A46112). The primers used in this study are summarized in **Table 2-1**. For analyses of gene expression, the gene CX6C1 was used as the reference because it was identified to be the most stable reference gene of those tested. The PBS vehicle control groups were used as the endogenous control in all qPCR experiments. Each qPCR reaction had a total volume of 10µl (performed in three technical replicates). Reactions consisted of 4.58µl of diluted cDNA, 0.42µl of primers (forward and reverse primer mix; 14.4µM), and 5µl of Power SYBR Green 2x Master Mix. The PCR reaction conditions were 50ºC for 2 minutes, then 95ºC for 10 minutes, followed by 40 cycles of 95ºC for 15s, then 60ºC for 1 minute. The melt curve stage consisted of 95ºC for 15s, then 60ºC for 1 minute and 95ºC for 15s. The qPCR was performed on a QuantStudio5 Real-Time PCR System (Thermo Fisher Scientific) and analyzed using the associated cloud-based Design and Analysis software (Thermo Fisher Scientific; version 2.5.1). Gene expression ($2^{-\Delta\Delta Ct}$) was calculated using fold change. PCR efficiencies were assessed using the LinRegPCR software version 2016.1 and determined to be above 1.80 **Table 2-1**.

Table 2-1. Summary of RT-qPCR primers used in this study.

Gene name	Accession number	Sequence (5'-3')	Amplicon size (bp)	Reference
CX6C1	FR904651.1	F: GCCTGCAATGCGAGGACTCC R: TTCCTTGGTTCTGTTACGCCGTAC	114	This study
IL-1b	NM_001123582	F: GCTGGAGAGTGCTGTGGAAGA R: TGCTTCCCTCCTGCTCGTAG	73	Wang *et al.* 2020
IL-6	NM_001124657.1	F: GTTCTGGGTGAGGTGTCTA R: GGTGTCAACCAGGAAGTTAC	93	Schug *et al.* 2019
IL-8	NM_001140710	F: ATTGAGACGGAAAGCAGACG R: CGCTGACATCCAGACAAATCT	136	Wang *et al.* 2020
IL-10	NM_001245099.1	F: CCATCAGAGACTACTACGAGGC R: TCTGTGTTCTGTTGTTCATGGC	165	Wang *et al.* 2020
IL-17a	GW574233	F: TGGTTGTGTGCTGTGTGTCTATGC R: TTTCCCTCTGATTCCTCTGTGGG	136	Wang *et al.* 2020
TGFb	EU082211	F: AGTTGCCTTGTGATTGTGGGA R: CTCTTCAGTAGTGGTTTGTCG	191	Wang *et al.* 2020
TNFa	AJ277604.2	F: GTGATGCTGAGTCCGAAAT R: GTCTCAGTCCACAGTTTGTC	97	(Semple *et al.* 2018)
JAM-1a	XM_021564368.2	F: TGAGGATGGAAGTCCGCAAC R: GTACCACAGTCCGAAGCACA	98	This study
Occludin	XM_021601275.2	F: GACAGTGAGTTCCCCACCAT R: AGCTCTCCCTGCAGGTCCTT	101	This study
Claudin 3	XM_021587920	F: AGGCAACGACGCTACATCAA R: GAAACCCAAGCAATGCGTCA	112	Wang *et al.* 2019
Claudin 12	XM_021621241	F: ATCATCGCCTTCATCTCCGT R: TAGCAGCCAGAGTAGCCATC	161	This study
E-cadherin	XM_021585993.2	F: ACTACGACGAGGAGGGAGGT R: TGGAGCGATGTCATTACGGA	107	This study
ZO-1	XM_036963739.1	F: CAAAGCCAGTGTATGCCCAG R: CAGCTTCATACTCGGCCTGA	119	Wang *et al.* 2020

2.3.6 Transepithelial electrical resistance

To determine the change in epithelial electrical resistance given by the effect of different treatments on the cell monolayer, a TEER experiment was carried out. Prior to seeding cells onto the Corning Transwell polyester membrane cell culture inserts (6.5 mm and 0.4µm pore size; catalog number CLS3470), baseline resistance was determined to be 107/0.33cm^2 using a STX2 chopstick electrode connected to a voltmeter. RTgutGC cells (passage number 20-30) were grown in T75 flasks, trypsinized when maximally (>90%)

confluent, and cell counts were performed using the trypan blue (0.04%; Thermo Fisher Scientific, catalogue number: C10312) exclusion assay and the automated *Countess cell counter* (Invitrogen, catalogue number: C10281) prior to seeding into the cell culture dishes. The cells were seeded on semipermeable Transwell membrane supports (Corning Costar Transwell, Millipore Sigma, catalogue number: CLS3470) at a density of approximately 2.6 x 10^5 cells/cm^2 or a final number of about 8,58 x 10^4 cells per insert (cell growth aera of 0.33 cm^2). The cells were cultured for at least 3 weeks prior to the experiment to ensure that they established the brush border membrane and tight junction complexes. To the apical and basolateral compartments 100µl or 500µl, respectively, of L-15 media supplemented with 10% FBS were added, and the medium was replaced every 4-5 days. Periodic inspection of the cell monolayers was carried out using a Nikon inverted light microscope.

Bacterial cultures were prepared as outlined in section 2.3.3 and bacteria-containing cell culture growth medium was added to the RTgutGC cells and incubated for 24h. At the end of the incubation time, culture medium was carefully removed so not to disturb the cell monolayer and the transwell inserts were transferred to a new 24-well plate containing sterile PBS on the basolateral compartment. To the apical compartment, 100µl of PBS were added. Cell monolayers were likewise washed two more times, 100µl of PBS was added to the apical compartment and 500µl to the base of the electrode, and measurements were recorded using a cup electrode collected to a voltmeter. The baseline reading (membrane only) was subtracted from the measurements and the resistance per cm^2 was determined. Statistical analyses were performed on the resistance values per area.

2.3.7 Statistical analyses

All statistical analyses were done using GraphPad Prism software version 7.0a. Nonparametric data were statistically compared with a one-way ANOVA (Kruskal-Wallis) and Dunn's multiple comparisons test. Experiments with two factors were compared with a two-way ANOVA and Tukey's multiple comparisons test.

2.4 Results

2.4.1 Effect of coincubation with LAB on the expression of tight junction and immune molecules in RTgutGC cells

After a 24h incubation period, *L. reuteri* LRE2 exhibited a nonsignificant increase in the expression of *zo-1* and *claudin-3* (**Figure 2-1 B, C**), whereas *L. reuteri* 830 and *L. rhamnosus* GR1 with *L. reuteri* RC14 caused a downregulation in all TJ molecules tested (**Figure2-1 B-D**). *L. rhamnosus* GG elicited a modest increase in *claudin-3* expression, but this was not observed with *zo-1* or *claudin-12* (**Figure 2-1**).

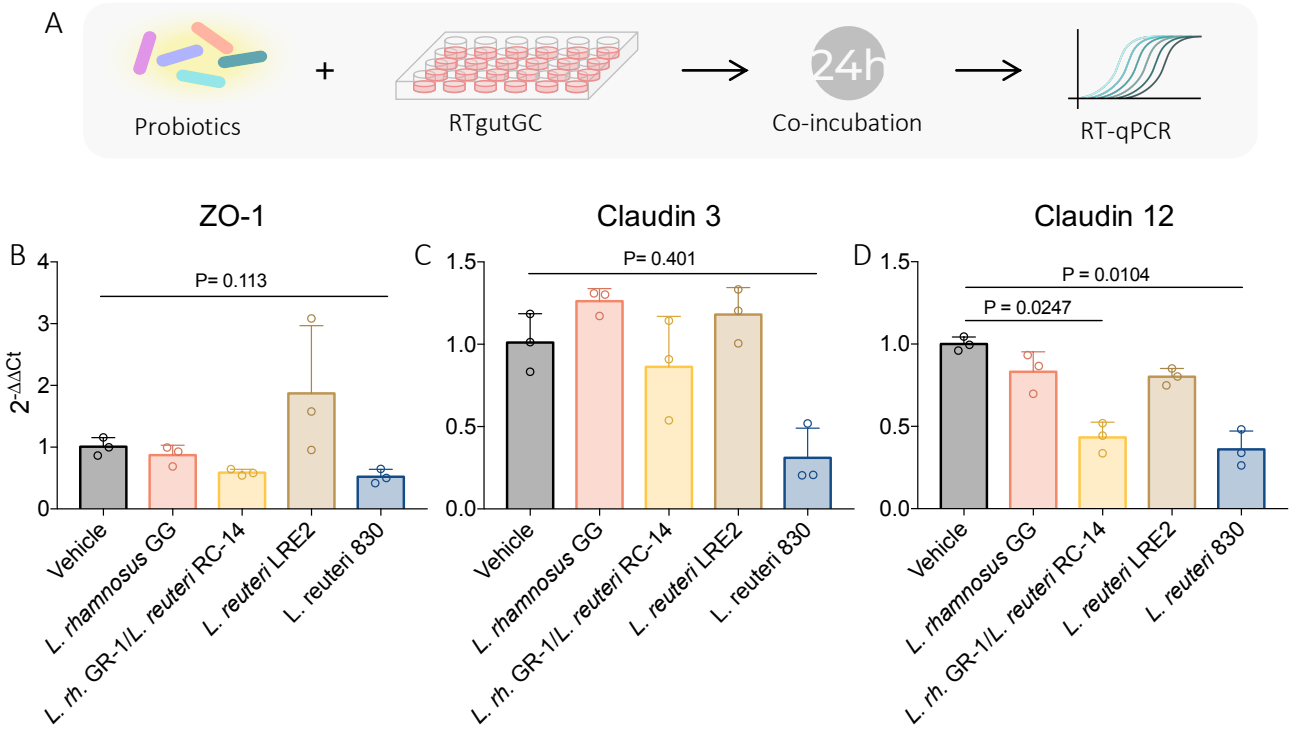

Figure 2-1. Effect of LAB exposure on the expression of key TJ molecules in rainbow trout gut cells. RTgutGC cells were incubated with lactobacilli (MOB 1:100; ~7.5x10^8 CFU/mL) for 24h and gene expression was measured using RT-qPCR. All data are expressed as mean values ± SD (n=3). Transcript abundance was normalized to b-Actin as per MIQE guidelines. Statistical significance was determined by a non-parametric one-way ANOVA (Kruskal-Wallis) and Dunn's multiple comparisons test.

To further examine whether other LAB could promote gut barrier integrity in the established RTgutGC *in vitro* model, a 48h endpoint coincubation experiment was performed using differentiated RTgutGC cells (Pumputis *et al.* 2018). The LAB strains were chosen based on reports suggesting their barrier-promoting properties (Del Piano *et al.* 2010), extensive use in aquaculture settings (Al-Hisnawi *et al.* 2019), as well as indigenous *Leuconostoc* isolates obtained from wild Chinook salmon guts. Namely, the strains selected were *Bifidobacterium breve* BR03, *Lacticaseibacillus rhamnosus* LR06, *Limosilactobacillus reuteri* LRE2, *Pediococcus acidilactici* 18MA/5M, *Leuconostoc mesenteroides* 8293, and *Leuconostoc mesenteroides* 1506. The exogenous LAB did not cause a significant change in the expression of the tight junction genes assessed (*claudin-3*, *zo-1*, and *claudin-12*). However, exposure to the indigenous *Leuconostoc* spp. resulted in a significant decrease in the expression of the TJ molecules examined (**Figure 2-2 B-D**). The exogenous LAB strains also did not induce changes in expression of key proinflammatory cytokines, whereas the *Leuconostoc* strains did (**Figure 2-2 E**).

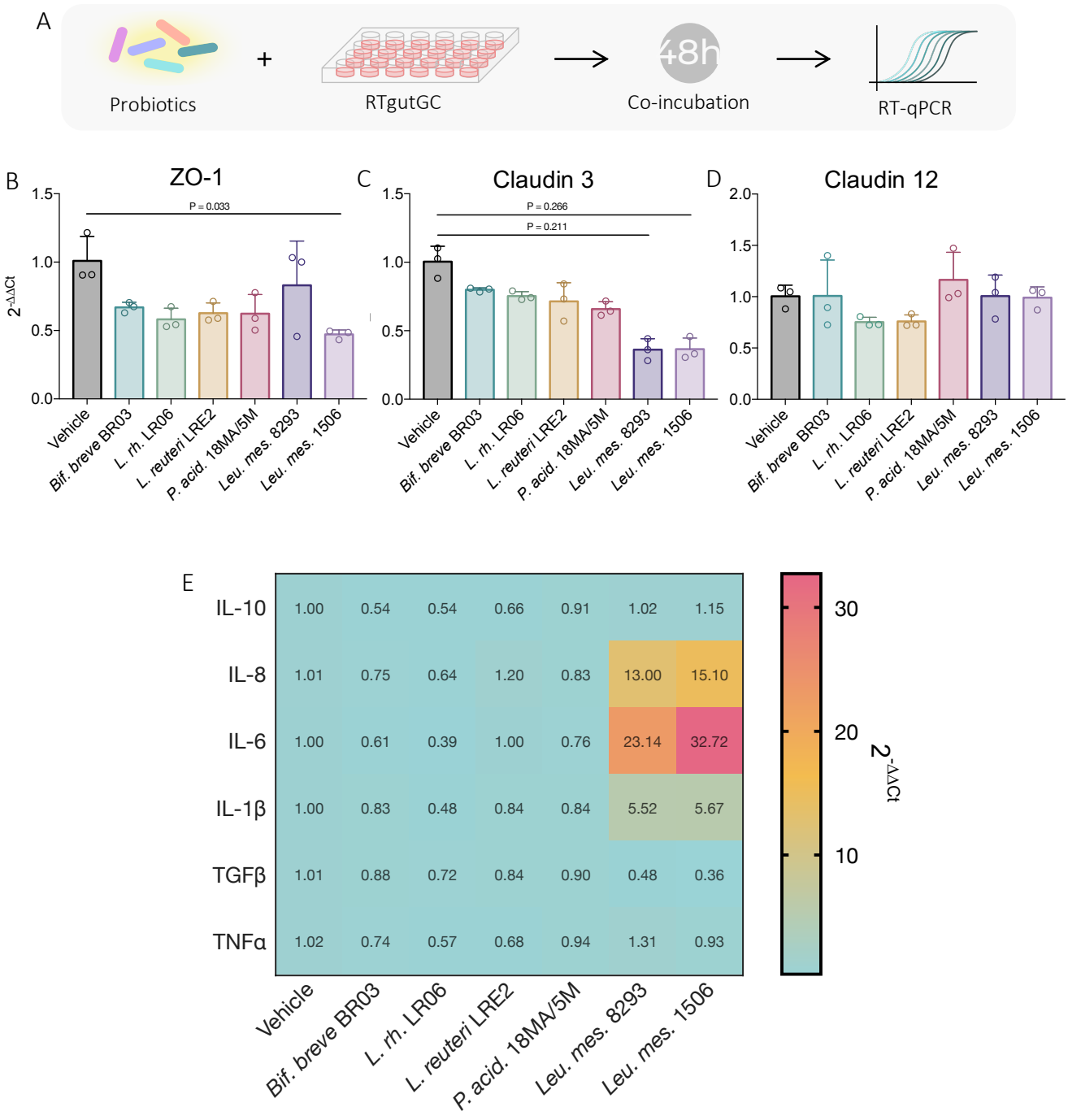

Figure 2-2. Effect of LAB coincubation on the expression of key TJ molecules in rainbow trout gut cells. RTgutGC cells were incubated with LAB for 48h (MOB 1:100; ~7.5x10^8 CFU/mL) and gene expression was measured using RT-qPCR. All data are expressed as mean values ± SD (n=3). Transcript abundance was normalized to CX6C1

as per MIQE guidelines. Statistical significance was determined by a non-parametric one-way ANOVA (Kruskal-Wallis) and Dunn's multiple comparisons test.

2.4.2 Changes in transepithelial electrical resistance in response to LAB and *V. anguillarum* coincubation

To investigate whether the candidate probiotics and/or *V. anguillarum* could modulate the integrity of the epithelial layer, a transepithelial electrical resistance (TEER) assay was performed (Srinivasan *et al.* 2015). Differentiated RTgutGC cells cultured on semipermeable Transwell polyester membrane supports (pore size 0.4µm) were exposed to suspensions of the LAB strains (MOB 1:100; ~$4x10^6$ CFU/mL) or *V. anguillarum* (MOB 2:1; ~$2x10^4$ CFU/mL) in L-15 cell culture media for 24h (**Figure 2-3 A**). *V. anguillarum*, but not the LAB strains, caused a significant decrease in resistance relative to the vehicle control (**Figure 2-3 B**).

To determine whether pre-treatment with the LAB strains could protect the cell monolayer against the pathogen-induced damage to the intercellular tight junctions, slight modifications to the aforementioned experimental design were performed. Briefly, the same LAB strains were grown and added to the apical compartment of the membrane inserts in like manner for 48h. Then, *V. anguillarum* (MOB 2:1; ~$2x10^4$ CFU/mL) was added for 24h and TEER measurements were taken at the end of the incubation period (**Figure 2-3 A**). There were no statistically significant differences in resistance in either of the LAB-pretreated groups, despite the addition of the pathogen. However, a statistically significant decrease in resistance ($P = 0.0265$) was observed in the group incubated with *V. anguillarum* only (**Figure 2-3 C**).

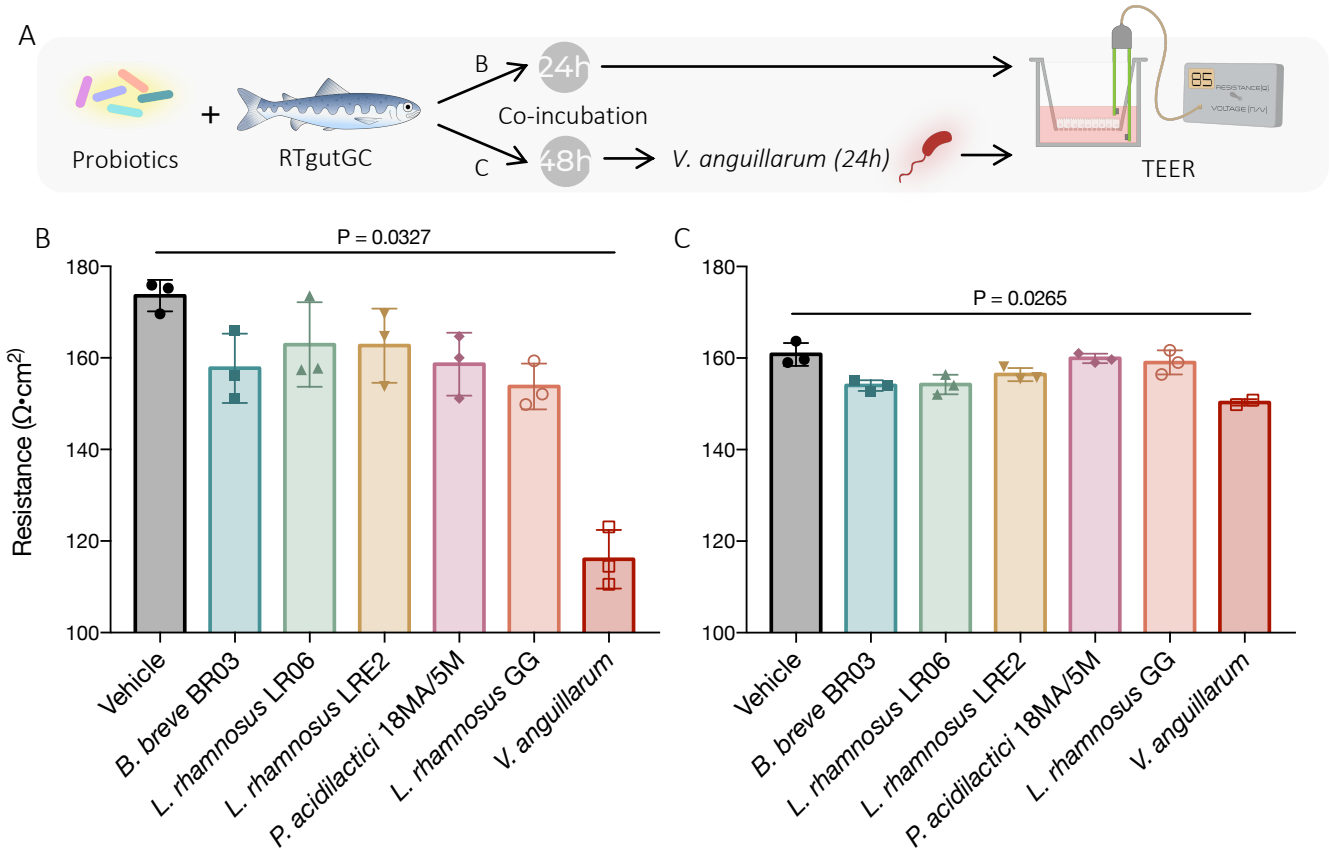

Figure 2-3. Transepithelial electrical resistance in response to exposure to candidate probiotic strains or *V. anguillarum*. RTgutGC cells were seeded on Transwell semipermeable transmembrane supports and bacteria were added to the apical compartment for 24h. The vehicle group was not exposed to bacteria at any time, and the *V. anguillarum* group was incubated only with the bacterium for the latter 24h of the experiment. TEER was determined based on the resistance given by the monolayer per area, normalized to the blank measurement. Statistical significance was determined by a non-parametric one-way ANOVA (Kruskal-Wallis) and Dunn's multiple comparisons test.

2.4.3 Effect of exposure to *Vibrio anguillarum* on the expression of tight junction and immune molecules

To characterize the response of RTgutGC cells to live or heat-killed (HK) *V. anguillarum*, a time-course coincubation experiment was carried out. Samples were

collected at 0, 3, 6, and 24h. Of the TJ-related molecules assessed, there was a significant downregulation of *e-cadherin* and *JAM-1a*, but not *occludin*, which had a puzzling upregulation by 24h (**Figure 2-4 B-D**). Of the cytokines assessed, all exhibited a time-dependent upregulation, which was statistically significant at the 24h timepoint relative to the 0h control group (**Figure 2-4 E-G**). In the case of *il-8*, but not *il-17a* or *tgf-β*, the upregulation was observed in cells exposed to both live and HK bacteria. In an independent replicate of this experiment, a similar time-dependent response was observed, in which there was a significant upregulation of *il-1β*, *il-8*, and *tnfα* at the 24h timepoint for groups exposed to live bacteria (data not shown).

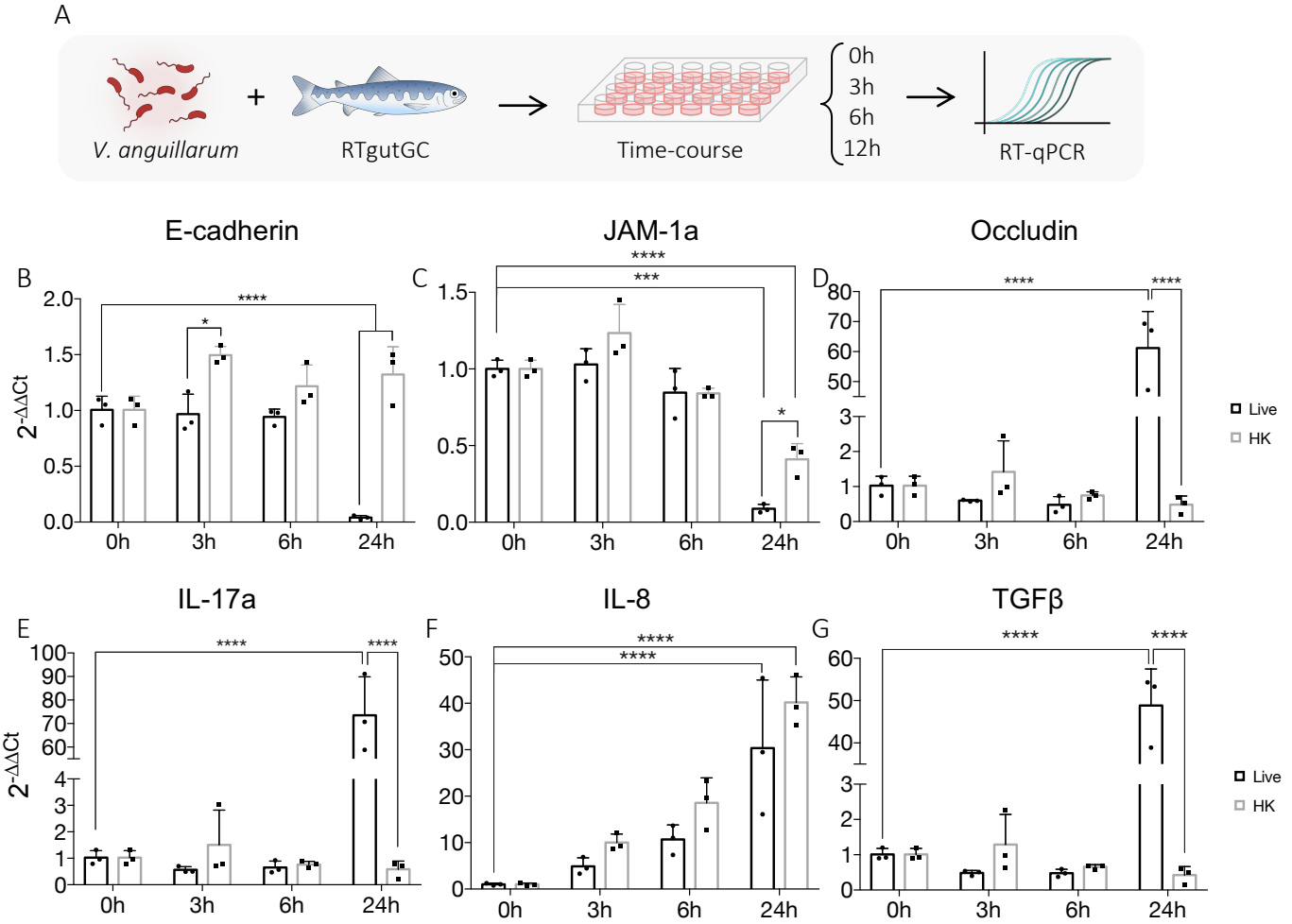

Figure 2-4. Time-course analysis of salmonid intestinal cells to live or heat-killed *V. anguillarum.* RTgutGC cells were exposed to either live or HK bacteria (2:1 MOB at the time of inoculation) and gene expression was measured using RT-qPCR. All data are expressed as mean values ± SD (n=3). Transcript abundance was normalized to CX6C1 as per MIQE guidelines. Statistical significance was determined by a non-parametric two-way ANOVA and Dunn's multiple comparisons test. (**** P<0.0001, *** P=0.0001, * P=0.0493)

2.4.4 Effect of pre-treatment with LAB and exposure to *V. anguillarum* on the expression of immune and tight junction molecules

To examine the potential use of LAB as a disease prevention strategy by stimulating immunity and gut barrier function, a time-course coincubation experiment was executed. Briefly, differentiated RTgutGC cells were pre-treated with LAB strains for 48h and then exposed to *V. anguillarum*. Samples were taken at 0h, 3h, 6h, and 12h after infection with the pathogen and the relative expression of key TJ and immune molecules was assessed through RT-qPCR (**Figure 2-5 A**). The LAB strains selected were *B. breve* BR03, *L. rhamnosus* LR06, *L. reuteri* LRE2, *L. rhamnosus* GG, *L. rhamnosus* GR1, *P. acidilactici* 18MA/5M, and the indigenous isolate *L. mesenteroides* 1506.

The expression of *zo-1* was significantly increased at the 12h timepoint in cells pre-treated with *L. reuteri* LRE2 and indigenous isolate *L. mesenteroides* 1506 relative to the baseline control (T=0h pre-treatment control group). Additionally, *zo-1* was significantly upregulated at the 12h timepoint relative to the expression level at 6h in groups pre-treated with *L. rhamnosus* GG, *P. acidilactici* 18MA/5M, and *L. mesenteroides* 1506 (**Figure 2-5 B**). The expression of *claudin-3* was significantly increased at the 6h timepoint relative to levels at 0h in groups pre-treated with *B. breve*

BR03, *L rhamnosus* LR06, *L. reuteri* LRE2, and *L. rhamnosus* GR1, as well as in the control group. There was also a significant decrease in expression between the 6h and 12h timepoints in the control and *L. reuteri* LRE2 groups (**Figure 2-5 C**). There were no statistically significant differences in the expression of *e-cadherin* at between treatment groups or timepoints (**Figure 2-5 D**). The expression of *jam-1a* was significantly increased between the 3h and 6h timepoints in the *L. rhamnosus* LR06 group, but subsequently decreased by 12h. There was also a significant downregulation of the molecule between 0 and 12h in the *P. acidilactici* 18MA/5M group (**Figure 2-5 E**).

The expression of the pro-inflammatory cytokines IL-8 and IL-17a was also assessed. There was an extremely significant upregulation of *il-8* in all groups at the 12h timepoint relative to the baseline control (**Figure 2-6 A**). The expression of *il-17a* was upregulated at the 3h timepoint in the *L. rhamnosus* LR06 and *L. rhamnosus* GG groups relative to the baseline control and to the levels at 0h (**Figure 2-6 B**).

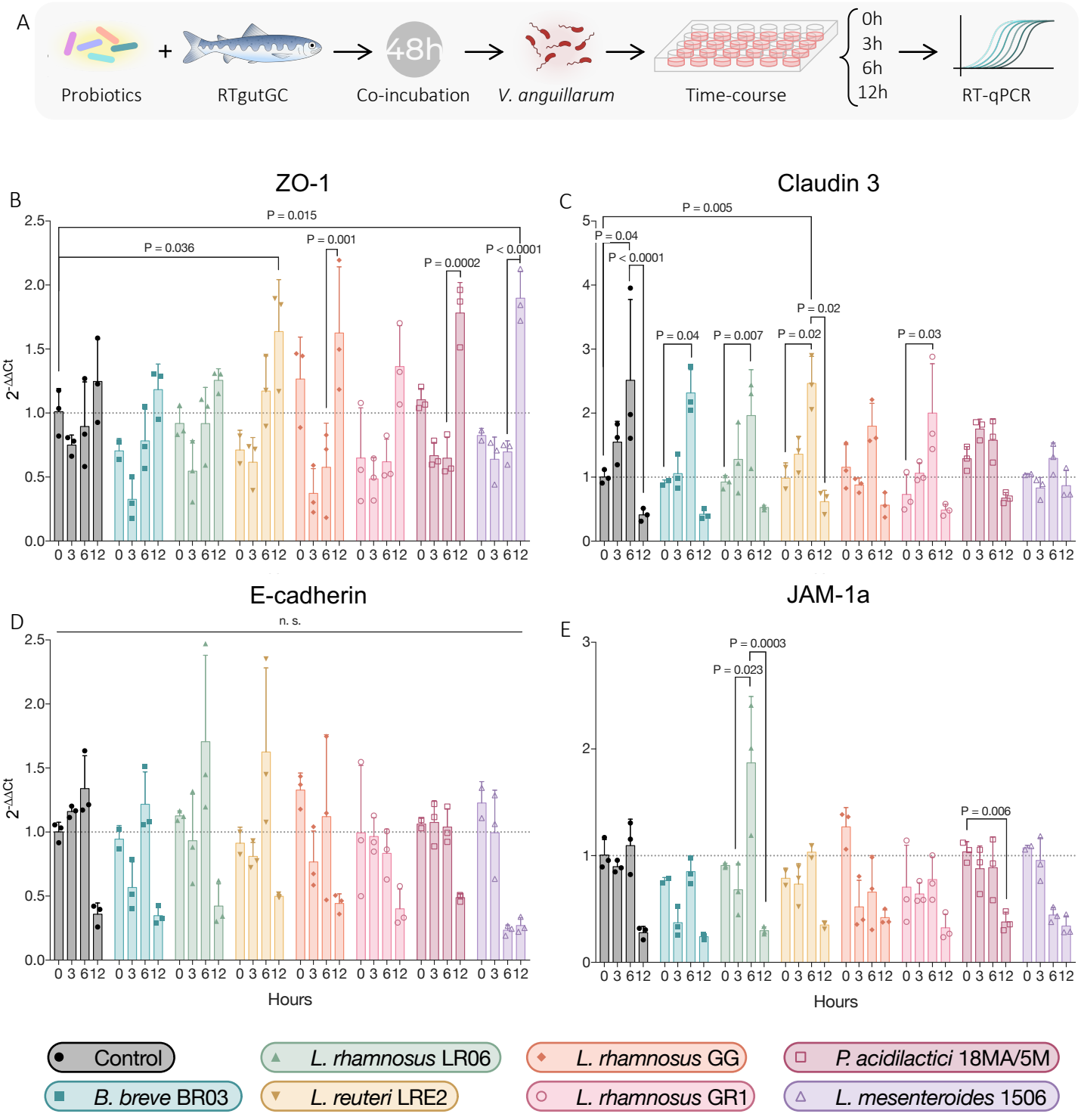

Figure 2-5. Effect of pre-treatment with select LAB strains and *V. anguillarum* on the expression of key tight junction molecules. RTgutGC intestinal epithelial cells were incubated with LAB for 48h (MOB 1:100; ~7.5x10^8 CFU/mL), then infected with *V. anguillarum* (2:1 MOB at the time of inoculation) and samples were collected at 0, 3, 6,

and 12h post-infection. The control group was not pretreated with LAB at any time and was only exposed to *V. anguillarum* for the latter 12h of the experiment. Gene expression was assessed using RT-qPCR. All data are expressed as mean values ± SD (n=3). Transcript abundance was normalized to CX6C1 as per MIQE guidelines. Statistical significance was determined by a two-way ANOVA and Tukey's multiple comparisons test.

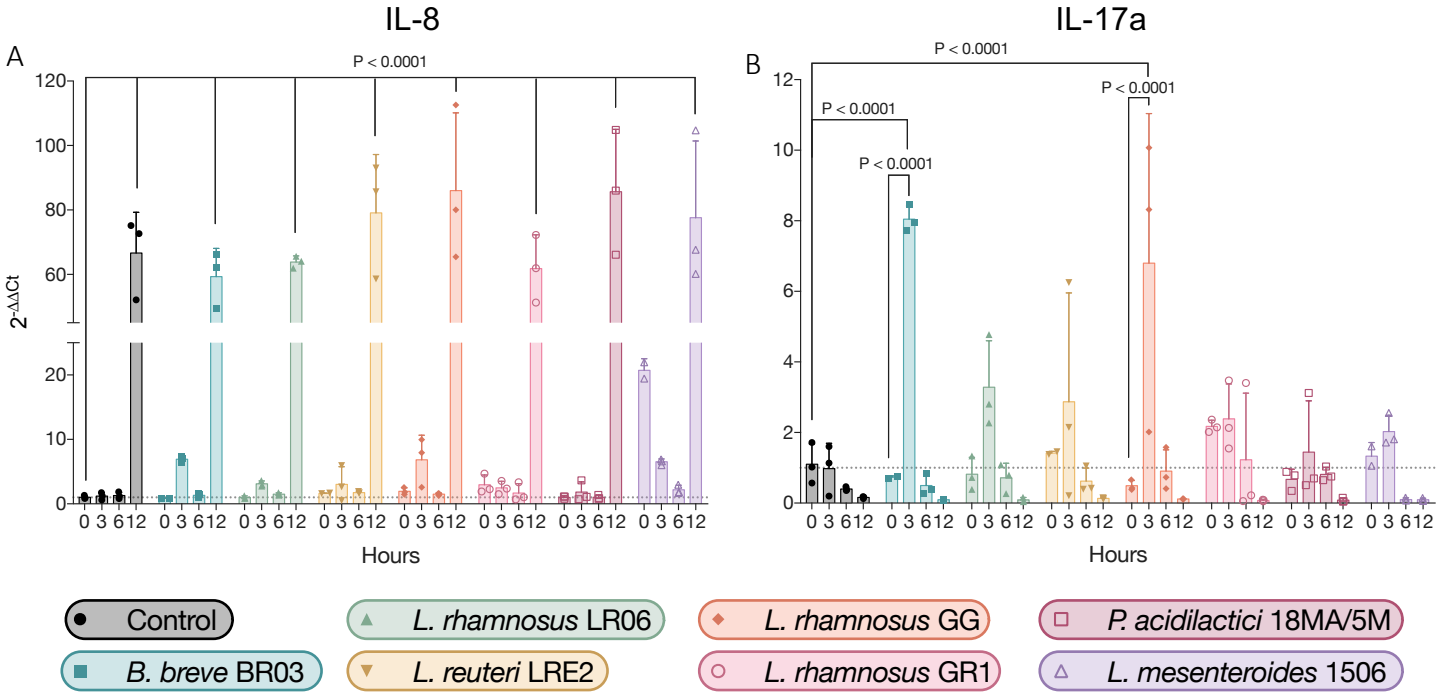

Figure 2-6. Effect of pre-treatment with select LAB strains and *V. anguillarum* on the expression of proinflammatory cytokines. RTgutGC intestinal epithelial cells were incubated with LAB for 48h (MOB 1:100; ~7.5x10^8 CFU/mL), then infected with *V. anguillarum* (2:1 MOB at the time of inoculation) and samples were collected at 0, 3, 6, and 12h post-infection. The control group was not pretreated with LAB at any time and was only exposed to *V. anguillarum* for the latter 12h of the experiment. Gene expression was assessed using RT-qPCR. All data are expressed as mean values ± SD (n=3). Transcript abundance was normalized to CX6C1 as per MIQE guidelines. Statistical significance was determined by a two-way ANOVA and Tukey's multiple comparisons test.

2.5 Discussion

Host-microbe interactions are central to fish health and aquaculture's profitability. However, studies to date are predominantly disease-centric and lack the resolution necessary for elucidating mechanistic links between microbes (beneficial or otherwise) and their aquatic hosts. The pressing need for novel sustainable solutions for mitigating infections makes the use of models appealing as a rapid and cost-effective tool for screening candidate beneficial microbes and for investigating underlying mechanisms. This knowledge is useful because it can inform *in vivo* studies and can provide actionable insights for targeted solutions. The purpose of the present study was to assess the suitability of the salmonid intestinal epithelial cell line RTgutGC as a tool for investigating host-microbe interactions and to evaluate the potential benefits of candidate fish probiotic strains *in vitro*.

The effects of select strains of LAB and the fish pathogen *V. anguillarum* were assessed in RTgutGC cells grown on conventional culture plates and Transwell semipermeable membranes. Co-incubation experiments were performed to study the effects of the bacteria on the host transcriptional response of immune and tight junction molecules. Additionally, transepithelial electrical resistance (TEER) was performed to evaluate the integrity and barrier function of the cells.

This study showed that *L. reuteri* 830, known for its probiotic activity in humans, could significantly decrease in the expression of *zo-1* and *claudin-3* in fish intestinal cells, while *L. rhamnosus* GR1/*L. reuteri* RC14 could decrease the expression of *claudin-12* after 24h (**Figure 2-1**). This indicates that the origin of the lactobacilli may not be critical for their activity to be conferred, at least in relation to the human and fish

intestinal epithelium. A comparative genomic analysis of *L. reuteri* strains suggested that human strains have undergone reductive evolution whereas rodent isolates possess a large and adaptable pan-genome (Frese *et al.* 2011). No fish isolates of *L. reuteri* were examined, but core effects on intestinal epithelia may be conserved across isolates suggesting an important role played by lactobacilli in barrier function irrespective of the host. This implies that use of human probiotic strains still has merit in applications to salmonids.

To further examine the effect of additional exogenous and endogenous LAB strains on the immune and gut barrier properties of the cells, a 48h endpoint coincubation experiment was performed. Interestingly, there were properties unique to the fish isolates. Only the *L. mesenteroides* 1506 and 8293 strains caused significant differences in the expression of barrier molecules *zo-1*, *claudin-3*, and *claudin-12*, which were decreased relative to the control group. The expression of the proinflammatory cytokines *il-8*, *il-6*, and *il-1b* were significantly upregulated by the *L. mesenteroides* strains (**Figure 2-2**).

It is not clear why *Leuconostoc* alone would influence ZO-1 and claudin-3, since these are important in scaffolding and transmembrane TJ proteins, respectively (Tipsmark and Madsen 2012). Claudin-12 is expressed in the salmonid gill and skin (Chasiotis *et al.* 2012; Gauberg, Kolosov and Kelly 2017). But its role in the gut remains unknown (Sundell and Sundh 2012). *Leuconostoc* are human gut constituents, often obtained through dairy consumption (Study *et al.* 2020), but their properties do not appear to be particularly suitable to human applications (Argyri *et al.* 2013).

The upregulation of proinflammatory cytokines IL-6, IL-8, and IL-1β elicited exclusively by the indigenous *Leuconostoc* isolates is suggestive of a response to a

bacterial threat. This observation begs the question of whether these strains are suitable as probiotic candidates for salmonids or if these are potentially harmful. The latter scenario is unlikely, as *Leuconostoc* have not been associated with pathogenic outbreaks in fish. However, the data herein presented indicates that these strains might not be the most appropriate for supplementation, as the excessive immune activation can in turn be detrimental to the barrier integrity and contribute to infection by pathobionts (Suzuki, Yoshinaga and Tanabe 2011). Although some strains with beneficial properties in fish have reportedly elicited an upregulation of proinflammatory markers in key mucosal and immune organs, it is thought that this response is part of an orchestrated effect that ultimately leads to homeostasis. Whether this is the case for the LAB strains tested here is unclear, but future studies would do well to elucidate the mechanism underlying the physiological benefits observed in aquatic species.

Barrier formation and integrity was assessed by TEER measurements, which were in line with levels reported previously (Geppert, Sigg and Schirmer 2016; Minghetti *et al.* 2017; Wang *et al.* 2019). A significant decrease in resistance was observed in cells exposed only to *V. anguillarum* for 24h (**Figure 2-3 B**), but pre-treatment with select LAB strains for 48h prior to exposure to the pathogen mitigated these effects (**Figure 2-3 C**). These results are supportive of using LAB to protect the epithelial barrier against pathogen-induced insults. Improvements in barrier function has been reportedly associated with increased levels of related tight junction gene (*cldn3* and *cdh1*) and protein (Claudin-3) expression (Geppert, Sigg and Schirmer 2016; Minghetti *et al.* 2017; Wang *et al.* 2019). Although the data in the present study is apparently in contrast with

these findings, the effect observed is modest and incubation with LAB protected against pathogen-induced damage but did not increase resistance after a 48h incubation period.

Since the expression of barrier-forming TJ molecules was unchanged, an increase in resistance was not expected (**Figure 2-1 B-D**; **Figure 2-2 B-D**). The significant decrease in resistance given by *V. anguillarum* is expected since the arsenal of virulence factors expressed by this bacterium are believed to degrade the epithelial barrier to gain access to the circulation (Frans *et al.* 2011). Further investigation of this phenomenon through orthogonal methods (such as Lucifer Yellow dye translocation studies) would be useful to ensure that the changes observed are physiologically relevant.

It has been proposed that *V. anguillarum* induces changes in the barrier function by producing proteases and toxins to invade the lamina propria (Olsson *et al.* 1996). Following a time-course coincubation experiment with live or heat-killed *V. anguillarum*, it was found that *e-cadherin* and *jam-1a* were significantly downregulated by 24h post-infection in the live group relative to the heat killed group and the baseline control. Interestingly, the expression of *occludin* was significantly increased in the live group at the 24h timepoint (**Figure 2-4 D**). These data seem to suggest that *V. anguillarum* not only impairs the barrier integrity, but the pathogen can also inhibit the expression of key barrier-forming TJ molecules.

The role of occludin in fish is not well understood, but studies in other organisms suggest that this protein is not only an integral component of tight junctions in various tissues, but that it can also participate in tight junction remodeling in response to cytokines (Van Itallie *et al.* 2010; Sawada 2013). High levels of proinflammatory cytokines, such as TNF-α and IFN-γ, promote the endocytosis of occludin in the tight

junction complexes, which coincides with increases in tight junction permeability (Yu and Turner 2008). Moreover, cytokine-induced changes in TEER and flux are directly proportional to occludin levels (Van Itallie *et al.* 2010). Paradoxically, the results in the present study appear to be at odds with the observations previously reported, in which the increased expression of *occludin* given by live *V. anguillarum* (**Figure 2-4 D**) exposure is associated with a decrease in resistance (**Figure 2-3 B, C**). Further validation of this observation is required, but these results potentially indicate that occludin might potentiate the inflammatory response and exacerbate the damage to the epithelial lining during infection in the salmonid gut (Van Itallie *et al.* 2010).

Tight junctions have complex regulatory networks that dynamically respond to physiological stimuli (Sawada 2013). Therefore, post-transcriptional and post-translational modifications can profoundly impact the biological function of the junctions. Analyses that consider not only the molecular phenomena impacting barrier function, but also the dynamic nature of these intercellular junctions, would be instrumental in understanding how the gut epithelium responds to threats and activates immune defence mechanisms.

There was a robust upregulation of *il-8* and *tgfb* assessed by the 24h timepoint for cells incubated with live *V. anguillarum*. Notably, there was also a time-dependent increase in the expression of *il-8* for both live and heat-killed groups, and these levels were significantly higher by 24h. These results indicate that IL-17a and TGFβ are involved in the response to secreted virulence factors, whereas IL-8 might be more implicated in the response to cell wall components such as lipopolysaccharides (LPS).

The upregulation of results are in line with the proposed mechanism of IL-8 induction given by LPS in other organisms (Yan *et al.* 2017).

The two human isolates, *L. rhamnosus* LR06 and *L. rhamnosus* GG upregulated expression of *il-17a* (**Figure 2-6 B**). In mammals, IL-17a is produced by a subset of T helper cells that induce the production of antimicrobial peptides, among other proinflammatory molecules (Iwakura *et al.* 2008). Host stimulation by LPS, peptidoglycans, and other antigens through pattern recognition receptors enables antigen-presenting cells to activate naïve T cells that mediate the adaptive immune response to the threat (Iwakura *et al.* 2008). Increased expression of IL-17a is also related to increased permeability of the blood brain barrier and small intestinal epithelial barrier (Rahman *et al.* 2018). In the context of the present study, pathogen-induced upregulation of IL-17a might enhance the damage to the epithelial barrier and thus contribute to the establishment of the infection. Therefore, it is puzzling that two probiotic strains also upregulated *il-17a* in intestinal epithelial cells.

Pretreatment with LAB has been associated with protection from pathogen-induced injury to the intestinal epithelium *in vitro* (Karimi *et al.* 2018). The data presented here suggest that the LAB strains tested have a mild effect in preventing pathogen-induced changes in the expression of key barrier proteins. Slight differences in trends of expression of *zo-1* were observed in groups pre-treated with *L. reuteri* LRE2, *L. rhamnosus* GG, *P. acidiliactici* 18MA/5M, and *L. mesenteroides* 1506 relative to the pre-treatment control group (ie. *Vibrio* only), indicating that these strains can potentially induce the expression of this key tight junction molecule in spite of the presence of the pathogen. The trends in *claudin-3* expression were different to the control only in the *L.*

mesenteroides 1506 group. Further studies to explore *L. mesenteroides* and its exopolysaccharide's ability to induce intestinal IgA or the ratio of CD4+ T-cells/CD8+ T cells would have been interesting (Matsuzaki *et al.* 2015) but were outside the scope of this thesis.

There were no noteworthy differences in the trends of expression of the transmembrane glycoprotein *e-cadherin* relative to the control group, but a 50-60% non-significant reduction in the expression of this molecule was observed by the 12h timepoint in all groups. E-cadherin is important for barrier formation in the gut, though its role in immune mediation in fish remains largely unclear. The glycoprotein functions as the receptor for induced phagocytosis and internalization of *Listeria monocytogenes* into Caco-2 epithelial cells (Mengaud *et al.* 1996). The human pathogen *Vibrio cholerae* produces a toxin that impairs recycling of cadherins to cell-cell junctions, thereby disrupting the barrier function (Ireton 2018). Viruses can also affect the gut lining. In Atlantic salmon, single nucleotide polymorphisms (SNPs) in an epithelial cadherin gene were associated with resistance to infectious pancreatic necrosis virus (IPNV), suggesting that cadherin can be targeted by bacterial and viral threats in the process of establishing an infection (Moen *et al.* 2015). These interactions happen at the protein level, which would likely not be reflected by changes in transcript abundance of this molecule. Moreover, there were minor differences in trends in the expression of *jam-1a* relative to the control and a similar downregulation of the expression levels was observed by the 12h timepoint.

The composition of tight junction complexes exhibit tissue-specific properties (Gauberg, Kolosov and Kelly 2017). The knowledge of the physiological function and

mapping of the expression of TJ protein isoforms is predominantly obtained from studies in terrestrial organisms. It is, therefore, plausible that the target TJ molecules assessed in the present studies have diverged evolutionarily and now exert biological functions distinct from that in mammals. For example, claudin-3 is a vital barrier-forming molecule present in tight junction complexes in the distal intestine (Feng *et al.* 2018). However, in finfish, claudin-3b has been found to be highly expressed in the kidney, but not the intestine, during saline adaptation in Atlantic salmon (Tipsmark and Madsen 2012). In RTgutGC cells, claudin-3 primarily localizes to the cell-cell interfaces, indicating that it likely plays a role in barrier formation. Likewise ZO-1, which was found around the cell boundaries as continuous ribbons, possibly adjacent to claudin-3 (Pumputis *et al.* 2019), which supports the use of the RTgutGC model.

The expression of *il-8* was highly increased by the 12h timepoint, indicating that the LAB strains tested were unable to dampen the excessive immune activation caused by *V. anguillarum* that can lead to epithelial injury and further contribute to the infection. The trends in the expression of *il-17a* show an upregulation of this molecule by the 3h timepoint in groups pre-treated with *B. breve* BR03 and *L. rhamnosus* GG, although there was considerable variability between biological replicates in the latter.

Overall, the human LAB strains tested here had properties potentially suitable for application to salmonids, but these did not translate to significant protection of gut barrier proteins. As such, these strains might be ill-suited to thrive in and promote host health in distantly related and physiologically distinct organisms. The use of endogenous strains showed more promising results, but safety assessments would be required given the propensity to increase responses normally deemed to be associated with inflammation

(Van Doan *et al.* 2020). The *in vitro* coculture system was a powerful and cost-effective tool for the investigation of host-microbe interactions. The advent of the Transwell system physiologically mimics the intestinal epithelial environment, in which the apical and basolateral compartments recapitulate the intestinal lumen and portal blood, respectively (Hubatsch, Ragnarsson and Artursson 2007).

The present study is the first of its kind to employ a tissue culture of the salmonid intestine for investigating host-microbe interactions and to evaluate the potential of LAB strains as candidate fish probiotics offsetting pathogen insults to the epithelial barrier. The RTgutGC model system provides a high-throughput tool to identify suitable probiotic strains worthy of further testing in live salmonids as long as the delivery vehicle allows the organisms to become metabolically active during intestinal transit.

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Chapter 3

3. Chinook salmon field study: Investigating the effects of probiotic supplementation in the context of a pathogenic infection with *Vibrio anguillarum*

3.1 Abstract

Aquaculture is the fastest-growing sector in the livestock industry, and it has become a critical contributor to the global food supply. However, the high incidence of infectious diseases threatens productivity and causes financial instability in the industry. Current management strategies consist of antibiotics, but efforts are being made to explore the use of beneficial bacteria as an eco-friendly alternative. This study aimed at investigating the effects of lactic acid bacteria (LAB) supplementation on the survival, growth, and expression of key tight junction and immune molecules in the hindgut tissue of Chinook salmon (*Oncorhynchus tshawytscha*) over the course of a disease challenge with *Vibrio anguillarum* – a common but deadly aquatic pathogen. In total, 1800 fish from five families were evenly allotted into six dietary treatments in a four-week supplementation trial. The dietary treatments included a basal diet (control), a sodium alginate vehicle control, and diets containing 1 x 10^8 CFU/g feed of the beneficial microbes: *Limosilactobacillus reuteri* LRE2, *Limosilactobacillus reuteri* 830, *Lacticaseibacillus rhamnosus* GR1 and *Limosilactobacillus reuteri* RC14, and *Pediococcus acidilactici* 18MA/5M. Supplementation was started two weeks prior to infection and was maintained over the course of the challenge. A total of 1x10^4 CFU/fish of *V. anguillarum* was injected intraperitoneally and samples were collected on days 0, 1, 3, 7, and 14 post-infection. Weight, length, and tissue were obtained on five occasions

and mortality was recorded daily. No negative effects in growth or survival were observed prior to or during infection in either of the LAB-supplemented or control groups. Gene expression analysis of the intestine on days 0, 3, 7, and 14 post-infection revealed no significant change in the expression of tight junction molecules *zo-1*, *jam-1a*, *ocldn*, *ecadh*, *marveld2*, *cldn15*, *cldn28b*, *muc2*, or *vil1*. Likewise, the expression of immune markers *il8*, *il10*, *il17a*, *tgfb*, and *myd88* were unchanged. Consequently, this field trial did not provide evidence to support the use of these strains to promote growth, disease resistance, or to modulate gut barrier function and intestinal mucosal immunity in Chinook salmon challenged with *V. angullarum*. Alternative strains or delivery systems or infecting the fish orally may still be worth testing, along with use of specific families of fish, before concluding there is no role for these strains in fish management.

3.2 Introduction

Fueled by growing global demands of high-quality alternative protein sources, aquaculture has become the fastest-growing sector in the livestock industry and its productivity has far surpassed that of hatcheries (Martin 2017; FAO 2020). The Food and Agriculture Organization (FAO) of the United Nations forecasts that an additional of at least 40 million tonnes of fish protein will be needed to meet demands by the end of the decade (FAO 2020). Concerted efforts have focused on the sustainable intensification of production; however, the high incidence of infectious diseases threatens to jeopardize the projected productivity goals (Lafferty *et al.* 2015). The substantial financial losses and instability caused by these diseases has fueled an interest in effective and eco-friendly solutions to tackle this challenge. In contrast to antibiotics, which enrich for antimicrobial resistant organisms (He *et al.* 2017), and vaccines, which are largely ineffective in fish

(Dixon 2012), probiotics have been investigated as an eco-friendly alternative (Langlois *et al.* 2021).

Probiotics are defined as 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host' (Hill *et al.* 2014). In some fish species, probiotic supplementation has been associated with a better immune performance, survival to pathogenic challenges, improved nutrient assimilation, and growth (Langlois *et al.* 2021). Although the precise mechanism of action is unknown, studies in other terrestrial and aquatic species seem to indicate that probiotics have primarily two modes of action: modulation of the host response (direct) and indirect modulation of the microbial community towards a state that promotes host health (indirect) (Merrifield *et al.* 2010).

It is possible that the immune modulation is mediated by components in the cell wall or metabolites that directly interact with the host (Bron, Van Baarlen and Kleerebezem 2012). The survival to pathogenic challenges might be due to direct antagonism with the pathogen or indirect modulation of the host fitness and immune response (Ringø *et al.* 2018; Doan, Soltani and Ringø 2021). The improved nutrient assimilation and growth might be due to the metabolism of otherwise indigestible nutrients facilitated by the beneficial microbes (Sealey *et al.* 2009). These concepts hold merit based on research in other systems and the investigation of the mechanism of action of these microbes is invaluable in order to devise targeted approaches to promote fish health.

The intestinal epithelium is the most prominent mucosal interface for host- and microbe-microbe interactions in terms of microbial richness and abundance, as well as an

extensive surface area for microbial contact with the host. The gut plays a vital role not only in nutrient absorption and osmoregulation, but it is also a key immune organ (Lazado and Caipang 2014). In fact, differences in immune competence between finfish have been attributed to the ability to prevent pathogen attachment and proliferation in the gut (Palaksha *et al.* 2008; Rajan *et al.* 2011; Gao *et al.* 2016). As such, strategies that promote optimal gut health locally can also have substantial systemic benefits for the host.

At the core of the intestinal mucosa is a single layer of epithelial cells and their intercellular junctions (ie. tight junctions - TJ, gap junctions, and adherens juctions) that enable the selectively permeable entry of luminal contents into the lamina propria. A breach in this barrier can allow access of pathogens and other harmful compounds that thus trigger infection and inflammation in the host (Bron *et al.* 2017). Although the mechanism is unknown, studies in terrestrial organisms indicate that certain probiotic strains can promote gut barrier function by upregulating the expression of TJ molecules (Wang *et al.* 2018). This phenomenon has not previously been investigated in fish, but the concept warrants attention, as the identification of a microbial-based strategy to bolster fish immunity, enhance growth, and prevent infections through the modulation of gut barrier health would be ground-breaking for the field.

Several classes of microbes have been proposed to benefit finfish, but the most prominent group are lactic acid bacteria (LAB), which includes lactobacilli and members of the *Lactococcus*, *Leuconostoc*, *Enterococcus Streptococcus*, *Pediococcus*, *Carnobacterium*, and *Weisella* genera (Ringø *et al.* 2018). These LAB are common residents of the finfish gut microbiota and some fish-associated strains have been shown

to promote host health (Ringø *et al.* 2018). However, research to date has been slow to determine a high-throughput framework for identifying and characterizing novel fish-associated beneficial microbes, and in North America, there is no fish-derived probiotic product available for large-scale use. As such, preparations containing exogenous strains of LAB have been used in aquaculture settings with promising outcomes (Hoseinifar *et al.* 2018).

Research in mammalian systems indicates that certain probiotic strains can promote intestinal barrier integrity, which has been linked with the attenuation of gastrointestinal disorders (Ukena *et al.* 2007; Patel *et al.* 2012; Wang *et al.* 2018). The selected strains used in the present field trial were *Limosilactobacillus reuteri* LRE2, *Limosilactobacillus reuteri* 830, *Lacticaseibacillus rhamnosus* GR1 and *Limosilactobacillus reuteri* RC14, and *Pediococcus acidilactici* 18MA/5M. The rationale for the selection of these candidate probiotics is based on the genetic similarities between *L. reuteri* 830 and LRE2 as well as benefits in reported in other organisms (Mogna *et al.* 2014). Furthermore, *L. rhamnosus* GR1 and *L. reuteri* RC14 were selected as this combination of strains has shown several health benefits in humans through interactions at mucosal surfaces (Reid 2020; Cunningham *et al.* 2021). Numerous reports in the literature have investigated *P. acidilactici* 18MA/5M for its health-promoting effects in several salmonid species (Vasanth *et al.* 2015; Jaramillo-Torres *et al.* 2019).

Vibriosis, caused by the Gram-negative bacterium *Vibrio anguillarum*, is one of the most prevalent bacterial diseases in aquaculture and incurs substantial losses to the industry (Lafferty *et al.* 2015). This organism is a halophilic aquatic bacterium that infects over 50 finfish and shellfish species worldwide. This pathogen is equipped with

an arsenal of virulence factors, that include a siderophore-mediated iron acquisition system, hemolysins, lipopolysaccharides and exopolysaccharides, toxins, secreted proteases, and a polar flagellum that aids in motility (Frans *et al.* 2011). The pathogen infects the host by entering the bloodstream primarily through the gut and gills (Grisez *et al.* 1996; Olsson *et al.* 1996, 1998; O'Toole *et al.* 1999), and the fish quickly succumb to haemorrhagic septicaemia (Grisez *et al.* 1996). The mortality of infected Chinook salmon can reach up to 50% (Ching *et al.* 2010). In terms of disease control strategies, there have been several commercially-available vaccines developed against *V. anguillarum* consisting of inactivated whole cells or live attenuated bacteria (Frans *et al.* 2011). Although these products do offer some degree of protection against the pathogen (Angelidis, Karagiannis and Crump 2006), not even the best vaccines can completely prevent the occurrence of disease (Austin and Austin 2007), which creates the need for more effective antimicrobial strategies.

Salmonids are the most economically important class of farmed finfish in the aquaculture world trade (FAO 2020). Although the farming of Atlantic salmon (*Salmo salar*) dominates the market, the farming of indigenous species is preferred on the West coast due to concerns of exogenous farmed escapees disrupting the native ecological niche (Noakes, Beamish and Kent 2000). The farming of Chinook salmon (*Oncorhynchus tshawytscha*) is favoured due to this species large size and high market value. Additionally, indigenous farmed escapees could potentially mate with the wild population, thereby reducing the biodiversity and decreasing the genetic variability of the population (McGinnity *et al.* 2003). The use of sterile triploid salmon effectively circumvents this issue, with the added benefit that triploid fish do not mature sexually,

thus preventing losses in flesh quality (Pandian 1998; Benfey 2001). Triploids, however, are more susceptible to infectious diseases (Ching *et al.* 2010) and exhibited 10-30% greater mortality compared to diploid siblings in a bacterial disease challenge (Ching *et al.* 2010; Semple *et al*., *unpublished*). Thus, improvements are needed to mitigate deficits in immune function in triploid fish.

For the current study, it was hypothesised that improving intestinal barrier function can reduce losses in aquaculture, given that nutrient adsorption, immunity, and prevention of infection occur at this interface. The objective was to examine the mechanisms through which certain probiotic bacteria can promote health in the context of a live bacterial infection with *Vibrio anguillarum* in triploid Chinook salmon.

3.3 Materials and methods

3.3.1 Spawning and animal husbandry

Chinook salmon (*Oncorhynchus tshawytscha*) were bred and reared at Yellow Island Aquaculture Ltd. (YIAL), a Chinook salmon farm that follows organic standards and has both freshwater hatchery and saltwater netpen facilities. The farm has been in operation since 1985 and is located on Quadra Island, British Columbia, Canada (latitude: N 50° 7' 59.124"; longitude: W 125° 19' 51.834"). To generate crosses, milt and eggs were collected from inbred production fish and the quality of the gametes was monitored to ensure a high level of fertilization. Fertilized eggs were then placed in a hydraulic pressure system to induce triploidization (Johnson *et al.* 2004). The embryos were then reared in vertical-stack incubation trays supplied with flow-through untreated spring water (temperature was between 7–9ºC). Hatching took place approximately 10 weeks post-fertilization. Alevins were then transferred to 160L rearing barrels supplied with flow-through spring water at 1.0L/minute. The water temperature was approximately 8ºC (temperature range 7–10ºC) and the dissolved O_2 saturation was regularly monitored and maintained at above 80%. Tanks received light from 7am to 5pm daily to maintain normal circadian rhythms. The fish density was between 50-150 per barrel, and each barrel contained individuals of a single family during the growth phase.

3.3.2 Fish diets

The animals were fed *ad libitum* two to three times a day with a fishmeal diet (Taplow Feeds, FirstMate Animal Nutrition; **Supplementary table 3-1**) during the growth phase. Probiotic supplementation was initiated 14 days prior to infection and

animals received the LAB until the end of the disease trial, for a total of 4 weeks of supplementation. Briefly, the probiotic-containing diets were prepared by adding the lyophilized probiotics (**Table 3-1**) to 10mL of a sodium alginate solution (0.001% (w/w) sodium alginate powder (Millipore Sigma, catalogue number: W201502) in tap water). Next, 100g of fish feed were added to the suspension, and finally 10mL of a 100mM $CaCl_2$ (Thermo Fisher Scientific, catalogue number: C614500) solution were added to the mix. The probiotic feed preparations were stored at 4ºC for no more than 72h.

Table 3-1. Amounts of lyophilized probiotic to be added to the fish feed for a total of 1 x 10^8 CFU/g feed.

Organism name	Supplier	CFU/g	Amount/100g feed
L. reuteri SD-LRE2-IT	Probiotical/SeedLabs	1 x 10^{11}	100mg
L. reuteri SD-RD830-FR	N/a	4.94 x10^{10}	200 mg
L. reuteri RC14 & *L. rhamnosus* GR1	RepHresh-ProB	1 x 10^9/cap	10 caps
Pediococcus acidilactici MA18/5M	BioPowerPA/Lallemand animal nutrition	1 x 10^{10}	1 g

3.3.3 PIT tagging

To be able to track individual fish over the course of the experiment, passive integrated transponder (PIT) identification tags were used. Fish were collected from their respective barrels and anesthetized using a clove oil bath (ThermoFisher, catalogue number: 10459550). Sedated individuals were then swiftly injected with a PIT tag intramuscularly, positioned caudal to the tip of the pectoral fin. Weight was then recorded, and fish were placed in an aerated recovery bucket for approximately 5 minutes to ensure that the injection and/or the clove oil bath were not lethal to the animals. Following recovery, the fish were transferred to flow-through freshwater tanks at a flow

rate of 1.0L/minute. The water temperature was between 7–10ºC and the dissolved O_2 saturation was regularly monitored and maintained at above 80%.

3.3.4 Bacterial cultures

Vibrio anguillarum serotype O1 was generously provided by Dr. Brian Dixon's laboratory at the University of Waterloo. The *V. anguillarum* strain was originally obtained from Pacific Biological Station (PBS) in Nanaimo, BC from Dr. Simon Jones. This strain was isolated from a diseased winter steelhead trout (*Oncorhynchus mykiss irideus*) obtained from Little Campbell River, BC. *V. anguillarum* was routinely cultured in tryptic soy broth or agar (TSB/TSA) supplemented with 2% (w/v) sodium chloride. For the infection challenge, a streak plate was prepared and incubated at room temperature (23˚C) for 48h. A single colony was used to inoculate 5mL of TSB+2% NaCl, which was then incubated for 24h at room temperature on an orbital shaker at approximately 220 rpm. The starter culture was then used to inoculate a total volume of 50mL of TSB+2%NaCl, which was incubated in like manner. Dilutions were then prepared in sterile PBS to a final concentration of $1x10^5$ CFU/mL. The bacterial suspension was immediately used and maintained at 4˚C until injection.

3.3.5 Disease trial design

Triploid Chinook salmon of five distinct crosses (more specifically, 60 fish per family for a total of 300 fish per treatment, split evenly into in 2 technical replicate tanks) were fed with either the candidate probiotics (**Table 3-1**; $1x10^8$ CFU/g), regular feed with sodium alginate coating (vehicle control), or regular feed, for a total of four weeks. The trial was conducted in flow-through freshwater tanks at a flow rate of 1.0L/minute.

Outflow water underwent UV sterilization (493.5 mJ/cm^2) to ensure the biocontainment of the pathogen. The water temperature ranged from 7–10ºC and the dissolved O_2 saturation was regularly monitored and maintained at above 80%. Mortality was recorded daily, and samples were collected on days 0, 1, 3, 7, and 14 by overdosing fish in a clove oil bath. Full-body weight and length were then recorded, and PIT tags were removed through a lateral incision to access the site of insertion. Whole fish were stored long-term in 50mL tubes (Sarstedt, catalogue number: 50809218) filled with RNAlater (Thermo Fisher Scientific, catalogue number: AM7021) at -20ºC until further sample processing.

3.3.6 Infection with live V. anguillarum

After 2 weeks of supplementation with the diets, fish were collected from their respective tanks and anesthetized in a clove oil bath. Although the pathogen infects via the intestine and gills, for practical reasons, all animals received an intraperitoneal 100µl injection of live *V. anguillarum* in sterile PBS for a total infectious dose of approximately 1x10^4 CFU/fish. This dose has been previously tested to induce approximately 50% mortality in Chinook salmon, with deaths initiating at around day 4-5 (Semple et al., *unpublished*). Animals were then placed in an aerated recovery bucket for approximately 5 minutes to ensure that the injection and/or the clove oil bath were not lethal to the fish. Following recovery, the fish were returned to their designated tanks.

3.3.8 Growth parameters

- Weight gain (WG): W_{DN} – Fam. avg. W_{D0}, where N is the sampling day
- Percent weight gain (%WG): 100*(W_{D0}- W_{D-14})/ W_{D-14}
- Specific growth rate (SGR): 100*[ln(W_{DN}) – ln(Fam. avg. W_{D0})]/N

3.3.9 RNA extraction and cDNA synthesis

Hindguts were dissected from all individuals and stored in an aliquot of the RNAlater solution. RNA was extracted by removing the tissue from the RNAlater solution with sterile forceps and blotting out excess solution by pressing the hindgut tissue between two sheets of sterile laboratory tissue paper (Kimberly-Clark Professional Kimwipes, catalogue number: KC34120). RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, catalogue number: 74134). Briefly, hindguts were placed in a screw-cap 2mL micro tubes (Thermo Fisher Scientific, catalogue number: 3469NK) containing 2mm and 0.5mm zirconia beads and the supplied homogenizing buffer. Samples were homogenized using a BioSpec3110BX Mini Beadbeater 1 (Fisher Scientific, catalogue number: NC0251414) beating five times for 90s at 7,000rpm. Tubes were then centrifuged for 30s at ≥8,000 rpm at room temperature to pellet debris and transferred to the spin column, following manufacturer's instructions. RNA was eluted in 30µl of elution buffer and quantified using a NanoDrop spectrophotometer. cDNA was synthesized from 500ng of the freshly isolated RNA using a High-Capacity cDNA Reverse Transcription Kit, following manufacturer's instructions (Thermo Fisher, catalogue number: 4368814), for a total volume of 20µl per reaction. Remaining RNA was stored at -80ºC.

3.3.10 Real-time quantitative PCR analysis

Reverse-transcribed cDNA was diluted 10x and used in qPCR reactions with PowerTrack SYBR Green Kit (Thermo Fisher, catalogue number: A46113). The primers used in this study are summarized in **Table 3-2**. For analyses of gene expression, the gene RPS20 was used as the reference because it was identified to be the most stable reference

gene of those tested (**Supplementary Figure 3-1**). Each qPCR reaction had a total volume of 10µl (performed in three technical replicates). Reactions consisted of 4.58µl of diluted cDNA, 0.42µl of primers (forward and reverse primer mix; 14.4µM), and 5µl of PowerTracker SYBR Green 2x Master Mix. PCR reaction conditions were 50ºC for 2 minutes, then 95ºC for 10 minutes, followed by 40 cycles of 95ºC for 15s, then 60ºC for 1 minute. The melt curve stage consisted of 95ºC for 15s, then 60ºC for 1 minute, then 95ºC for 15s. qPCR was performed on a QuantStudio5 Real-Time PCR System (Thermo Fisher Scientific; serial number: 272530299) and analyzed using the associated cloud-based Design and Analysis software (Thermo Fisher Scientific; version 2.5.1). Gene expression ($2^{-\Delta\Delta Ct}$) was calculated using fold change. PCR efficiencies were assessed using the LinRegPCR software version 2016.1 and determined to be above 1.80. An endogenous control was included in each 384-well plate to ensure that run efficiencies were comparable and that normalizations to the reference gene could be made between plates (**Supplementary figure 3-2**).

Table 3-2. Summary of RT-qPCR primers used in this study.

Gene name	Accession number	Sequence (5'-3')	Amplicon size (bp)	Reference
RPS20	NM_001124364.1	F: CCGCAATGTCAAGTCTCTGG R: ACTGTGCAGGTCGATCAAAC	202	This study
EF1a_2	NM_001124339	F: TGCCCCTGGACACAGAGATT R: CCCACACCACCAGCAACAA	90	Semple *et al.* 2018
EF1a_3	NM_001124339	F: CGCACAGTAACACCGAAACTAATTAAGC R: GCCTCCGCACTTGTAGATCAGATG	134	This study
18S	XM_021588520.1	F: CCCAAATCAAGTCCAATTCACA R: CTGTCTTCTCCTCCCCTCCA	106	This study
GAPDH	NM_001124246	F: TGACCACAGTCCACGCCTAC R: GCAGGGATGATGTTCTGGTG	103	This study
CX6C1	FR904651.1	F: GCCTGCAATGCGAGGACTCC R: TTCCTTGGTTCTGTTACGCCGTAC	114	This study

IL-8	NM_001140710	F: ATTGAGACGGAAAGCAGACG R: CGCTGACATCCAGACAAATCT	136	Wang *et al.* 2020
IL-17a	GW574233	F: TGGTTGTGTGCTGTGTGTCTATGC R: TTTCCCTCTGATTCCTCTGTGGG	136	Wang *et al.* 2020
TGFb	EU082211	F: AGTTGCCTTGTGATTGTGGGA R: CTCTTCAGTAGTGGTTTGTCG	191	Wang *et al.* 2020
IL-10	NM_001245099.1	F: CCATCAGAGACTACTACGAGGC R: TCTGTGTTCTGTTGTTCATGGC	165	Wang *et al.* 2020
MYD88	NM_001136545	F: GACAAAGTTTGCCCTCAGTCTCT R: CCGTCAGGAACCTCAGGATACT	110	Wang *et al.* 2020
JAM-1a	XM_021564368.2	F: TGAGGATGGAAGTCCGCAAC R: GTACCACAGTCCGAAGCACA	98	This study
Occludin	XM_021601275.2	F: GACAGTGAGTTCCCCACCAT R: AGCTCTCCCTGCAGGTCCTT	101	This study
Muc2	XM_036968565.1	F: GCACTCCGCACTTTTACCT R: TTCACATGGTTGGACTGGCG	144	Wang *et al.* 2020
Tricellu-lin	XM_036977099.1	F: TTTAGCAGGGGCAAAGGTGA R: TTCACACGCAGTCACTCAGC	112	This study
E-cadherin	XM_021585993.2	F: ACTACGACGAGGAGGGAGGT R: TGGAGCGATGTCATTACGGA	107	This study
Villin 1	XM_021579240.1	F: CATGTGGAGTGGAGGGAAGT R: TCCTCTTTCTTGGTGGGGTC	234	This study
Claudin 15	XM_036987534.1	F: GGCACGTCTGAGAAACAACC R: TAGGAAGTGGCAGCCTGACT	92	This study
Claudin 28B	NM_001195160.1	F: CTCACTCTACATCGGCTGGG R: CACAGAACTAGCAGCCTTGGA	124	This study
ZO-1	XM_036963739.1	F: CAAAGCCAGTGTATGCCCAG R: CAGCTTCATACTCGGCCTGA	119	Wang *et al.* 2020

3.3.11 Statistical analyses

All statistical analyses were done using GraphPad Prism software version 7.0a. Survival analysis was done using the Mantel-Cox test. Nonparametric data were statistically compared with a one-way ANOVA and Dunn's multiple comparisons test. Experiments with two factors were compared with a two-way ANOVA and Tukey's multiple comparisons test. Gene expression data were compared with a two-way ANOVA and Sidak's multiple comparisons test.

3.4 Results

3.4.1 Supplementation of Chinook salmon with LAB strains does not significantly improve survival to *Vibrio anguillarum* challenge.

To assess the physiological response of fish to probiotic supplementation in the context of a pathogenic challenge, a field trial was designed (**Figure 3-1**).

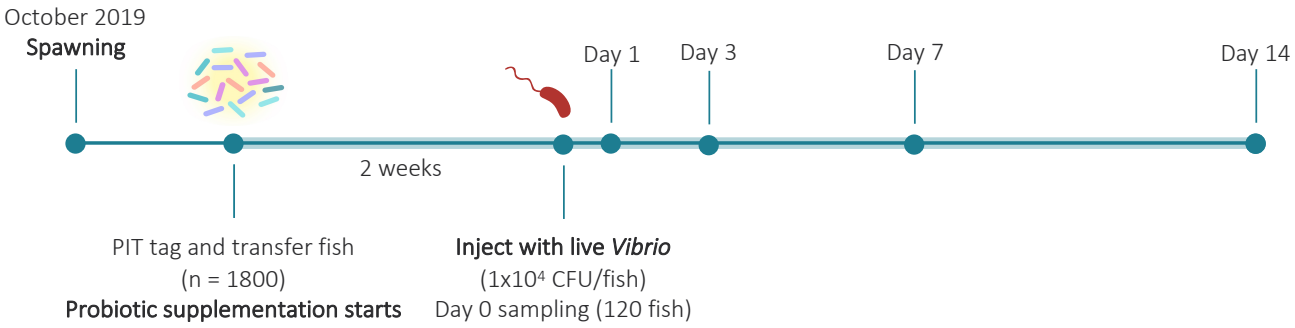

Figure 3-1. Experimental timeline of the disease trial. Triploid Chinook salmon spawned in the fall of 2019 (150 fish per tank per treatment, 2 technical replicate tanks) were fed with either the candidate probiotics (Table 1; $1x10^8$ CFU/g), regular feed with sodium alginate coating (vehicle control), or regular feed, for two weeks. Fish were then injected intraperitoneally with live *V. anguillarum* (~$1x10^4$ CFU/fish). Samples were taken at 0, 3, 7, and 14 dpi; mortality was recorded daily.

There was no statistically significant difference in survival between control-, vehicle, or probiotic-fed fish (**Figure 3-2 A**). Of note, however, are the inter-family differences in survival given the different probiotic strains supplemented (**Figure 3-2 B-F**). Families 56 and 101, in particular, had a survival advantage of approximately 15% in the LRE2-fed group compared to the regular and vehicle control groups (**Figure 3-2 C, F**). Similarly, families 57 and 100 had a comparable improvement in survival given by *P. acidilactici* 18MA/5M (**Figure 3-2 D, E**). One of the families, 55, did not seem to

respond to either of the probiotic supplements. Statistical analyses revealed that the survival curves were not significantly different according to the Mantel-Cox test.

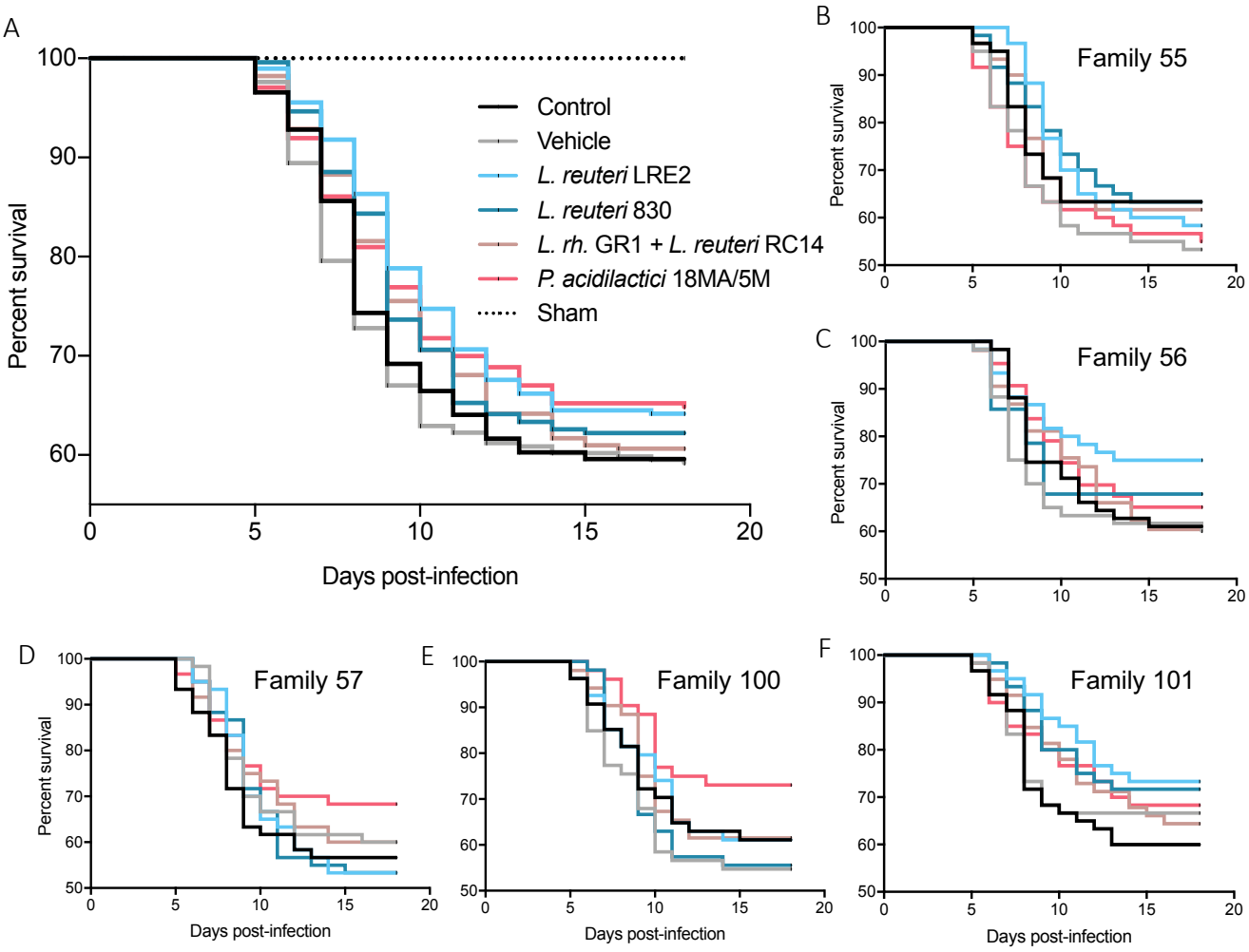

Figure 3-2. Survival of Chinook salmon supplemented with lactobacilli strains to a *Vibrio anguillarum* challenge. (A) Probiotic supplementation appears to have little effect on the survival of fish from all five families to the disease challenge; however, modest family- and strain-specific effects can be observed. **(B)** Family 55 failed to respond to all probiotic treatments; whereas *L. reuteri* LRE2 confers a survival advantage of ~15% in families 56 and 101 **(C, F)**. *P. acidilactici* 18MA/5M confers a slight survival advantage to families 57 and 100 relative to the vehicle and regular feed controls **(D, E)**. Differences were not statistically significant (Mantel-Cox test).

3.4.2 Lactic acid bacteria supplementation does not increase weight of fish prior to or during an active infection.

To assess whether the strains used in this study had a significant impact in the growth of Chinook salmon, weight was measured prior to the beginning of supplementation and after two weeks of probiotic administration, though prior to infection (**Figure 3-3 A**). The percent weight gain for each individual was determined and the median percent weight gain plotted. Individuals supplemented with *P. acidilactici* 18MA/5M had a significantly higher percent weight gain compared to the *L. reuteri* 830-supplemented group, although differences were not statistically significant between either probiotic group with the control or vehicle groups, as determined by a non-parametric one-way ANOVA (Kruskal-Wallis) and Dunn's multiple comparisons test. During the infection challenge, the weight of the sampled individuals was recorded, and the specific growth rate was determined relative to the average weight per family on day 0. There were no statistically significant differences between or within groups (two-way ANOVA and Tukey's multiple comparison test; **Figure 3-4 B**), and family of origin had no substantial impact on weight gain, as determined by the specific growth rate (**Supplementary figure 3-3**).

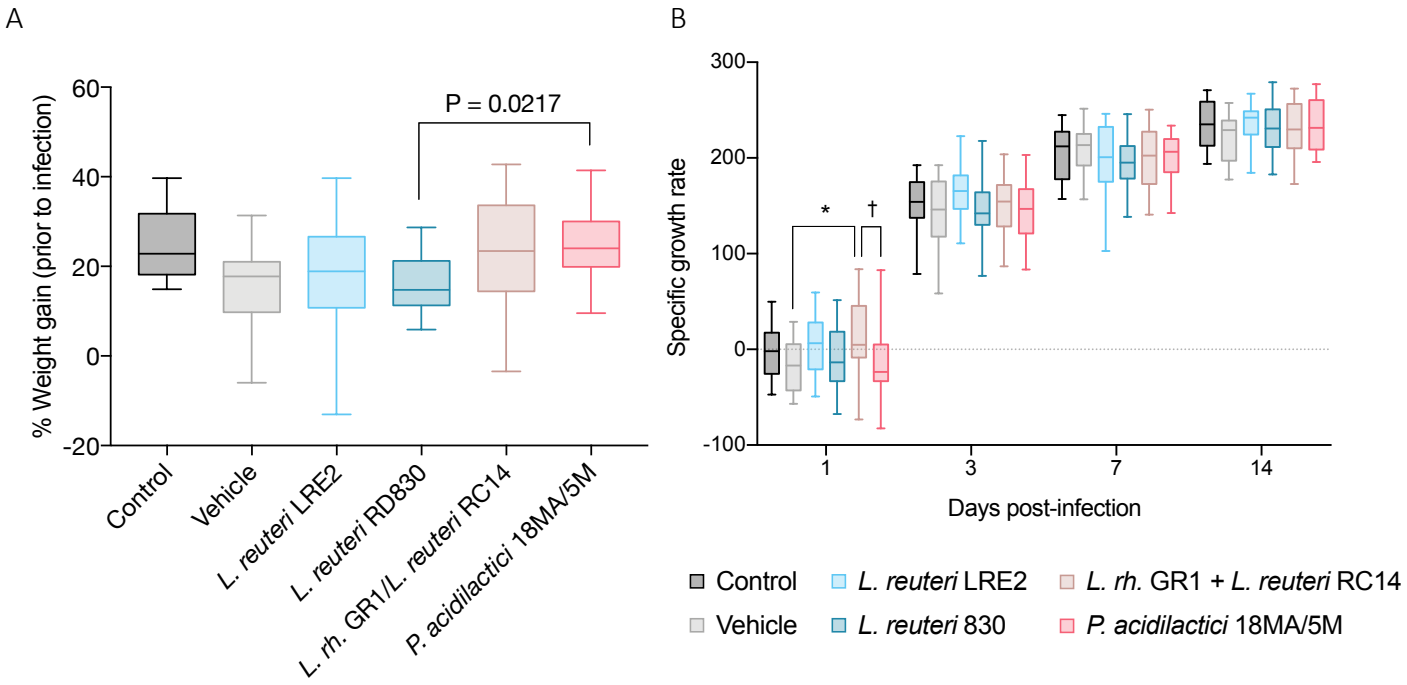

Figure 3-3. Weight of Chinook salmon is not significantly improved by probiotic treatment. (A) Percent weight gain prior to infection. Fish were weighed prior to probiotic supplementation (two weeks before the infection trial, probiotic supplementation was then started), then weighed again (repeated measures; N = 120) before injections with *V. anguillarum* (day 0). Statistical significance was determined by a non-parametric one-way ANOVA (Kruskal-Wallis) and Dunn's multiple comparisons test. **(B)** Fish weight was measured on five occasions (0, 1, 3, 7, 14 dpi) and the average weight per family at day 0 was used to establish an initial baseline. Specific growth rate (SGR) was determined as $100*[\ln(W_{DN}) - \ln(\text{Fam. avg. } W_{D0})]/N$, where N is the sampling day. Statistical significance was determined by a two-way ANOVA and Tukey's multiple comparison test (* P = 0.0309; † P = 0.0276). Boxes represent first and third quartile values, horizontal lines denote medians, and whiskers encompass maximum and minimum values.

3.4.3 Expression of tight junction and immune mRNAs was unchanged in the hindgut tissue during infection.

To investigate whether the LAB strains supplemented could modulate gut barrier function and immunity at the transcript level, the expression of a panel of tight junction

molecules was assessed through RT-qPCR (**Figure 3-5**). Given that there were apparent family-specific differences in survival, priority was given to individuals of family 56, as this family displayed a 15% survival advantage in the *L. reuteri* LRE2-supplemented group compared to the control and vehicle groups (**Figure 3-2 C**). Hindguts of individuals sampled on days 0, 3, 7, and 14 were included in the study to assess the short-term (3 dpi) and medium- to long-term (7 and 14 dpi, respectively) effects on tight junction and immune markers relative to the day 0 baseline. The expression of barrier-forming molecules, such as ZO-1, JAM-1a, Occludin, E-cadherin, Tricellulin, and Claudin 15 was not significantly changed over the course of the infection challenge (**Figure 3-5 A-F**). Similarly, the expression of Mucin 2, which is the major gel-forming molecule in the intestinal mucus, and Villin 1, which regulates intestinal epithelial morphology, was unchanged (**Figure 3-5 H, I**; Van der Sluis *et al.* 2006; Ubelmann *et al.* 2013). There is an apparent increasing trend in the expression of the pore-forming molecule Claudin 28B in the group supplemented with *L. rhamnosus* GR1 and *L. reuteri* RC14 on day 3, however the biological significance of these trends is questionable due to the high variability between individuals (**Figure 3-5 G**). Statistical significance was assessed with a 2-way ANOVA and Sidak's multiple comparisons test.

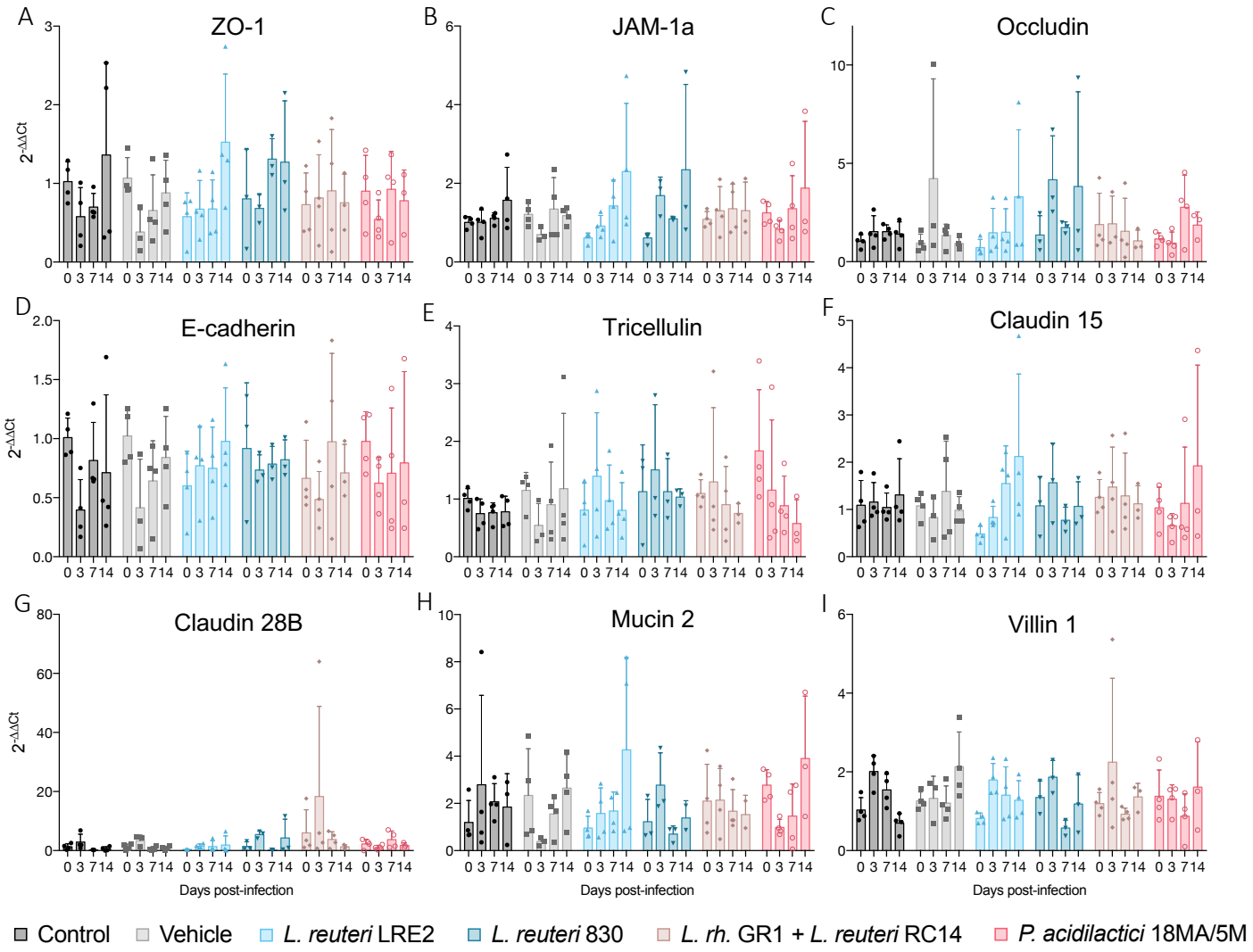

Figure 3-4. Relative expression of tight junction molecules in the hindgut tissue. Gene expression data points are displayed as mean fold change (normalized to RPS20) of individual biological replicates. Statistical significance was assessed with a 2-way ANOVA and Sidak's multiple comparisons test. Error bars represent means ± SD.

The expression of several immune markers was assessed to investigate the effect LAB supplementation on pro- and anti-inflammatory molecules during the infection challenge. The expression of IL-8 is moderately increased in the groups supplemented with *L. reuteri* 830, *L. rhamnosus* GR1 and *L. reuteri* RC14, and *P. acidilactici* 18MA/5M on day 7 (**Figure 3-5 A**). The expression of IL-10 was significantly increased in the group supplemented with *L. reuteri* 830 on day 7 post-infection (**Figure 3-5 B**); statistical differences were not present within or between groups otherwise (2-way ANOVA and Sidak's multiple comparisons test, $P > 0.05$). The expression of proinflammatory molecules IL-17a, TGFβ, and MyD88 were likewise unchanged between and within groups (**Figure 3-5 C-E**).

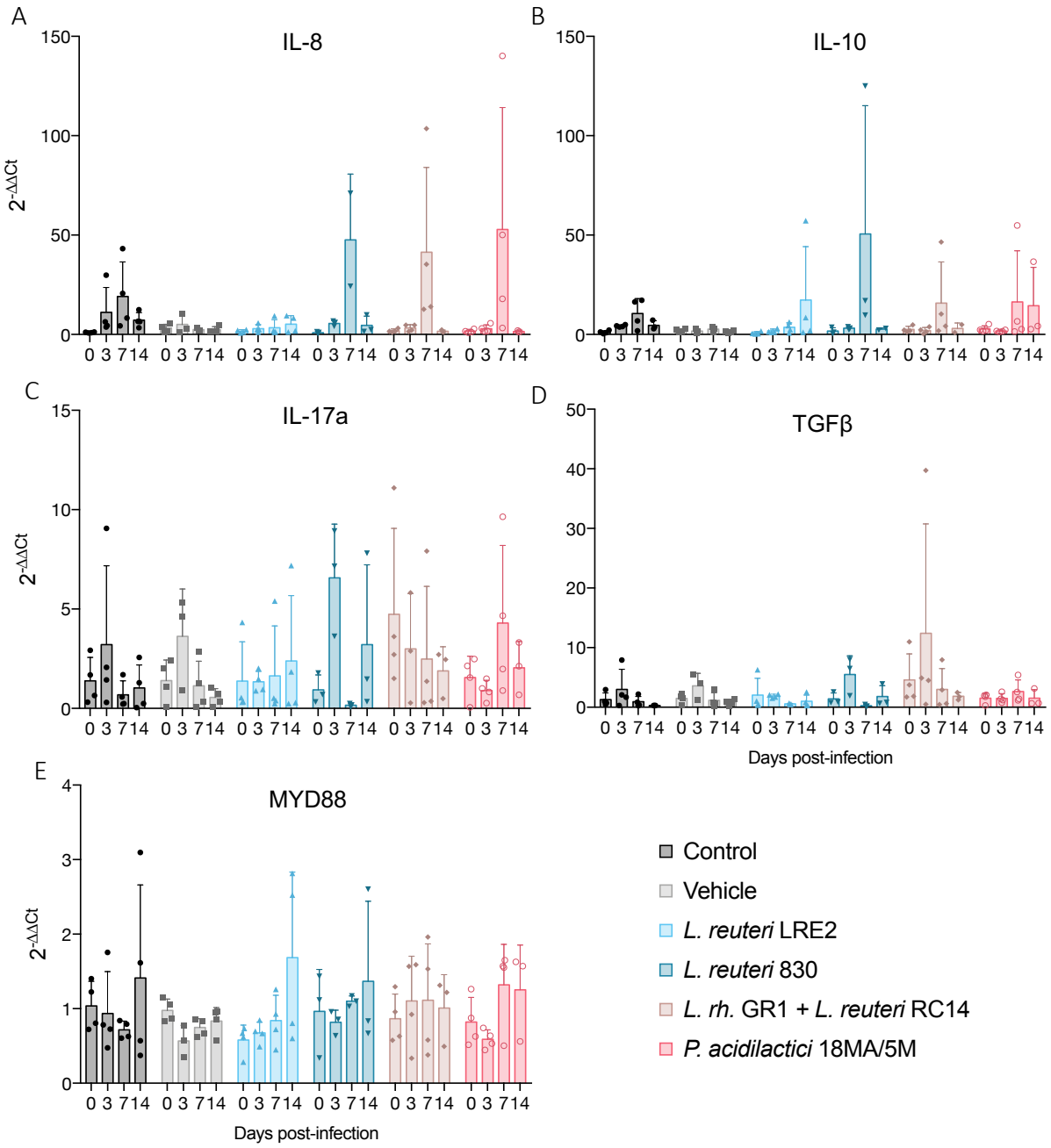

Figure 3-5. Relative expression of immune molecules in the hindgut tissue. Gene expression data points are displayed as mean fold change (normalized to RPS20) of individual biological replicates. Statistical significance was assessed with a 2-way ANOVA and Sidak's multiple comparisons test. Error bars represent means ± SD.

3.5 Discussion

This field trial showed that supplementation with candidate probiotic strains herein tested did not lead to an increase in weight gain, survival advantage, nor an upregulation in tight junction and immune molecules in the hindgut tissue of Chinook salmon challenged intraperitoneally with *V. anguillarum* over several weeks of study duration. When juvenile Chinook salmon were challenged with *V. anguillarum*, there was variable survival across the four candidate probiotics tested, with *L. reuteri* LRE2 and *P. acidilactici* 18MA/5M conferring a non-statistically significant survival advantage of 15% relative to the control and vehicle groups in families 56 and 100, respectively. This highlighted family-specific differences in survival in response to the different probiotic treatments (**Figure 3-2 B-F**). Though not statistically significant, these data suggest that the benefits due to probiotic supplementation, at least in the context of a bacterial infection, might be influenced by fish genetic factors and disparities in immune competence.

Family-specific differences in survival, fitness, and immune competence have been described in the literature (Bonnet *et al.* 1999; Johnson *et al.* 2004; Yáñez *et al.* 2013; Semple *et al.* 2018). For instance, genetic polymorphisms impacting major histocompatibility (MHC) loci in Atlantic salmon and rainbow trout have been associated with disease resistance and susceptibility (Miller *et al.* 2004). Additionally, epigenetic modifications such as DNA methylation patterns, histone modifications and variants, noncoding RNAs, as well as chromatin architectural remodelling can impact host fitness and coping mechanisms (Granada *et al.* 2017). Transgenerational epigenetic inheritance has been described in several vertebrate species, including some fish species (Knecht *et*

al. 2017). Changes in DNA methylation patterns has been linked to phenotypic variation, including early sexual maturation and development, salt adaptation, and growth performance (Gavery and Roberts 2017). It is, therefore, plausible that epigenetic differences can contribute to inter-family organismal development and immune function, as well as their response to probiotic supplementation. In practical terms, this may suggest propagation of only families that have a higher chance of growth and survival.

Additionally, inter-female variation in egg size, ovulation timing, and fertilization can also contribute to differences in the uniformity of triploidization. Genotypic factors that impact the surface-to-volume ratio of the egg, meiotic timing, and susceptibility for retention of the polar body, can affect the uniformity of the induced polyploidy (Johnson *et al.* 2004). The result is genetic mosaicism, in which a proportion of the cells of an individual are triploid and some are diploid (Johnson *et al.* 2004). This can also impact survival and how well a given family performs in response to probiotic supplementation. As such, additional measurements that confirm the induced polyploidy in the tissues of interest, such as flow cytometry, can be helpful in determining whether the inter-family variability is a result of lack of uniformity in the ploidy of the cells of an organism.

No appreciable increase in weight or specific growth rate was observed either prior to (**Figure 3-3 A**) or over the course of the infection trial (**Figure 3-3 B**) in either one of the probiotic treatments relative to the control or vehicle groups. Although there are some examples of growth promotion mediated by LAB supplementation in salmonids (Nikoskelainen et al., 2001; Vendrell et al., 2007; Ramos et al., 2015), there are also cases in which improvements in weight gain were not found (Zokaeifar *et al.* 2012).

Although the underlying mechanism through which beneficial microbes promote weight gain is not known, it has been proposed that these bacteria enhance nutrient absorption by facilitating the metabolism of otherwise indigestible nutrients (Langlois *et al.* 2021). As such, it is likely that the candidate probiotics herein tested lacked the metabolic repertoire necessary to facilitate digestion to the degree necessary to appreciably affect weight gain within the experimental window. Alternatively, the method of infecting the fish with the pathogen results in an invasive disease not amenable to orally ingested probiotic amelioration. Another explanation could be that the probiotic strains did not have sufficient time to reach an active metabolic state to influence the host. These hypotheses warrant further investigation.

There is currently no consensus in the literature regarding the ideal window for probiotic administration in salmonids. Some studies have reported a significant reduction in mortality and morbidity in the context of a bacterial challenge and robust stimulation of the innate immune response (cellular and humoral parameters) in as few as 14 days of supplementation (Langlois *et al.* 2021). However, in rainbow trout supplemented with *Enterococcus casseliflavus* NC0209951 and challenged with *S. iniae*, the serum IgM and C3 levels were found to be increased only after 8 weeks of supplementation (Safari *et al.* 2016). Similarly, a reduction in *A. salmonicida* burden in the hindgut of brown trout was reported after 4 weeks of supplementation with *L. lactis* CFLP 100 and *L. mesenteroides* CLFP 196 (Balcázar *et al.* 2009). These findings beg the question of what an appropriate supplementation window would be given the experimental design and research question that this study aimed to address, which can only be answered empirically. As such, future studies should conduct preliminary trials examining the parameters of interest (*ie.* growth,

survival, morbidity, immune stimulation, stress) in various supplementation timelines to further improve the study design and help inform practices for the industry.

Although there were little to no differences in the relative expression of the tight junction molecules assessed at any of the timepoints (**Figure 3-4**), it is important to note the considerable variability between individuals. This is a common pattern in trials using outbred fish (Semple *et al.* 2018), but the reason why this is observed in siblings of the same cross is unclear. One potential explanation is that even within families, disparities exist in terms of growth, as some individuals might be more aggressive feeders, resulting in an ingestion of larger amounts of the beneficial microbes compared to its siblings. Due to resource constraints, the analysis of the hindgut tissue of all five fish families was not feasible, but a more comprehensive study might shed light on inter-family differences that potentially explain the differences in survival observed. In addition, the knowledge about the physiological function of the TJ molecules assessed in this study is based on detailed functional studies in mammals, but similar studies in fish are still limited.

The complex milieu of the salmonid gut is comprised of several inter-dependent factors. These include a rich microbial community, mucosal epithelial cells, secreted antimicrobials and antibodies, mucus, microbial metabolites, and resident host immune cells, which together greatly impact organismal health (Merrifield *et al.* 2010). The integrity of the intercellular junctions between adjacent epithelial cells has been proposed as a key determinant of mucosal homeostasis, and damage to the barrier is increasingly being recognized as a hallmark of the pathophysiology of several chronic gastrointestinal disorders in humans (Bron *et al.* 2017). Since probiotic-mediated improvement of gut barrier function has been positively associated with health benefits in several vertebrate

species (Bron *et al.* 2017), we sought to investigate whether the strains herein tested could modulate gut barrier function and immunity at the transcript level in Chinook salmon.

Tight junctions consist of large, dynamic protein complexes that form the circumferential seal between adjacent epithelial cells. Some of the main protein families found in TJs are claudins, occludins, junction-associated membrane proteins (JAM), and zonula occludens (ZO-1). In order to orchestrate the preclusion of luminal contents and enable the passage of ions and nutrients, the epithelial barrier must have both 'fence' and 'gate' capabilities. The selectively permeable transport of molecules across the epithelial barrier is facilitated by claudin isoforms, as well as occludin and other adjacent molecules, and their selectivity is based on molecule size and electrical charge (Vikström *et al.* 2008; Cummins 2012; Sundell and Sundh 2012).

Therefore, the panel of TJ molecules selected was representative of four main characteristics of the intestinal epithelium. The first is the barrier-forming cell-cell connections represented by *zo-1*, *jam-1a*, *ocldn*, *ecadh*, and *mald2* (tricellulin; Schug *et al.* 2019). The second is the pore-forming ability that enables the selectively permeable transport of compounds across the epithelial barrier, represented by *cldn15* and *cldn28b* (Bagnat *et al.* 2007; Tipsmark *et al.* 2010). The third is mucus secretion, represented by *muc2*, which is a key structural component of the colonic mucus layer (Van der Sluis *et al.* 2006). The fourth characteristic of interest is the apical surface projections that form the brush border membrane, represented by villin (*vil1*), which is a Ca^{2+}-dependent actin-binding protein involved in the structural remodelling and nucleation of microvilli (Friederich *et al.* 1999; Ubelmann *et al.* 2013).

The intestinal mucosa is a prime target organ for immunomodulation given by oral probiotic administration. Although numerous studies over the last decades have examined the potential for promoting immune homeostasis locally and systemically through the supplementation of beneficial microbes, the underlying mechanism remains poorly understood. The present study sought to better understand the relationship between immune modulation in the hindgut and candidate probiotics in during a bacterial infection. The selection of the immune genes assessed was based on their function in bacterial stimulation (*il8*, *myd88*), promoting pathogen clearance and autoimmunity (*il17a*), as well as anti-inflammatory markers (*il10*, *tgfb*).

In spite of extensive inter-individual differences in relative expression, there was no clear trend indicating that administration of the candidate probiotics had a significant impact on the immune markers assessed compared to the control and vehicle groups (**Figure 3-5**). Our data are in contrast with several studies in salmonid species as well as finfish in general, which largely suggest that supplementation with LAB results in an upregulation of cellular and humoral immune parameters at the intestinal mucosal interface (Hoseinifar *et al.* 2018; Ringø *et al.* 2018; Langlois *et al.* 2021). For instance, *P. acidilactici* 18MA/5M has been shown to significantly upregulate the expression of proinflammatory markers IL-1β, TNFα, and IL-8 in Atlantic salmon (Abid *et al.* 2013; Jaramillo-Torres *et al.* 2019). The same strain was also used in a study in rainbow trout, which reported the upregulation of IL-1 and IL-8 and a decrease in the expression of IL-10, while simultaneously protecting the intestinal epithelium from inflammation and damage to the tissue (Al-Hisnawi *et al.* 2019).

In rainbow trout, dietary *Lacticaseibacillus rhamnosus* GG (ATCC 53103) resulted in an increase in respiratory burst activity, serum complement activity, and serum immunoglobulin levels, which was associated with a survival advantage against *Aeromonas salmonicida* infection (Nikoskelainen *et al.* 2001, 2003). Similarly, administration of *Lactiplantibacillus plantarum* subsp. *plantarum* CLFP 3 to rainbow trout led to an upregulation in the expression of IL-1β, TNFα, and IL-10 in the head kidney and IL-8 and IgT were upregulated in the intestine during infection with *Lactococcus garviae*. The supplemented fish also had a significantly lower mortality rate compared to the control group, suggesting that probiotic-mediated modulation of immune parameters might be a mechanism for improvements in survival to bacterial pathogens (Pérez-Sánchez *et al.* 2011).

Nevertheless, a cautionary note must follow that the observed effects are only descriptive of the host and microbe species in which these studies were conducted. The vast genetic and physiological differences in both hosts and beneficial microbes preclude sweeping generalizations and encourages the technical use of the term 'probiotic' exclusively in the cases for which reputable investigations have provided evidence for the alleged benefits. Furthermore, a more comprehensive analysis including a larger pool of sampled individuals can enhance the power and inference ability of these analyses. Also, the investigation of the innate and adaptive immune response in other relevant immune organs in teleosts (such as head kidney, spleen, gills, and skin) would provide a clearer picture of the effects of LAB supplementation in Chinook salmon during infection.

In considering why our study did not show the effects reported by others, the delivery method comes into question. The transit time for food and the bacteria

embedded in the pellet through the intestine of the salmon is approximately 12h (Sveier, Wathne and Lied 1999). This means the dried organisms must hydrate, become metabolically active and 'escape' their sodium alginate covering then make an impact via the microbiota and epithelial layer before being excreted. Moreover, the primary energy source of LAB strains are carbohydrates and an increase in the abundance of these genera has been associated with a carbohydrate-rich fish diet (Ringø *et al.* 2016). This thus raises the question of whether the fishmeal diet had the nutritional requirements to support LAB growth. Future studies should examine whether the inclusion of a prebiotic growth stimulant and a coating other than alginate could provide a faster and better delivery system for these fish.

In conclusion, it is hoped that this research will help inform future studies in order to optimize the benefits of probiotic strains and gain insights into their mechanisms, such as via improving the intestinal barrier function. Ideally, once strains and delivery are selected and feed conversion rates are proven, a better field trial than deliberate intraperitoneal infection, would be to feed fish in an aquaculture set-up and monitor the incidence and severity of naturally occurring infections over time. This would then demonstrate the validity of the concept and gather information on the cost-benefit of probiotic supplementation in salmonid aquaculture.

3.6 References

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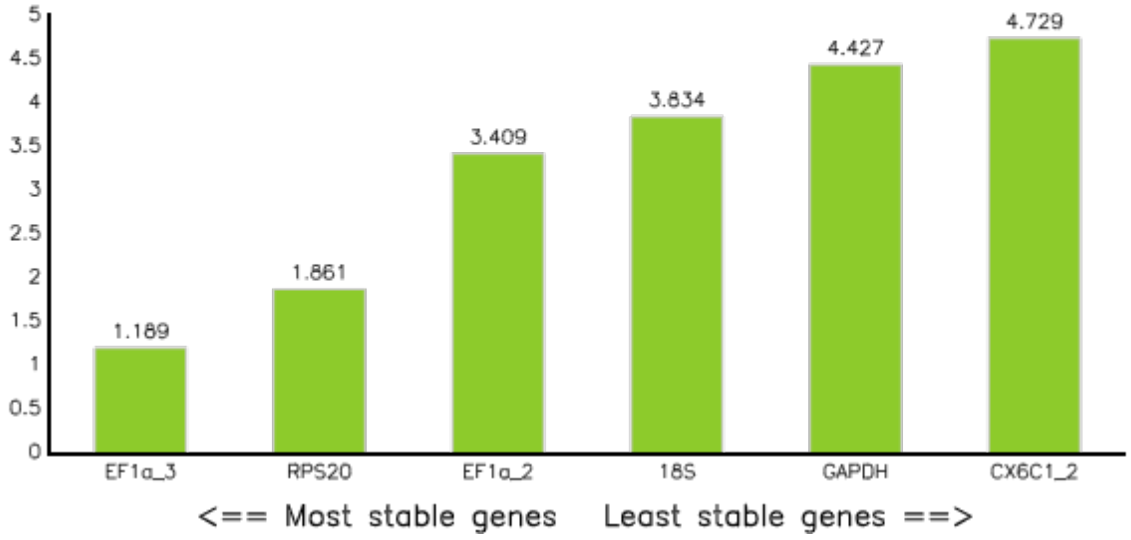
3.7 Supplementary material

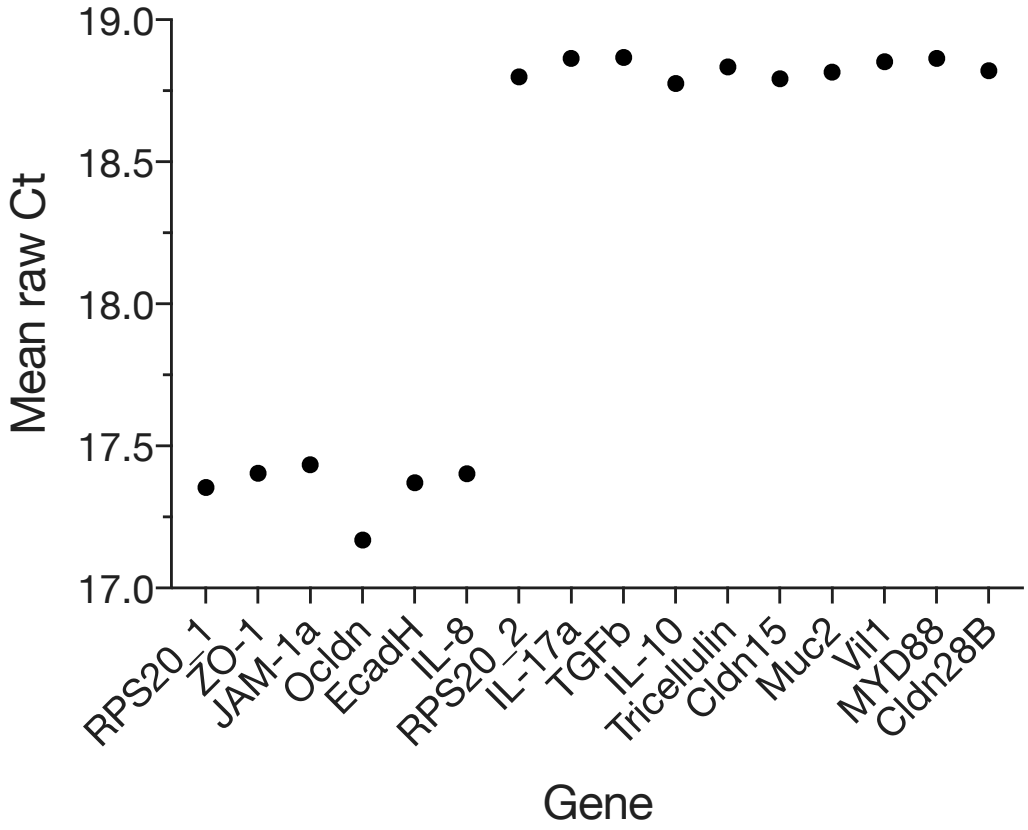
Supplementary table 3-1. Nutrition facts of the fish feed administered to Chinook salmon as provided by Taplow Feeds, FirstMate Animal Nutrition.

		Particle diameter			
		6.5mm		8.5mm	
	Units	As Fed	Dry Matter Basis	As Fed	Dry Matter Basis
Moisture	%	6		6	
Total protein	%	42.49	45.20	40.88	43.49
Fish meal	%	51.8		54.5	
Total fat	%	28.47	30.29	25.37	26.99
Fish oil	%	21.7		18.9	
Amino acids					
Methionine	%	1.39	1.48	1.33	1.41
Cystine	%	0.56	0.60	0.56	0.60
Lysine	%	3.55	3.78	3.38	3.60
Tryptophan	%	0.52	0.55	0.51	0.54
Threonine	%	1.82	1.94	1.75	1.86
Isoleucine	%	1.97	2.10	1.91	2.03
Histidine	%	1.2	1.28	1.14	1.21
Valine	%	3.61	3.84	3.46	3.68
Leucine	%	3.31	3.52	3.21	3.41
Arginine	%	3.56	3.79	3.4	3.62
Phenylalanine	%	1.72	1.83	1.69	1.80
Taurine	%	0.35	0.37	0.33	0.35
Vitamins					
Vitamin A	IU/kg	2007.76	2135.91	2087.76	2221.02
Thiamine	mg/kg	5.98	6.36	6.52	6.94
Riboflavin	mg/kg	9.54	10.15	9.47	10.07
Niacin	mg/kg	218.37	232.31	222.18	236.36
Pantothenic acid	mg/kg	29.09	30.95	29.8	31.70
Pyridoxine	mg/kg	4.52	4.81	4.7	5.00
Biotin	µg/kg	945.78	1006.15	977.8	1040.21
Folic Acid	µg/kg	2413.79	2567.86	2505.26	2665.17
Vitamin B12	µg/kg	369.39	392.97	349.75	372.07
Vitamin C	mg/kg	29.4	31.28	31.3	33.30
Vitamin D3	IU/kg	1927.45	2050.48	2004.25	2132.18
Vitamin E	IU/kg	53.64	57.06	55.73	59.29
Choline	mg/kg	3348.84	3562.60	3222.98	3428.70

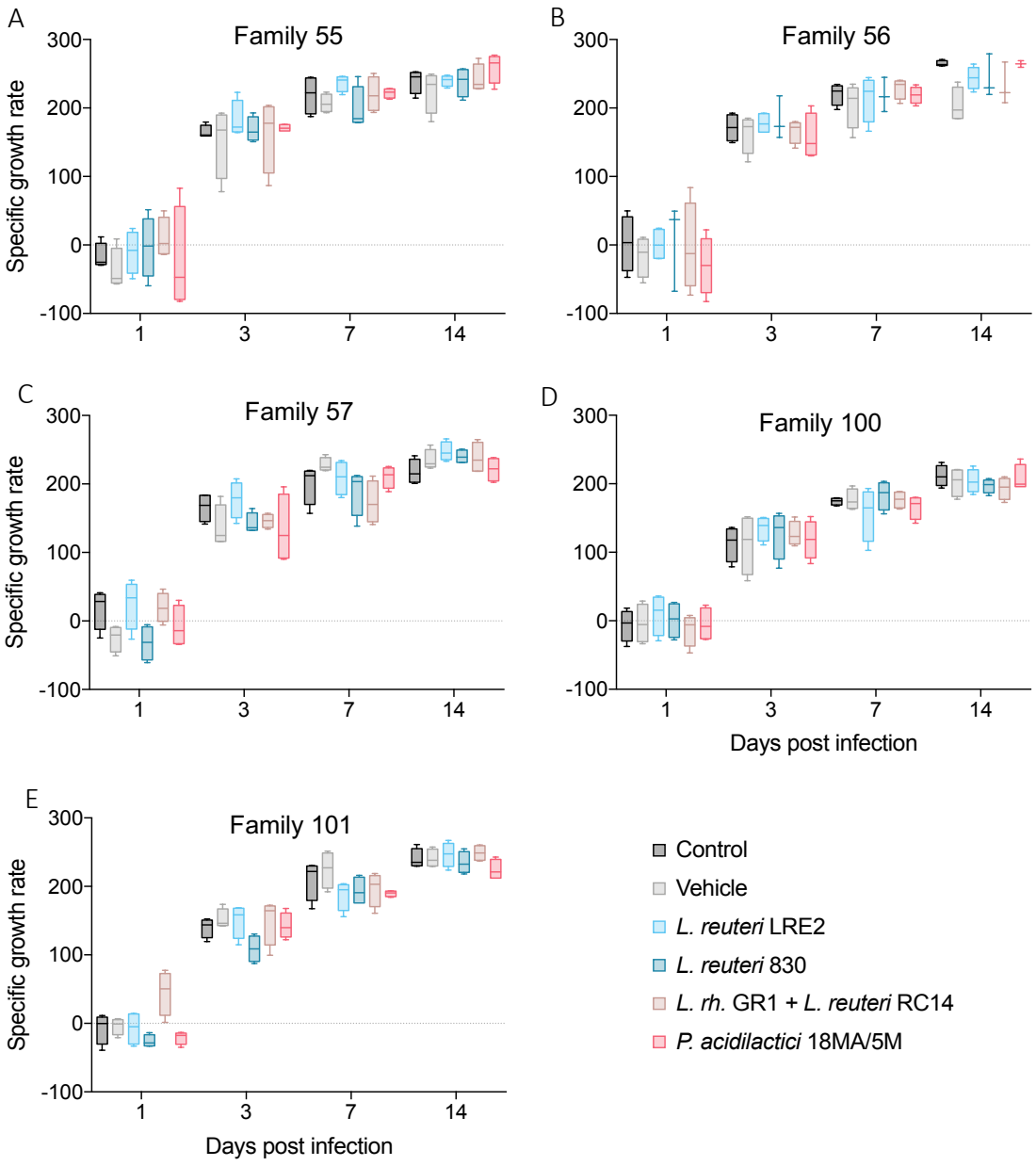
Minerals					
Calcium	%	2.01	2.14	1.9	2.02
Phosphorous	%	1.66	1.77	1.6	1.70
Sodium	%	1.55	1.65	1.47	1.56
Potassium	%	1.02	1.09	1	1.06
Magnesium	%	0.13	0.14	0.13	0.14
Manganese	mg/kg	27.55	29.31	30.77	32.73
Iron	mg/kg	126.6	134.68	128.23	136.41
Copper	mg/kg	9.29	9.88	10.01	10.65
Zinc	mg/kg	128.19	136.37	129.5	137.77
Selenium	mg/kg	1.34	1.43	1.29	1.37
Chloride	%	2.21	2.35	2.09	2.22
Iodine	mg/kg	4.02	4.28	4.18	4.45



Supplementary figure 3-1. Comprehensive gene stability as assessed using the RefFinder software (Xie, Xiao and Chen 2012; https://www.heartcure.com.au/reffinder/). The reference gene RPS20 was selected due to its combined stability and efficiency compared to the other potential reference genes.



Supplementary figure 3-2. Endogenous RPS20 quality control to ensure that run efficiencies were comparable. Samples were run in technical triplicates and the mean raw Ct value was plotted. Two mastermix preparations were made, and genes were compared to the respective RPS20 normalization controls (ie. RPS20_1 for ZO-1, JAM-1a, Ocldn, EcadH, and IL-8; RPS20_2 for IL-17a, TGFb, IL-10, Tricellulin, Cldn15, Muc2, Vil1, MYD88, and Cldn28B).



Supplementary figure 3-3. Specific growth rate of Chinook salmon from five different family crosses during infection with *Vibrio anguillarum*. Differences within and between groups were not statistically significant (two-way ANOVA and Tukey's multiple comparisons test). Boxes represent first and third quartile values, horizontal lines denote medians, and whiskers encompass maximum and minimum values.

Chapter 4

4. General discussion

4.1 The importance of microbes in aquaculture and study summary

Microbes are ubiquitous in aquaculture systems, and their effective management and pathogen control are vital for maximizing production. The supplementation of beneficial microbes has been increasingly recognized as a promising eco-friendly microbial management strategy (Langlois *et al.* 2021). Although several recent reports emphasize the benefits of probiotic supplementation in finfish, very few have endeavoured to characterize their effects at a molecular level in the gut, particularly in salmonids.

The primary goal of this project was to identify promising probiotic candidate strains for supplementation in salmonid aquaculture through the modulation of intestinal mucosal immunity and gut barrier function, with the intent that these could improve growth and disease resistance. The study design employed a combinatorial approach that integrated both *in vitro* and *in vivo* analyses. First, the rainbow trout intestinal epithelial cell line RTgutGC was used as a model for understanding the molecular response of the host to several strains of lactic acid bacteria (LAB) and to assess whether these microbes could protect the intestinal epithelium from damage due to *Vibrio anguillarum*. The second objective was to examine these effects at the organismal level through a field trial conducted in British Columbia. On site, juvenile Chinook salmon (3.5-17g) were supplemented with select LAB strains for two weeks, then infected with *V. anguillarum*.

The growth and mortality were recorded, and the expression of several tight junction and immune genes in the intestine was assessed to determine whether probiotic supplementation could maintain or improve the barrier function of the gut in the context of a bacterial challenge.

The main conclusion from this project is that the LAB strains herein examined did not exhibit a strong ability to modulate barrier function on an intestinal cell line, or enhance disease resistance in live salmonids. Although no deleterious effects were observed, the benefits provided to fish must be of sufficiently large magnitude to offset the costs in sourcing and administering the beneficial microbes and therefore make probiotic supplementation a viable, sustainable, and affordable solution for the industry.

In Chapter 2, LAB co-incubation did not induce an increase in the expression of several key tight junction molecules assessed. The strains originating from humans did not appreciably upregulate immune molecules. Some of these isolates did increase the expression of pro-inflammatory cytokines, but the experimental evidence was too preliminary to predict how this would affect live fish and so the suitability of these strains as probiotic candidates must be investigated further. Moreover, exposure to *V. anguillarum* was associated with a pronounced decrease in the expression of key TJ molecules, except for *occludin*, which was unexpectedly increased, perhaps signaling a defensive reaction by the host. The results appeared to suggest that the pathogen potentially targets occludin as a potentiator of the inflammatory response. This in turn further exacerbates the damage to the intestinal barrier and enables pathogen invasion into the circulation (Van Itallie *et al.* 2010). Interestingly, LAB pre-treatment mitigated

the injury caused by the pathogen, even though the expression patterns of several tight junction and immune molecules were not substantially altered.

While the proposed approach is informative, future studies would benefit from validating these findings in systems that more closely mimic the physiological conditions of the fish gut. In particular, the use of gut-on-chip and organoid systems that can reconstruct the basic intestinal architecture allow for the study of host-microbe interactions *in vitro* in a more physiologically relevant environment (Drieschner *et al.* 2019a, 2019b). Furthermore, the study of the host response to bacteria is constrained by the optimal growth conditions of both organisms. While the RTgutGC cells are cultured at room temperature in an aerobic environment, most LAB are grown optimally at around 37˚C and in the absence of oxygen. As such, the differences in temperature and oxygen levels can affect the metabolic activity of the candidate probiotics, presumably limiting the potential benefits on the host. Not only that, the fish swim in water that is well below room temperature (around 4˚C). This means the intestinal environment is not conducive to LAB reaching logarithmic growth unless they were to colonize and have access to growth factors. This raises the question of delivering the strains with prebiotic compounds or in an active growing stage. The latter could be achieved on a fish farm by using large fermentation tanks, then allowing the bacteria in suspension to adsorb to food pellets, just prior to feeding the fish. Experiments like this are worthy of investigation in order to determine the potential for probiotic organisms to improve yields.

The present study also examined changes at the transcript level. However, these differences in mRNA abundance might not always correlate to changes at the protein level, as several post-transcriptional and post-translational modifications can affect the

physiological function of the tight junctions (Shen, Weber and Turner 2008). Future work would benefit from further validating these findings through methods such as Western blotting and immunofluorescence, coupled with automated quantification of the staining. Although there are few commercially available fish-specific antibodies, some studies have had success using human- or murine-specific antibodies that target the conserved protein epitopes (Pumputis *et al.* 2019).

In Chapter 3, while supplementation of select LAB strains did not lead to a significant survival advantage of Chinook salmon against an infection challenge with *V. anguillarum*, there were appreciable family-specific improvements in survival. Aquaculture farmers use different families of fish, making it theoretically possible to improve the results by matching responder families with probiotic strains.

We had hypothesized that the gene expression of several tight junction and immune molecules would be enhanced by the probiotic strains. The fact that there was little difference between the control and vehicle groups on the RTgutGC cells could have one of several explanations. For example, it is known that lactobacilli GG does modulate barrier proteins in Caco-2 intestinal cells (Johnson-Henry *et al.* 2008; Miyauchi, Morita and Tanabe 2009; Karczewski *et al.* 2010). So, either there are major differences between the cells and receptivity to this strain, or the suspending fluid and incubation environment somehow inhibited an interaction, or there are other factors not identified that interfered with the signaling. Nevertheless, based on the experimental data, the candidate fish probiotic strains showed no substantial evidence of being able to confer health benefits to Chinook salmon in the context of this experiment.

Although the use of models has been instrumental for foundational advances in the understanding of the physiology of related organisms, there are no substitutes for investigating the intended target organism directly, whenever possible. The present study examined the suitability of LAB supplementation in Chinook salmon in the context of a pathogen challenge. The advent of ethically conducting experiments in the target host species enables the direct observation of the effects of the treatments in the context of the complexities of the organism's physiology. This is especially important as it pertains to investigating host-microbe interactions. For example, it is not uncommon that host-microbe studies conducted in rodent models do not hold true for human hosts (Nguyen *et al.* 2015). As such, aquaculture research can greatly benefit from studies of fish in tanks or the wild. For the latter, feeding non-pathogenic LAB into the river or sea should not constitute an environmental hazard, unlike adding *Vibrio* pathogens. The LAB will not survive for long in the water, and if they are ingested by fish or other mammals, they will not colonize or persist. Therefore, their activity is short-acting and unlikely to have negative environmental consequences. This makes it all the more important to deliver physiologically active organisms that are taken up rapidly during the feeding frenzy.

In addition to ethical issues, a drawback of animal trials is that they are time- and resource-intensive and the high complexity of the system does not lend itself to a fine control of variables that can affect the fish response to microbial management strategies. Therefore, some effects can be masked. To reduce the risk of this happening, gnotobiotic models could be considered if they are feasible in a research laboratory (Perez-Pascual *et al.* 2021).

Clearly, the mode of delivery and the metabolic state of the living microbes are crucial. Although some studies have detected the presence of the supplemented microbes in the fish intestine, none have investigated whether the microbes are functionally and metabolically active in the fish gut. A powerful approach that could be employed to address this knowledge gap is the integration of multi-omic datasets, which integrate metagenomic and metabolomic tools to identify enriched pathways and the key molecules being produced by the microbiota as a snapshot of the functional state of the system (Li *et al.* 2017; Uengwetwanit *et al.* 2020). Furthermore, the development of novel delivery vehicles appropriate for aquaculture systems can ensure the targeted release of the living microbes in the intended organ (*ie*. the gut).

4.2 Final considerations

The complex network of interactions between microbes and their hosts exists on a continuum from pathogenicity to mutualism and can be described as an interdependent triad of host-microbe, host-pathogen, and microbe-microbe interactions (Boutin *et al.* 2013). As such, the importance of microbes in aquaculture systems cannot be overlooked. A better understanding of the structure and function of organisms in aquatic settings will help guide strategies to optimize fish health and their productivity as a food supply.

This thesis investigated the development of tools for the assessment of host-microbe interactions in salmonids systems. Although further investigation of the phenomena discussed here is necessary, it is hoped that the findings will contribute to greater investment in microbial management strategies that are effective, economically sound, and environmentally sustainable. Future studies that focus on the nexus of

industry goals and academic interests can bridge the gap between the technologies needed and the applicability necessary for meeting production goals. Pathogens will always exist; but rather than try to eradicate them using antimicrobial agents which themselves can be toxic and destroy commensal organisms, the use of probiotic strains should continue to be explored to improve host immune performance and animal welfare and support a healthy gut microbiota.

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Appendices

Appendix A.

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Curriculum vitae

Name: Luana S. Langlois

Post-secondary education and degrees:
The University of Western Ontario
London, Ontario, Canada
BSc. (Hons.) Genetics & Biochemistry
2015 – 2019

Honours and awards:
Natural Sciences and Engineering Research Council/Canada Graduate Scholarship – Master's
2020 – 2021

Queen Elizabeth II Graduate Scholarship in Science and Technology/Ontario Graduate Scholarship – offered but denied
2020 – 2021

Western Graduate Research Scholarship
2020 – 2021

Mitacs/Lab2Market Research Commercialization Program – offered but denied
2020 – 2021

Schulich School of Medicine and Dentistry/Peadiatrics Summer Studentship Award
2019

Faculty of Science/Dean's Honour Roll
2017 – 2018, 2018 – 2019

Related work experience:
Research assistant, Bérubé Lab
Lawson Health Research Institute, Victoria Hospital, LHSC
May 2019 – August 2019

Honours research thesis student, Bérubé Lab
Lawson Health Research Institute, Victoria Hospital, LHSC
September 2018 – April 2019

Undergraduate research assistant, Bérubé Lab
Lawson Health Research Institute, Victoria Hospital, LHSC
May 2018 – August 2018

Undergraduate research assistant, Karas Lab
Department of Biochemistry, Western University
September 2017 – April 2018

Undergraduate research assistant, Reid Lab
Lawson Health Research Institute, St. Joseph's Hospital, LHSC
May 2017 – February 2018

Undergraduate research assistant
Department of Biology, Western University
September 2016 – April 2017

Leadership experience:

Co-director, co-founder
Canadian Genetically Engineered Machine Conference 2020
September 2018 – October 2020

Communications associate
Forest City SynBio, London, ON
February 2020 – July 2020

President, founder
Western iGEM team, Western University
February 2019 – November 2019

President
Western Synthetic Biology Research Program, Western University
May 2017 – November 2019

Peer mentor
Leadership and Academic Mentorship Program, Western University
September 2016 – April 2019

Membership coordinator
Western Heads East, Western International, Western University
September 2017 – April 2018

VP Communications, co-founder
Western Synthetic Biology Research Program, Western University
September 2016 – April 2017

Summer Academic Orientation Leader
The Student Success Centre, Western University
June 2016 – August 2016

Community involvement:

Worship Leader and Children's Ministry Leader
New Life Fellowship Church, Mt. Brydges, ON
August 2014 – present

London Health Science Centre Outreach volunteer
Victoria Hospital, LHSC
November 2018

SciHike Outreach Program Coordinator
Western Synthetic Biology Research Program, Western University
August 2017 – November 2019

Biology Open House Outreach Volunteer
Department of Biology, Western University
March 2018

Chemistry Open House Outreach Volunteer
Department of Chemistry, Western University
November 2016, June 2017

Alzheimer's Outreach Program Recreation Volunteer
McCormick Home, London, ON
Summer 2015, 2016, 2017

Publications:

Langlois L, Akhtar N, Tam KC *et al.* Fishing for the right probiotic: Host-microbe interactions at the interface of effective aquaculture strategies. *FEMS Microbiol Rev* 2021:1–19.

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