Serological diversity in Flavobacterium psychrophilum: A critical update using isolates retrieved from Chilean salmon farms

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Serological diversity in *Flavobacterium psychrophilum*: a critical update using isolates retrieved from Chilean salmon farms

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Running title: Updated serotyping for Chilean *F. psychrophilum*

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Abstract

Chile is currently the second largest producer of farmed salmon worldwide, but *Flavobacterium psychrophilum,* as one of the most detrimental pathogens, is responsible for major losses during the freshwater culturing step in salmonid fish farms. An antigenic study conducted 10 years ago reported four serological groups using 20 *F. psychrophilum* Chilean strains. To reduce disease outbreaks and to develop vaccine candidates, antigenic knowledge needs to be regularly updated using a significant number of additional recent *F. psychrophilum* isolates. The present study aimed at investigating the serological diversity of 118 *F. psychrophilum* isolates collected between 2006-2018 from farmed Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), and coho salmon (*Oncorhynchus kisutch*). The current study supports an expansion of the known antigenic groups in Chile from 4 to 14. However, the use of the slide-agglutination technique for serotyping is costly, labor-intensive, and requires significant technical expertise. Addressing these points, the mPCR-based procedure was a very useful tool for serotyping the collected Chilean *F. psychrophilum* isolates. This technique revealed the presence of diverse mPCR serotypes (i.e., types 0, -1, -2, and 4). Therefore, mPCR should be employed to select the bacterial strain(s) for vaccine development and to conduct follow-up, selective breeding, or epidemiological surveillance in Chilean fish farms. Given the presented findings, changes to Chilean fish-farming practices are vital for ensuring the continued productivity and wellbeing of farmed salmonids.

Keywords: serological diversity, serotype PCR, *Flavobacterium psychrophilum*, Chilean salmon farms.

1. INTRODUCTION

Flavobacterium psychrophilum is the etiological agent of bacterial cold-water disease and rainbow trout fry syndrome, two conditions that are responsible for major losses during the culturing step in freshwater salmonid fish farms worldwide (Nematollahi, Decostere, Pasmans & Haesebrouck, 2003). Though variable, mortalities resulting from these conditions without intervention generally range from 2–30% (Wiens, Palti, & Leeds, 2018), but in extreme cases, mortalities can be as high as 50–90% (Cipriano & Holt 2005; Nilsen, Olsen, Vaagnes, Helleberg, Bottolfsen, Skjelstad & Colquhoun, 2011). These diseases affect essentially salmonids but non-salmonid freshwater or anadromous fish may also be affected (Elsayed, Eissa, & Faisal, 2006; Verma & Prasad, 2014; Soares, Walker, Elwenn, Bayliss, Garden, Stagg, & Munro, 2019).

In Chile, which is currently the second largest producer of farmed salmon worldwide, these conditions have been observed since 1993 in facilities for freshwater rainbow trout (*Oncorhynchus mykiss*) and the incidence of *F. psychrophilum* has since dramatically increased in Atlantic salmon (*Salmo salar*) and coho salmon (*Oncorhynchus kisutch*) (Bustos, Calbuyahue, Montaña, Opazo, Entrala & Solervisenc, 1995; Avendaño-Herrera, Ilardi & Fernández, 2009). This pathogen causes significant to high mortality rates (i.e. 5-70%) in fingerlings, making it one of the most detrimental pathogens for Chilean freshwater aquaculture facilities (Godoy & Avendaño-Herrera, 2012). Despite the severe impact of this pathogen, antimicrobial therapies are currently the only control method in farmed fish. Outbreak control on Chilean farms using estimated tons of florfenicol and oxytetracycline has provoked detrimental environmental consequences (Henríquez-Núñez, Evrard, Kronvall, & Avendaño-Herrera, 2012; Avendaño-Herrera, 2018).

The development of a sustainable aquaculture requires better epidemiological knowledge of circulating pathogens and of intraspecific genetic/antigenic pathogen diversity. To reduce disease outbreaks and to develop vaccine candidates, epidemiological research was conducted on different Chilean *F. psychrophilum* isolates recovered from disease outbreaks. Valdebenito and Avendaño-Herrera (2009), using RAPD, 16S rRNA alleles, and REP-PCR, reported relative genetic homogeneity among 20 Chilean *F. psychrophilum* isolates collected from farmed Atlantic salmon and rainbow trout. The same year, Avendaño-Herrera, Araya, and Fernández (2009) proposed that the disease outbreaks in Chilean farms were dominated by a closely related cluster of strains, as determined by pulsed-field gel electrophoresis with 12 isolates. However, Avendaño-Herrera, Houel, Irgang, Bernardet, Godoy, Nicolas, and Duchaud (2014) using multi-locus sequence typing on 94 Chilean isolates, revealed a countrywide distribution of 15 genotypes closely related to those most prevalent in European and North American fish farms, in addition to overlapping host species for the different lineages.

While genotypic information about Chilean *F. psychrophilum* strains is arguably abundant, antigenic classification studies are starkly lacking. Only a single investigation on 20 isolates has been published for Chile (Valdebenito & Avendaño-Herrera, 2009), the results of which contributed to the tentative licensing of a commercial vaccine in 2013. The so-termed Flavomune vaccine (SAG N∘2160-BP) contains whole cells inactivated with formaldehyde (Solis, Poblete-Morales, Cabral, Valdés, Reyes, Avendaño-Herrera & Feijóo, 2015). In the seven years since the initial applications of this vaccine, sales and use in aquaculture facilities have decreased as a result of efficiency concerns (publication in process). Therefore, it is essential to update antigenic knowledge using a high number of recently collected *F. psychrophilum* isolates, in addition to applying up-to-date

methodological tools for comparative purposes (e.g. molecular serotyping via mPCR [Rochat, Fujiwara-Nagata, Calvez, Dalsgaard, Madsen, Calteau, & Duchaud, 2017]). In the present study, 118 *F. psychrophilum* isolates retrieved from Chilean salmonid farms were subjected to serological and genetic analyses to provide a 10-year update in knowledge, better elucidate pathogen origin and propagation, and propose control and management practices.

2. MATERIALS AND METHODS

2.1. Bacterial isolates and growth conditions

A total of 118 *F. psychrophilum* isolates were examined (Table 1). These isolates were recovered between 2006 and 2018 from rainbow trout, Atlantic salmon, and coho salmon directly sampled in the field or sent to diagnostic laboratories. Most specimens presented typical clinical signs of bacterial cold-water disease or rainbow trout fry syndrome. The *F. psychrophilum* type strain NCIMB 1947^{T} (serotype Fp^{T}) was included for comparative purposes in all analyses. Each isolate was confirmed as *F. psychrophilum* by using standard phenotyping procedures (Bernardet, Nakagawa, & Holmes, 2002), including analyses of colony morphology and pigmentation, cell morphology, gliding motility, Gram-staining, cytochrome oxidase and catalase activities, oxidation/fermentation reactions, the presence of cell wall-associated flexirubin-type pigments, and the absorption of Congo red.

For all experiments, *F. psychrophilum* strains were routinely grown on the tryptone yeast extract salts medium (0.4% tryptone, 0.05% yeast extract, 0.02% anhydrous calcium chloride, 0.05% magnesium sulphate heptahydrate, pH 7.2) in either a liquid or solid state (tryptone yeast extract salts medium supplemented with 1% (w/v) bacteriological agar). Bacteria were aerobically incubated at 18 °C for 3-5 days. Stock cultures were maintained frozen at -80 °C in Cryobille tubes (AES Laboratory) in tryptone yeast extract salts broth with 15% glycerol.

2.2 DNA extraction and confirmation of bacterial species

Chromosomal DNA was extracted using the InstaGene™ Matrix (Bio-Rad Laboratories) following the manufacturer's recommendations. DNA concentration was adjusted using 1X Tris-EDTA buffer (Thermo Fisher Scientific) to 5 or 50 ng μL^{-1} (depending on the genetic

analysis) with a NanoDrop™ Lite (Thermo Fisher Scientific). Extracted DNA was maintained at -20 °C until use in PCR analyses. Amplification reactions used 2 µL of each DNA solution.

In addition to phenotypic tests, each isolate was genetically confirmed as *F. psychrophilum* prior to antigenic and genetic typing using two different PCR tests (Urdaci, Chakroun, Faure, & Bernardet, 1998). The amplification cycles used for denaturation, primer annealing, and primer extension were carried out according to published PCR protocols (Urdaci et al., 1998). Negative controls, consisting of the same reaction mixture but with sterile distilled water instead of template DNA, were included in each PCR batch. Each reaction was prepared using 0.5 µL of each primer (10 µM) and the GoTaq® Green Master Mix (Promega), according to the manufacturer's instructions. Positive results included the presence of a single product with a size comparable to strain NCIMB 1947^T (i.e. 1088 base pairs [bp] for primers FP1 and FP2) (Urdaci, Chakroun, Faure, & Bernardet, 1998). Aliquots (5 µL) of the PCR product were separated on a 1% (w/v) agarose gel for 60 min at 100 V in 1× TAE and 1× GelRed® Nucleic Acid Gel Stain (Biotium). The bands were photographed under UV light and computer digitized (Gel Doc 100, Bio-Rad). The AccuRuler 100bp Plus DNA Ladder (100- to 3000-bp, MaestroGen) served as a molecular mass marker.

2.3 Serological characterization using antisera

Antigenic analyses were carried out using slide agglutination as described by Valdebenito and Avendaño-Herrera (2009). Briefly, thermostable antigens of each *F. psychrophilum* isolate were obtained by heat-killing the bacterial suspension (10^9 cells mL^{-1}) in phosphate buffered saline (pH 7.4) at 100 °C for 60 min, followed by a single wash in the same saline solution and maintenance at 4 °C until required. Slide agglutination employed the anti-rabbit

polyclonal antibody against representative Atlantic salmon strains (i.e. 1739 and 1196) and rainbow trout isolates (i.e. 1731 and 1150), as well as antisera against strain NCIMB 1947^T (serotype Fp^T) (Valdebenito & Avendaño-Herrera, 2009). Each serological analysis was performed with the unabsorbed rabbit serum. For slide agglutination, 10 µL of each *F. psychrophilum* serum were mixed with a similar volume of thermostable antigen suspensions on a slide using a gentle rocking motion. When cross reactions were observed with more than two antisera prepared with isolates from different hosts, assays used absorbed antisera with the heterologous antigen (Romalde, Magariños, Barja & Toranzo 1993). The reaction was recorded macroscopically against a dark background after 1 to 2 minutes. Controls for autoagglutination were performed in a saline buffer.

2.4 Multiplex PCR-based serotyping

The Chilean isolates were typed using the multiplex PCR (mPCR)-based serotyping method described by Rochat et al. (2017). In order to maximize the discriminatory power of the scheme, an additional primer pair, able to identify a supplementary mPCR serotype (namely type-4) was included. This primer pair [Type-4_fw (5'-TGAAGCAAAAGCAACAAACA-3') and Type-4_rev (5'-CCCCAAACTGCTTACCTAAT-3')] resulted in an amplification product of 992 bp.

PCR analyses were performed in a total volume of 25 µL using the GoTaq® Green Master Mix (Promega) and 10 Mm of each primer. The mPCR amplification mix was heated at 95 °C for 5 min, followed by 30 cycles at 95 °C for 30 s, 52 °C for 30 s, 72 °C for 60 s, and a final extension at 72 °C for 10 min. The amplified products were electrophoresed on 2% agarose gels run in 1× TAE (0.04 M Tris, 1 mM EDTA, pH 8.0). The gels were photographed under

UV light and computer digitized (Gel Doc 100, Bio-Rad), and the AccuRuler 100bp Plus DNA Ladder (MaestroGen) was used as the molecular size standard.

2.5 PCR- restriction fragment length polymorphism (RFLP)

Two primer pairs were used (Izumi, Aranishi, & Wakabayashi, 2003); a degenerate universal primer pair (GYR-1/GYR-1R) and a specific primer pair for the gyrB gene of *F. psychrophilum* (PSY-G1F/PSY-G1R). The expected amplification products were 1178 bp and 290 bp for the universal primer pair and 1017 bp for the specific primer pair. Thermocycle programs for the amplifications were carried out according to Izumi et al. (2003). The PCR products were digested according to the manufacturer's instructions with two restriction enzymes, *RsaI* (Promega, GYR-1/GYR-1R) and *HinfI* (New England BioLabs Inc., PSY-G1F/PSY-G1R). The digested product (10 µL) was analyzed by horizontal electrophoresis.

2.6 16S rRNA allele PCR assays

PCR analyses were used to differentiate the presence of a single or both 16S rRNA alleles, which provide information about polymorphisms or genetic lineages I and II (Soule, LaFrentz, Cain, LaPatra, & Call, 2005). Distribution of these alleles among the different isolates were determined with two independent PCR tests using primers for the allele of strain CFS259-93 and that of strain ATCC 49418^T (equivalent to NCIMB 1947^T). These primers respectively amplify products of 600 and 298 bp. The PCR program included one denaturation cycle at 95 ºC for 5 min, followed by 30 cycles at 95 ºC for 30 s, 61 ºC for 60 s, and 72 ºC for 60 s, and a final extension of one cycle at 72 ºC for 10 min (Ramsrud, LaFrentz, LaFrentz, Cain, & Call, 2007).

3. RESULTS AND DISCUSSION

All isolates were biochemically homogeneous regardless of source (i.e. salmon or trout) and year of isolation, and all were identical to the type strain NCIMB 1947^{T}. Regarding phenotypic testing, all bacterial isolates were long, slender, Gram-negative rods with gliding motility that were catalase positive and weakly cytochrome-oxidase positive. All isolates contained cell wall-associated flexirubin-type pigments, but did not absorb Congo red. The FP1-FP2 primer pairs produced a unique PCR product of the expected length (i.e. 1088 bp), thereby providing another indication that all studied isolates belongs to the species *F. psychrophilum* (data not shown).

A total of 118 *F. psychrophilum* isolates were serotyped and genotyped (Table 1). Of these, 84 isolates were obtained from rainbow trout, 33 from Atlantic salmon, and 1 from coho salmon. Salmon farming take place in a wide spatial distribution covering from central to southern Chile (~1700 km), with most freshwater farm and hatcheries located in the Biobío, La Araucanía, Los Ríos and Los Lagos regions also known as VIII, IX, XIV and X regions, respectively (Quiñones, Fuentes, Montes, Soto, & León-Muñoz, 2019). Our isolates originated from the following regions in Chile, listed geographically from north to south: Valparaíso, 1 isolate; Metropolitana de Santiago, 6 isolates; Maule, 6 isolates; Biobío, 34 isolates; La Araucanía, 49 isolates; Los Ríos, 2 isolates; Los Lagos, 5 isolates and Aysén del General Carlos Ibáñez del Campo, 10 isolates. The precise geographical origin was unavailable for 5 isolates (Table 1). The quantity of isolates collected per year was as follows: 9 isolates in 2006; 3 in 2007; 13 in 2011; 19 in 2013; 24 in 2014; 12 in 2015; 20 in 2017 and 18 in 2018. Importantly, the number of *F. psychrophilum* isolates included per region and per year does not reflect disease prevalence.

3.1 Serological characterization using antisera

Based on the slide agglutination test proposed by Valdebenito and Avendaño-Herrera (2009) for Chilean *F. psychrophilum*, serotyping was performed on the 118 isolates. Eight of the included isolates (i.e. 1733, 1658, 1793, 1779, 19250, 1150, 19443, and 1731) were originally reported in Valdebenito and Avendaño-Herrera (2009). Unfortunately, isolates 1196 and 1739 could not be included in the study. Nevertheless, the results still stand as isolate 1733 presents the same profile as isolates 1196 and 1739 from Atlantic salmon (Valdebenito & Avendaño-Herrera, 2009). Slide agglutination assays using O-antigens confirmed the existence of antigenic heterogeneity, but while the 2009 study reported four main patterns (groups 1-4) of serological reactions, the current investigation defined a total of 14 serological combinations (Supplementary Table 1). These groups included five isolates without a serological reaction to any the four antisera used, which were prepared against two isolates recovered from Chilean rainbow trout and Atlantic salmon, respectively. However, these isolates showed strong reaction with the anti-serum obtained with the type strain. The four previously described patterns of serological groups (Valdebenito & Avendaño-Herrera, 2009) represented 59.66% of the currently assessed isolates. The first group dominated, comprising 35 of the 118 *F. psychrophilum* isolates that showed cross-reactions with the unabsorbed antisera raised against the two Atlantic salmon and rainbow trout isolates.

3.2 Multiplex PCR-based serotyping

The mPCR-based serotyping method also showed antigenic heterogeneity among the Chilean isolates. Worth noting, the present study proposed the design of an updated mPCR protocol able to discriminate an additional serotype (Type-4), which was not previously described by Rochat et al. (2017). The distribution of PCR-serotypes for rainbow trout, Atlantic salmon,

and coho salmon isolates are shown in Figure 1. *F. psychrophilum* isolates obtained from rainbow trout and Atlantic salmon in La Araucanía region were mixed, regardless of serotype. Therefore, the 49 Chilean isolates from this region were classified as type-0, 2, and 4. During the early development of the industry in Chile, freshwater fish farms were mainly located in the Los Lagos region (X region). Given the growing demand for eggs, fingerlings and smolts, the industry then began to build freshwater fish farms in more northern areas (Quiñones et al., 2019). The Araucanía region (IX region) is referred to as the "cradle" of Chilean salmon farming as it is where reproductive farms are located and where spawning occurs. After initial growth (> 5 g on average), fish are distributed to different regions in Chile. The most proximal is the Biobío region (VIII), where *F. psychrophilum* type-2 isolates were also observed in rainbow trout and Atlantic salmon.

In the present study, six Chilean *F. psychrophilum* isolates belonged to type-0. Applying the guidelines described in Rochat et al. (2017), type-0 isolates correspond to Fp^T serotype according to the Lorenzen and Olesen (1997) serotyping scheme and did not contain any genes related to FI056_50102, DK002_320117, or FPC840_340035 encoding different O-antigen polymerases (Cisar, Bush, & Wiens, 2019). Type-0 isolates were obtained from rainbow trout and Atlantic salmon over a wide geographical distribution and many years of isolation (between 2006 and 2017) (Figure 1a). Moreover, 15 isolates belonged to type-1 (corresponding to the Fd serotype according to the Lorenzen and Olesen serotyping scheme), and 64 isolates belonged to type-2 (corresponding to the Th serotype according to the Lorenzen and Olesen serotyping scheme) (Figure 1b & c). In addition, no Chilean *F. psychrophilum* isolate was classified as serotype-3. This serotype has been specifically described for isolates recovered from ayu (*Plecoglossus altivelis*) or ayu eggs in Japan (Rochat et al., 2017), but, in Chile, this fish species is neither cultivated nor endemic. The

lack of serotype-3 isolates was consistent with the results of PCR-RFLP analyses, which did not find genotype A among the 118 Chilean isolates.

Furthermore, the presently obtained data clearly show that 33 of the 118 isolates belong to serotype-4 (Figure 1d). Based on host distribution, serotype-1 was detected exclusively from rainbow trout in perfect accordance with previous findings (Rochat et al. 2017), while the remaining serotypes were not associated with or exclusive to a certain fish host (i.e. Atlantic salmon or rainbow trout). In particular, Rochat et al. (2017) reported that 22 of 23 strains retrieved from coho salmon belonged to type-0, being the Chilean isolate MHC 1710K, an exception. Therefore, two Chilean isolates obtained from coho salmon, MHC 1710K obtained in 2001 (Avendaño-Herrera et al., 2014) and LM-10-Fp from the present study belonged to serotype-2. Nevertheless, the present data are limited in relation to serotyping in coho salmon since only one sample (isolate LM-10-Fp) from this species was available (Table 1), and more isolates will be needed to substantiate the initial conclusions drawn from the two Chilean isolates in coho salmon.

A final point worth highlighting is that *F. psychrophilum* isolates obtained from the same farms and years presented serological diversity, with more than one serotype found among the sampled isolates. For example, isolates recovered from outbreaks in 2014 at the EP aquaculture facilities were classified as type-0, type-1, and type-2. This reflects greater diversity than was found for the antigenic patterns reported by Valdebenito and Avendaño-Herrera (2009).

3.3 PCR-RFLP and 16S rRNA allele PCR assays

RFLP analysis of *gyrB* PCR products using the *HinfI* enzyme showed an identical cleavage pattern of the DNA fragment for all 118 *F. psychrophilum* isolates corresponding to genotype B. This finding aligns with previous descriptions by Valdebenito and Avendaño-Herrera (2009) for Chilean isolates. When RFLP tests were performed on PCR products amplified with the specific primer pair PSY-G1F and PSY-G1R, 24 isolates (20.3%) belonged to genotype R, while the remaining 94 isolates (79.7%) were grouped as genotype S. These results confirm a lack of relationship between PCR-RFLP genotyping and host fish species (i.e. Atlantic salmon or rainbow trout) in Chilean isolates. This contrasts with reports from Japan, where 90.3% of *F. psychrophilum* isolates recovered from rainbow trout were determined to be genotype R (Izumi et al., 2003).

The distribution of the two 16S rRNA alleles was also examined to determine if there was an association with the fish species (trout [genetic lineage I] vs Pacific salmon [genetic lineage II]), as described by Ramsrud et al. (2007). Of the studied isolates, 72% (n = 85) contained only the alleles found in strain CFS 259-93, while the remaining 33 isolates were positive for both the alleles found in the type strain and in strain CSF 259-93 (Table 1). While there was a clearly major cluster, no association could be found with the host fish species, in line with findings from the 2009 study (Valdebenito & Avendaño-Herrera, 2009). However, all Chilean *F. psychrophilum* isolates classified as type-2 contained only the alleles found in strain CSF 259-93 (corresponding to lineage II), while the majority of the type-4 isolates (i.e. 30 of 33) presented both allele sequences (Table 1).

The presently obtained results, from both slide agglutination and mPCR, evidence much greater antigenic heterogeneity than found in 2009, though analyses used the same four Chilean antisera. Differences in antigens, antisera, and serotyping techniques (e.g. slide

agglutination or ELISA) can result in a lack of consensus among serological groups for bacterial species, which can complicate vaccine development. By contrast, PCR-based serotyping proved greatly advantageous for serotyping the Chilean isolates, aligning with outcomes reported by Saticioglu, Duman, Wiklund, and Altun (2018) for Turkish rainbow-trout isolates.

The observed antigenic variability could have arisen from pressures exerted by overlapping host ranges, particularly as most fish farms in Chile house mixed stocks of different salmonids (e.g. Atlantic salmon, rainbow trout, and coho salmon) (Avendaño-Herrera et al., 2009; 2014). Furthermore, fish movements are an integral part of the Chilean salmon farming production cycle (Mardones, Martine-Lopez, Valdes-Donoso, Carpenter, & Perez, 2014). In general, most eggs are incubated and raised from fry until pre-smolt states at the hatchery facility, where movements of live fish to another freshwater facility occur. So, Chilean fish-farming practices mean that most fish are transported once or twice over their lifetime to complete development, often over long distances (> 380 km) (Godoy & Avendaño-Herrera, 2012) and in the different freshwater facilities (e.g. lake-based, tank and cage systems, estuary cage systems, stream-based flow-through systems, and recirculation tank systems).

In addition to transport during growth, use of the same commercial vaccine for more than five years has possibly provoked changes in the predominant serotypes or serological groups in Chile. In fact, routine use of the same vaccine and antigens can trigger vaccine breaks, as has been described for other freshwater diseases in Chile, such as enteric red mouth disease (Bastardo, Bohle, Ravelo, Toranzo & Romalde 2011). Apart from vaccination, Chilean aquaculture farms have, for years, combatted *F. psychrophilum* by using autochthonous immersion bacterins (Bravo & Midtlyng, 2007). Nevertheless, these bacterins

333 are made from single isolates, and, ultimately, information about treatment effectivity in
334 terms of relative survival is lacking.

335 The present study provides previously unknown information relevant to bacterin
336 treatments – a single farm could harbor *F. psychrophilum* isolates belonging to distinct
337 antigenic groups or serotypes, in line with the serotyping patterns reported by Valdebenito
338 and Avendaño-Herrera (2009) and Rochat et al. (2017). Taken together, the aforementioned
339 practices have likely further contributed to the serological diversity reported herein for *F.*
340 *psychrophilum.*

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344 **4. CONCLUSIONS**

345 The present study provides a critical update to the knowledge on serological diversity in *F.*
346 *psychrophilum*. While prior studies had already reported the presence different serological
347 groups of strains in Chile, the current study supports an expansion of the known antigenic
348 groups in Chile from 4 to 14. However, the use of the slide-agglutination technique for
349 serotyping is costly, labor-intensive, and requires significant technical expertise. Addressing
350 these points, the mPCR-based procedure was a very useful tool for serotyping the collected
351 Chilean *F. psychrophilum* isolates. It reveals the presence of diverse mPCR serotypes (i.e.,
352 types 0, -1, -2, and 4). Therefore, the application of mPCR and could be employed for the
353 selection of bacterial strain for vaccine development and follow up, selective breeding, or
354 epidemiological surveillance in Chilean fish farms. Given the presented findings, changes to
355 Chilean fish-farming practices are vital for ensuring the continued productivity and wellbeing
356 of farmed salmonids.

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CONFLICT OF INTEREST

The authors declare that research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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477

478 **Figure legends**

479 **Figure 1** Distribution of Chilean F. psychrophilum recovered rainbow trout, Atlantic salmon
480 and coho salmon isolates (n = 118) farmed in different geographical areas from
481 Chile according to the PCR serotyping method (i.e. type 0, 1, 2 and 4).

Table 1 *Flavobacterium psychrophilum* strains used in this study and their molecular serotypes, serological groups, and PCR-RFLP analysis for each isolate. Abbreviations: AtS, Atlantic salmon; Rt, rainbow trout; Cs, coho salmon; ++, positive reaction; -, negative reaction; ni, uninformed; RM, Metropolitana; V, Valparaíso; VII, Maule; VIII, Biobío; IX, La Araucanía; X, Los Lagos; XI, Aysén and XIV; Los Ríos.

								Agglutination with serum anti-					Genotypes using PCR-RFLP		
								Atlantic salmon		Rainbow trout		Control			
	Isolates	Host	Year	State	Farm	Organ	Serotype	1196	1739	1150	1731	ATCC 49418^T	A/B	R/S	16S RNA allele
1	1733	Rt	2006	VIII	AT	Kidney	Type-0	++	++	++	++	-	B	R	Only CSF
2	CUR-L4	AtS	2013	IX	CR	Ulcer	Type-0	-	++	-	-	-	B	S	Both
3	CC26	Rt	2014	VIII	EP	Spleen	Type-0	-	++	-	++	++	B	R	Only CSF
4	PF-38	AtS	2014	RM	PÑ	Kidney	Type-0	++	++	-	++	++	B	S	Both
5	P9	Rt	2015	IX	MP	Ulcer	Type-0	++	++	++	++	++	B	S	Both
6	Pto5	Rt	2017	VIII	ni	Ulcer	Type-0	-	-	++	-	-	B	R	Only CSF
7	1658	Rt	2006	X	RP	Kidney	Type-1	++	++	-	++	-	B	R	Only CSF
8	1793	Rt	2006	VIII	AT	kidney	Type-1	++	-	++	++	-	B	R	Only CSF
9	1779	Rt	2006	VIII	AT	Kidney	Type-1	-	++	++	-	-	B	R	Only CSF
10	19250	AtS	2006	XI	PC	Skin	Type-1	++	++	++	++	-	B	S	Only CSF
11	C7	Rt	2011	V	RB	Kidney	Type-1	++	++	++	++	-	B	S	Only CSF
12	C8	Rt	2011	VIII	KR	Ulcer	Type-1	++	++	++	++	++	B	S	Only CSF
13	C13	Rt	2011	VIII	KR	Kidney	Type-1	++	++	++	++	-	B	S	Only CSF
14	CC1	Rt	2014	VIII	EP	Liver	Type-1	-	++	-	-	-	B	S	Only CSF
15	CC5	Rt	2014	VIII	EP	Kidney	Type-1	-	++	-	-	-	B	S	Only CSF
16	CC8	Rt	2014	VIII	EP	Tail	Type-1	-	++	-	-	++	B	R	Only CSF
17	CC17	Rt	2014	VIII	EP	Spleen	Type-1	++	++	++	-	++	B	R	Both
18	CC50	Rt	2014	VIII	EP	Fresh water	Type-1	-	++	++	-	-	B	R	Only CSF
19	FP003	Rt	2015	IX	VR	ni	Type-1	++	-	++	++	-	B	R	Both
20	FP005	Rt	2017	XIV	PP	ni	Type-1	++	++	++	++	-	B	R	Only CSF
21	[4]	Rt	2017	ni	ni	Spleen	Type-1	-	-	++	-	-	B	S	Only CSF

Continued Table 1

	Isolates	Host	Year	State	Farm	Organ	Serotype	Agglutination with serum anti-					Genotypes using PCR-RFLP		16S RNA allele
								Atlantic salmon		Rainbow trout		Control			
								1196	1739	1150	1731	ATCC 49418^T	A/B	R/S	
22	1150	Rt	2006	IX	TM	Kidney	Type-2	++	++	++	++	++	B	R	Only CSF
23	19443	AtS	2006	X	LQ	Kidney	Type-2	++	++	++	++	-	B	S	Only CSF
24	1731	Rt	2006	VIII	AT	ni	Type-2	++	++	-	++	-	B	R	Only CSF
25	LM-22-Fp	Rt	2007	X	OS	Kidney	Type-2	++	++	++	++	++	B	S	Only CSF
26	LM-10-Fp	Cs	2007	XIV	PP	Kidney	Type-2	++	++	++	++	-	B	S	Only CSF
27	LM-30-Fp	Rt	2007	X	CT	Gill	Type-2	++	++	++	++	++	B	S	Only CSF
28	C1	Rt	2011	VIII	KR	Spleen	Type-2	++	-	++	++	-	B	S	Only CSF
29	C4	Rt	2011	VIII	KR	Spleen	Type-2	++	++	++	++	-	B	S	Only CSF
30	C5	Rt	2011	VIII	KR	Gill	Type-2	++	++	-	++	-	B	S	Only CSF
31	C6	Rt	2011	VIII	KR	Spleen	Type-2	++	++	++	++	++	B	S	Only CSF
32	C9	Rt	2011	VIII	KR	Spleen	Type-2	++	++	++	++	-	B	S	Only CSF
33	C10	Rt	2011	VIII	KR	Ulcer	Type-2	++	++	++	++	-	B	S	Only CSF
34	C12	Rt	2011	VIII	KR	Gill	Type-2	++	++	++	++	-	B	S	Only CSF
35	C14	Rt	2011	VIII	KR	Lesion	Type-2	++	++	++	++	++	B	S	Only CSF
36	C15	Rt	2011	VIII	KR	Gill	Type-2	++	++	-	++	-	B	S	Only CSF
37	C16	Rt	2011	VIII	KR	Spleen	Type-2	++	++	++	++	-	B	S	Only CSF
38	CC79	AtS	2013	VIII	EP	Kidney	Type-2	++	++	++	++	-	B	S	Only CSF
39	CC80	AtS	2013	VIII	EP	Ulcer	Type-2	-	++	-	-	-	B	S	Only CSF
40	CC81	AtS	2013	VIII	EP	Spleen	Type-2	-	++	-	-	-	B	S	Only CSF
41	CC78	AtS	2013	VIII	EP	Kidney	Type-2	++	++	++	++	-	B	S	Only CSF
42	CC82	AtS	2013	VIII	EP	Liver	Type-2	++	++	++	++	++	B	S	Only CSF
43	CC83	AtS	2013	VIII	EP	Liver	Type-2	-	-	++	++	-	B	S	Only CSF
44	CC84	AtS	2013	VIII	EP	Liver	Type-2	-	++	-	-	-	B	S	Only CSF

Continued Table 1

	Isolates	Host	Year	State	Farm	Organ	Serotype	Agglutination with serum anti-					Genotypes using PCR-RFLP		16S RNA allele
								Atlantic salmon		Rainbow trout		Control			
								1196	1739	1150	1731	ATCC 49418^T	A/B	R/S	
45	MEL 110	AtS	2013	IX	MP	Liver	Type-2	-	++	++	++	++	B	S	Only CSF
46	MEL111	AtS	2013	IX	MP	Liver	Type-2	++	++	++	++	++	B	S	Only CSF
47	MEL122	AtS	2013	IX	MP	Ulcer	Type-2	++	++	++	-	++	B	S	Only CSF
48	CC6	Rt	2014	VIII	EP	Liver	Type-2	-	-	++	-	-	B	S	Only CSF
49	CC14	Rt	2014	VIII	EP	Liver	Type-2	-	-	++	++	++	B	R	Only CSF
50	CC16	Rt	2014	VIII	EP	Liver	Type-2	++	++	++	-	-	B	S	Only CSF
51	MA-A5	Rt	2014	XI	DC	Spleen	Type-2	++	-	++	-	++	B	S	Only CSF
52	MA-A6	Rt	2014	XI	DC	Spleen	Type-2	-	++	-	-	-	B	S	Only CSF
53	MA-41	Rt	2014	XI	DC	Liver	Type-2	++	-	++	-	-	B	S	Only CSF
54	MA-44	Rt	2014	XI	DC	Liver	Type-2	++	-	++	++	-	B	S	Only CSF
55	MA-46	Rt	2014	XI	DC	Kidney	Type-2	-	++	-	-	-	B	S	Only CSF
56	MA-49	Rt	2014	XI	DC	Liver	Type-2	++	-	-	++	++	B	S	Only CSF
57	MA-50	Rt	2014	XI	DC	Liver	Type-2	-	++	-	-	-	B	R	Only CSF
58	MA-51	Rt	2014	XI	DC	Kidney	Type-2	-	++	++	++	-	B	S	Only CSF
59	MA-52	Rt	2014	XI	DC	Spleen	Type-2	-	++	++	-	-	B	S	Only CSF
60	FP004	Rt	2015	ni	ni	ni	Type-2	++	++	++	-	-	B	S	Only CSF
61	1.1	AtS	2017	ni	ni	ni	Type-2	++	++	++	++	++	B	S	Only CSF
62	4.1	AtS	2017	ni	ni	ni	Type-2	-	-	++	-	-	B	S	Only CSF
63	4.2	AtS	2017	ni	ni	ni	Type-2	-	-	++	-	-	B	S	Only CSF
64	A1B	Rt	2017	IX	PN	Spleen	Type-2	++	++	++	++	++	B	S	Only CSF
65	A3L	Rt	2017	IX	PN	Ulcer	Type-2	++	++	++	++	++	B	S	Only CSF
66	LBL1	Rt	2017	IX	PN	Ulcer	Type-2	-	-	++	-	-	B	S	Only CSF
67	LBL2	Rt	2017	IX	PN	Ulcer	Type-2	++	-	++	++	-	B	S	Only CSF

Continued Table 1

	Isolates	Host	Year	State	Farm	Organ	Serotype	Agglutination with serum anti-					Genotypes using PCR-RFLP		16S RNA allele
								Atlantic salmon		Rainbow trout		Control			
								1196	1739	1150	1731	NCIMB 1947^{T}	A/B	R/S	
68	LBL3	Rt	2017	IX	PN	Ulcer	Type-2	++	++	++	-	++	B	S	Only CSF
69	P1P1/Flp 007	Rt	2018	IX	VR	Kidney	Type-2	++	++	++	++	++	B	R	Only CSF
70	P1P4/FIp 008	Rt	2018	IX	VR	Spleen	Type-2	++	-	++	++	-	B	R	Only CSF
71	P2P3/Flp 009	Rt	2018	IX	VR	Spleen	Type-2	++	++	++	-	-	B	R	Only CSF
72	P3P1/Flp 010	Rt	2018	IX	VR	Spleen	Type-2	-	++	++	++	++	B	R	Only CSF
73	P3P3/Flp 011	Rt	2018	IX	VR	Spleen	Type-2	-	-	-	-	++	B	R	Only CSF
74	P3P5/Flp 012	Rt	2018	IX	VR	Spleen	Type-2	-	-	-	-	++	B	R	Only CSF
75	P4P1/Flp 013	Rt	2018	IX	VR	Kidney	Type-2	++	++	++	++	++	B	R	Only CSF
76	P4P2/Flp 014	Rt	2018	IX	VR	Kidney	Type-2	++	-	-	++	++	B	R	Only CSF
77	P4P3/Flp 015	Rt	2018	IX	VR	Spleen	Type-2	++	-	++	++	++	B	R	Only CSF
78	P4P4/Flp 016	Rt	2018	IX	VR	Spleen	Type-2	-	-	++	-	-	B	R	Only CSF
79	P5P4/Flp 018	Rt	2018	IX	VR	Spleen	Type-2	-	++	-	++	++	B	R	Only CSF
80	P5P5/Flp 019	Rt	2018	IX	VR	Spleen	Type-2	-	-	++	++	-	B	R	Only CSF
81	P6P2/Flp 020	Rt	2018	IX	VR	Spleen	Type-2	-	++	++	-	++	B	R	Only CSF
82	P6P3/Flp 021	Rt	2018	IX	VR	Spleen	Type-2	++	++	-	++	-	B	R	Only CSF
83	P6P4/Flp 022	Rt	2018	IX	VR	Spleen	Type-2	++	++	-	++	-	B	R	Only CSF
84	P6P5/Flp 023	Rt	2018	IX	VR	Spleen	Type-2	++	++	-	++	-	B	R	Only CSF
85	P9P3/Flp 027	Rt	2018	IX	VR	Kidney	Type-2	++	++	-	++	-	B	R	Only CSF
86	P9P15/Flp 028	Rt	2018	IX	VR	Spleen	Type-2	++	++	-	++	-	B	R	Only CSF

Continued Table 1

	Isolates	Host	Year	State	Farm	Organ	Serotype	Agglutination with serum anti-					Genotypes using PCR-RFLP		16S RNA allele
								Atlantic salmon		Rainbow trout		Control			
								1196	1739	1150	1731	ATCC 49418^T	A/B	R/S	
87	1779	Rt	2006	X	RP	ni	Type-4	-	++	-	-	-	B	R	Only CSF
88	CC72	AtS	2013	VIII	KÑ	ni	Type-4	-	-	-	++	++	B	S	Both
89	MEL 99	AtS	2013	IX	MP	Liver	Type-4	-	++	++	++	++	B	S	Both
90	TAC 3H	AtS	2013	VII	S	Liver	Type-4	++	++	++	-	-	B	S	Both
91	TAC 3R	AtS	2013	VII	S	Kidney	Type-4	++	++	++	++	-	B	S	Both
92	TAD SP1R	AtS	2013	VII	S	Kidney	Type-4	++	++	-	++	-	B	S	Both
93	TAD SP2R	AtS	2013	VII	S	Kidney	Type-4	++	++	++	-	-	B	S	Both
94	TAD3 SP1 B	AtS	2013	VII	S	Spleen	Type-4	++	++	-	++	-	B	S	Both
95	TAD3 SP1 H	AtS	2013	VII	S	Liver	Type-4	++	++	++	++	-	B	S	Both
96	PF-13	AtS	2014	RM	PÑ	Liver	Type-4	++	++	-	++	-	B	S	Both
97	PF-14	AtS	2014	RM	PÑ	Spleen	Type-4	++	++	++	++	++	B	S	Both
98	PF-16	AtS	2014	RM	PÑ	Kidney	Type-4	++	++	-	++	-	B	S	Both
99	PF-20	AtS	2014	RM	PÑ	Kidney	Type-4	++	++	-	++	-	B	S	Both
100	PF-37	AtS	2014	RM	PÑ	Spleen	Type-4	++	++	-	++	-	B	S	Both
101	P10	Rt	2015	IX	CR	Ulcer	Type-4	++	-	++	++	-	B	S	Only CSF
102	P11	Rt	2015	IX	CR	Ulcer	Type-4	-	-	++	++	-	B	R	Both
103	P13	Rt	2015	IX	MP	Ulcer	Type-4	++	++	++	-	-	B	S	Both
104	P14	Rt	2015	IX	CR	Ulcer	Type-4	++	++	++	++	-	B	S	Both
105	P15	Rt	2015	IX	CR	Ulcer	Type-4	++	-	++	++	-	B	S	Only CSF
106	P16	Rt	2015	IX	CR	Ulcer	Type-4	-	++	++	++	-	B	S	Both
107	P17	Rt	2015	IX	CR	Liver	Type-4	-	-	++	++	-	B	S	Both
108	P23	AtS	2015	IX	Ll	Liver	Type-4	++	-	++	++	++	B	S	Both
109	P24	AtS	2015	IX	Ll	Ulcer	Type-4	++	++	++	++	-	B	S	Both
110	S1L2	Rt	2017	IX	PN	Ulcer	Type-4	-	-	++	++	-	B	R	Both
111	S1R	Rt	2017	IX	PN	Kidney	Type-4	-	-	-	-	++	B	R	Both

Continued Table 1

	Isolates	Host	Year	State	Farm	Organ	Serotype	Agglutination with serum anti- Atlantic salmon 1196	Atlantic salmon 1739	Rainbow trout 1150	Rainbow trout 1731	Control ATCC 49418^T	Genotypes using PCR-RFLP A/B	Genotypes using PCR-RFLP R/S	16S RNA allele
112	S2L2	Rt	2017	IX	PN	Ulcer	Type-4	++	++	++	++	++	B	S	Both
113	S3L1	Rt	2017	IX	PN	Ulcer	Type-4	-	-	-	-	++	B	S	Both
114	S3L2	Rt	2017	IX	PN	Ulcer	Type-4	++	-	++	++	-	B	R	Both
115	S3R	Rt	2017	IX	PN	Kidney	Type-4	++	++	++	++	++	B	R	Both
116	S5L1	Rt	2017	IX	PN	Lesion	Type-4	-	-	++	++	-	B	R	Both
117	S5L2	Rt	2017	IX	PN	Lesion	Type-4	-	-	-	++	++	B	S	Both
118	S5R	Rt	2017	IX	PN	Kidney	Type-4	-	-	-	-	++	B	R	Both

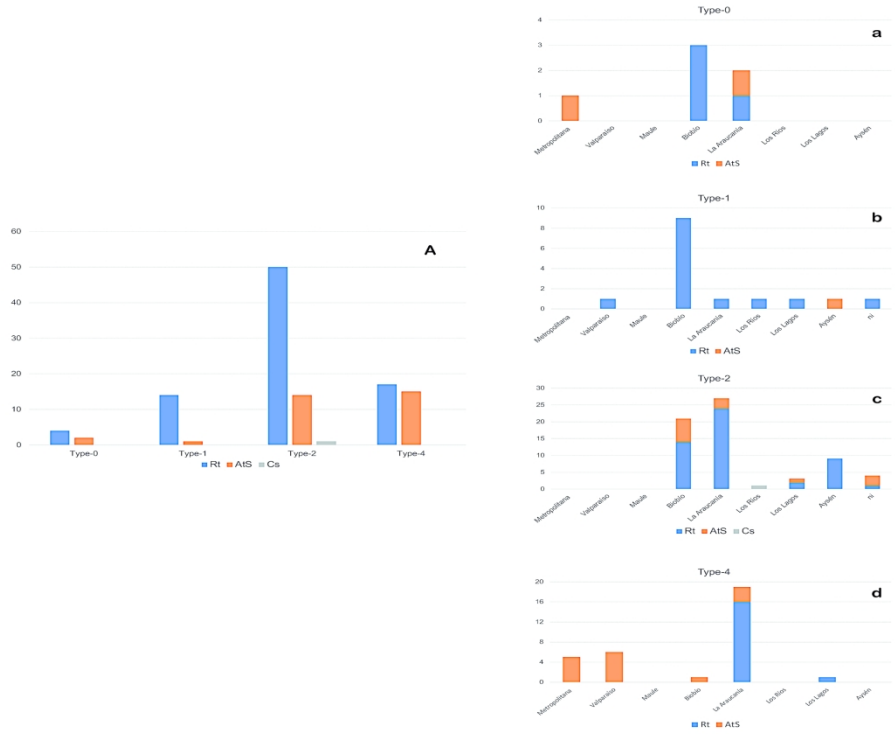

Figure 1

279x215mm (300 x 300 DPI)