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

4

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6

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13

14

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

16

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20 **Abstract**

21 Chile is currently the second largest producer of farmed salmon worldwide, but
22 *Flavobacterium psychrophilum*, as one of the most detrimental pathogens, is responsible for
23 major losses during the freshwater culturing step in salmonid fish farms. An antigenic study
24 conducted 10 years ago reported four serological groups using 20 *F. psychrophilum* Chilean
25 strains. To reduce disease outbreaks and to develop vaccine candidates, antigenic knowledge
26 needs to be regularly updated using a significant number of additional recent *F.*
27 *psychrophilum* isolates. The present study aimed at investigating the serological diversity of
28 118 *F. psychrophilum* isolates collected between 2006-2018 from farmed Atlantic salmon
29 (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), and coho salmon (*Oncorhynchus*
30 *kisutch*). The current study supports an expansion of the known antigenic groups in Chile
31 from 4 to 14. However, the use of the slide-agglutination technique for serotyping is costly,
32 labor-intensive, and requires significant technical expertise. Addressing these points, the
33 mPCR-based procedure was a very useful tool for serotyping the collected Chilean *F.*
34 *psychrophilum* isolates. This technique revealed the presence of diverse mPCR serotypes (i.e.,
35 types 0, -1, -2, and 4). Therefore, mPCR should be employed to select the bacterial strain(s)
36 for vaccine development and to conduct follow-up, selective breeding, or epidemiological
37 surveillance in Chilean fish farms. Given the presented findings, changes to Chilean fish-
38 farming practices are vital for ensuring the continued productivity and wellbeing of farmed
39 salmonids.

40

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

44 1. INTRODUCTION

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

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

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

81 While genotypic information about Chilean *F. psychrophilum* strains is arguably
82 abundant, antigenic classification studies are starkly lacking. Only a single investigation on
83 20 isolates has been published for Chile (Valdebenito & Avendaño-Herrera, 2009), the
84 results of which contributed to the tentative licensing of a commercial vaccine in 2013. The
85 so-termed Flavomune vaccine (SAG N°2160-BP) contains whole cells inactivated with
86 formaldehyde (Solis, Poblete-Morales, Cabral, Valdés, Reyes, Avendaño-Herrera & Feijóo,
87 2015). In the seven years since the initial applications of this vaccine, sales and use in
88 aquaculture facilities have decreased as a result of efficiency concerns (publication in
89 process). Therefore, it is essential to update antigenic knowledge using a high number of
90 recently collected *F. psychrophilum* isolates, in addition to applying up-to-date

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

Review Copy

97 2. MATERIALS AND METHODS

98 2.1. Bacterial isolates and growth conditions

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

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

116

117 2.2 DNA extraction and confirmation of bacterial species

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

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

124 In addition to phenotypic tests, each isolate was genetically confirmed as *F.*
125 *psychrophilum* prior to antigenic and genetic typing using two different PCR tests (Urdaci,
126 Chakroun, Faure, & Bernardet, 1998). The amplification cycles used for denaturation, primer
127 annealing, and primer extension were carried out according to published PCR protocols
128 (Urdaci et al., 1998). Negative controls, consisting of the same reaction mixture but with
129 sterile distilled water instead of template DNA, were included in each PCR batch. Each
130 reaction was prepared using 0.5 µL of each primer (10 µM) and the GoTaq® Green Master
131 Mix (Promega), according to the manufacturer's instructions. Positive results included the
132 presence of a single product with a size comparable to strain NCIMB 1947^T (i.e. 1088 base
133 pairs [bp] for primers FP1 and FP2) (Urdaci, Chakroun, Faure, & Bernardet, 1998). Aliquots
134 (5 µL) of the PCR product were separated on a 1% (w/v) agarose gel for 60 min at 100 V in
135 1× TAE and 1× GelRed® Nucleic Acid Gel Stain (Biotium). The bands were photographed
136 under UV light and computer digitized (Gel Doc 100, Bio-Rad). The AccuRuler 100bp Plus
137 DNA Ladder (100- to 3000-bp, MaestroGen) served as a molecular mass marker.

138

139 **2.3 Serological characterization using antisera**

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

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

155

156 **2.4 Multiplex PCR-based serotyping**

157 The Chilean isolates were typed using the multiplex PCR (mPCR)-based serotyping method
158 described by Rochat et al. (2017). In order to maximize the discriminatory power of the
159 scheme, an additional primer pair, able to identify a supplementary mPCR serotype (namely
160 type-4) was included. This primer pair [Type-4_fw (5'-TGAAGCAAAAGCAACAAACA-
161 3') and Type-4_rev (5'-CCCCAAACTGCTTACCTAAT-3')] resulted in an amplification
162 product of 992 bp.

163 PCR analyses were performed in a total volume of 25 µL using the GoTaq® Green Master
164 Mix (Promega) and 10 Mm of each primer. The mPCR amplification mix was heated at 95 °C
165 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
166 extension at 72 °C for 10 min. The amplified products were electrophoresed on 2% agarose
167 gels run in 1× TAE (0.04 M Tris, 1 mM EDTA, pH 8.0). The gels were photographed under

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

170

171 **2.5 PCR- restriction fragment length polymorphism (RFLP)**

172 Two primer pairs were used (Izumi, Aranishi, & Wakabayashi, 2003); a degenerate universal
173 primer pair (GYR-1/GYR-1R) and a specific primer pair for the *gyrB* gene of *F.*
174 *psychrophilum* (PSY-G1F/PSY-G1R). The expected amplification products were 1178 bp
175 and 290 bp for the universal primer pair and 1017 bp for the specific primer pair.
176 Thermocycle programs for the amplifications were carried out according to Izumi et al.
177 (2003). The PCR products were digested according to the manufacturer's instructions with
178 two restriction enzymes, *RsaI* (Promega, GYR-1/GYR-1R) and *HinfI* (New England BioLabs
179 Inc., PSY-G1F/PSY-G1R). The digested product (10 μ L) was analyzed by horizontal
180 electrophoresis.

181

182 **2.6 16S rRNA allele PCR assays**

183 PCR analyses were used to differentiate the presence of a single or both 16S rRNA alleles,
184 which provide information about polymorphisms or genetic lineages I and II (Soule, LaFrentz,
185 Cain, LaPatra, & Call, 2005). Distribution of these alleles among the different isolates were
186 determined with two independent PCR tests using primers for the allele of strain CFS259-93
187 and that of strain ATCC 49418^T (equivalent to NCIMB 1947^T). These primers respectively
188 amplify products of 600 and 298 bp. The PCR program included one denaturation cycle at
189 95 °C for 5 min, followed by 30 cycles at 95 °C for 30 s, 61 °C for 60 s, and 72 °C for 60 s,
190 and a final extension of one cycle at 72 °C for 10 min (Ramsrud, LaFrentz, LaFrentz, Cain,
191 & Call, 2007).

192 3. RESULTS AND DISCUSSION

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

201 A total of 118 *F. psychrophilum* isolates were serotyped and genotyped (Table 1). Of
202 these, 84 isolates were obtained from rainbow trout, 33 from Atlantic salmon, and 1 from
203 coho salmon. Salmon farming take place in a wide spatial distribution covering from central
204 to southern Chile (~1700 km), with most freshwater farm and hatcheries located in the Biobío,
205 La Araucanía, Los Ríos and Los Lagos regions also known as VIII, IX, XIV and X regions,
206 respectively (Quiñones, Fuentes, Montes, Soto, & León-Muñoz, 2019). Our isolates
207 originated from the following regions in Chile, listed geographically from north to south:
208 Valparaíso, 1 isolate; Metropolitana de Santiago, 6 isolates; Maule, 6 isolates; Biobío, 34
209 isolates; La Araucanía, 49 isolates; Los Ríos, 2 isolates; Los Lagos, 5 isolates and Aysén del
210 General Carlos Ibáñez del Campo, 10 isolates. The precise geographical origin was
211 unavailable for 5 isolates (Table 1). The quantity of isolates collected per year was as follows:
212 9 isolates in 2006; 3 in 2007; 13 in 2011; 19 in 2013; 24 in 2014; 12 in 2015; 20 in 2017 and
213 18 in 2018. Importantly, the number of *F. psychrophilum* isolates included per region and
214 per year does not reflect disease prevalence.

215

216 **3.1 Serological characterization using antisera**

217 Based on the slide agglutination test proposed by Valdebenito and Avendaño-Herrera (2009)
218 for Chilean *F. psychrophilum*, serotyping was performed on the 118 isolates. Eight of the
219 included isolates (i.e. 1733, 1658, 1793, 1779, 19250, 1150, 19443, and 1731) were originally
220 reported in Valdebenito and Avendaño-Herrera (2009). Unfortunately, isolates 1196 and
221 1739 could not be included in the study. Nevertheless, the results still stand as isolate 1733
222 presents the same profile as isolates 1196 and 1739 from Atlantic salmon (Valdebenito &
223 Avendaño-Herrera, 2009). Slide agglutination assays using O-antigens confirmed the
224 existence of antigenic heterogeneity, but while the 2009 study reported four main patterns
225 (groups 1-4) of serological reactions, the current investigation defined a total of 14
226 serological combinations (Supplementary Table 1). These groups included five isolates
227 without a serological reaction to any the four antisera used, which were prepared against two
228 isolates recovered from Chilean rainbow trout and Atlantic salmon, respectively. However,
229 these isolates showed strong reaction with the anti-serum obtained with the type strain. The
230 four previously described patterns of serological groups (Valdebenito & Avendaño-Herrera,
231 2009) represented 59.66% of the currently assessed isolates. The first group dominated,
232 comprising 35 of the 118 *F. psychrophilum* isolates that showed cross-reactions with the
233 unabsorbed antisera raised against the two Atlantic salmon and rainbow trout isolates.

234

235 **3.2 Multiplex PCR-based serotyping**

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

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

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

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

266 Furthermore, the presently obtained data clearly show that 33 of the 118 isolates
267 belong to serotype-4 (Figure 1d). Based on host distribution, serotype-1 was detected
268 exclusively from rainbow trout in perfect accordance with previous findings (Rochat et al.
269 2017), while the remaining serotypes were not associated with or exclusive to a certain fish
270 host (i.e. Atlantic salmon or rainbow trout). In particular, Rochat et al. (2017) reported that
271 22 of 23 strains retrieved from coho salmon belonged to type-0, being the Chilean isolate
272 MHC 1710K, an exception. Therefore, two Chilean isolates obtained from coho salmon,
273 MHC 1710K obtained in 2001 (Avendaño-Herrera et al., 2014) and LM-10-Fp from the
274 present study belonged to serotype-2. Nevertheless, the present data are limited in relation to
275 serotyping in coho salmon since only one sample (isolate LM-10-Fp) from this species was
276 available (Table 1), and more isolates will be needed to substantiate the initial conclusions
277 drawn from the two Chilean isolates in coho salmon.

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

284

285 3.3 PCR-RFLP and 16S rRNA allele PCR assays

286 RFLP analysis of *gyrB* PCR products using the *HinfI* enzyme showed an identical cleavage
287 pattern of the DNA fragment for all 118 *F. psychrophilum* isolates corresponding to genotype
288 B. This finding aligns with previous descriptions by Valdebenito and Avendaño-Herrera
289 (2009) for Chilean isolates. When RFLP tests were performed on PCR products amplified
290 with the specific primer pair PSY-G1F and PSY-G1R, 24 isolates (20.3%) belonged to
291 genotype R, while the remaining 94 isolates (79.7%) were grouped as genotype S. These
292 results confirm a lack of relationship between PCR-RFLP genotyping and host fish species
293 (i.e. Atlantic salmon or rainbow trout) in Chilean isolates. This contrasts with reports from
294 Japan, where 90.3% of *F. psychrophilum* isolates recovered from rainbow trout were
295 determined to be genotype R (Izumi et al., 2003).

296 The distribution of the two 16S rRNA alleles was also examined to determine if there
297 was an association with the fish species (trout [genetic lineage I] vs Pacific salmon [genetic
298 lineage II]), as described by Ramsrud et al. (2007). Of the studied isolates, 72% (n = 85)
299 contained only the alleles found in strain CFS 259-93, while the remaining 33 isolates were
300 positive for both the alleles found in the type strain and in strain CSF 259-93 (Table 1). While
301 there was a clearly major cluster, no association could be found with the host fish species, in
302 line with findings from the 2009 study (Valdebenito & Avendaño-Herrera, 2009). However,
303 all Chilean *F. psychrophilum* isolates classified as type-2 contained only the alleles found in
304 strain CSF 259-93 (corresponding to lineage II), while the majority of the type-4 isolates (i.e.
305 30 of 33) presented both allele sequences (Table 1).

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

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

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

326 In addition to transport during growth, use of the same commercial vaccine for more
327 than five years has possibly provoked changes in the predominant serotypes or serological
328 groups in Chile. In fact, routine use of the same vaccine and antigens can trigger vaccine
329 breaks, as has been described for other freshwater diseases in Chile, such as enteric red mouth
330 disease (Bastardo, Bohle, Ravelo, Toranzo & Romalde 2011). Apart from vaccination,
331 Chilean aquaculture farms have, for years, combatted *F. psychrophilum* by using
332 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*.

341

342

343

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.

357

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363

364 CONFLICT OF INTEREST

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

367

368

369 DATA AVAILABILITY STATEMENT

370 The data that support the findings of this study are available on request from the
371 corresponding author. The data are not publicly available due to privacy or ethical
372 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).

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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.

Isolates	Host	Year	State	Farm	Organ	Serotype	Agglutination with serum anti-				Genotypes using PCR-RFLP		16S RNA allele		
							Atlantic salmon		Rainbow trout		Control	A/B		R/S	
							1196	1739	1150	1731	ATCC 49418 ^T				
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	A/B	R/S		
							1196	1739	1150	1731	ATCC 49418 ^T				
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	A/B	R/S		
							1196	1739	1150	1731	ATCC 49418 ^T				
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	A/B	R/S		
							1196	1739	1150	1731	NCIMB 1947 ^T				
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/Flp 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		Contro l	A/B	R/S		
							1196	1739	1150	1731	ATCC 49418 ^T				
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	LI	Liver	Type-4	++	-	++	++	++	B	S	Both
109	P24	AtS	2015	IX	LI	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-					Genotypes using PCR-RFLP		16S RNA allele	
							Atlantic salmon		Rainbow trout		Control	A/B	R/S		
							1196	1739	1150	1731	ATCC 49418 ^T				
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

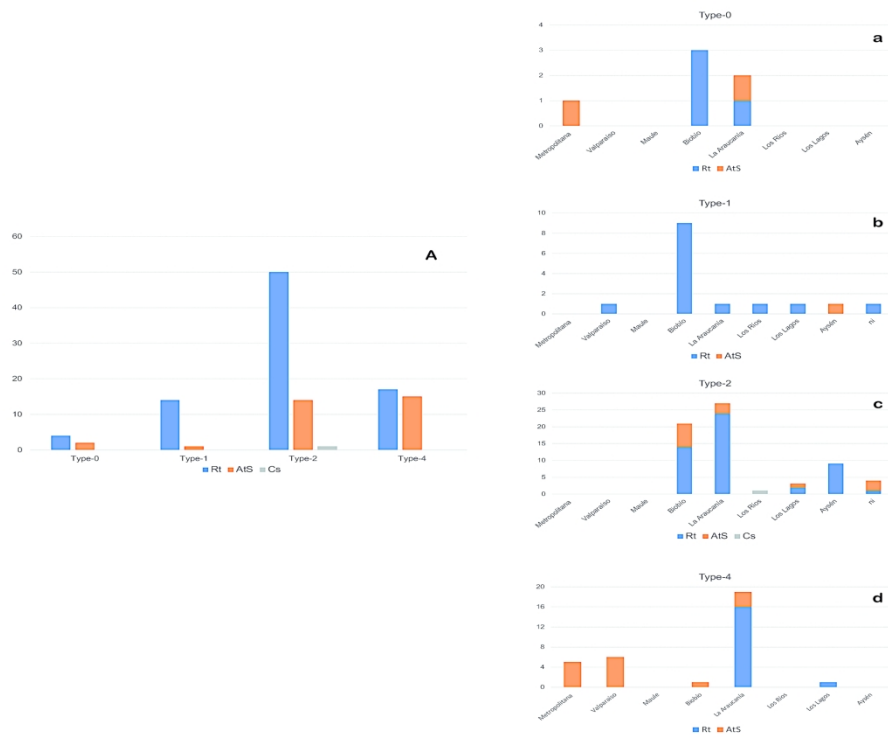


Figure 1

279x215mm (300 x 300 DPI)