Journal of Industrial Microbiology & Biotechnology (2020) 47:299–309
https://doi.org/10.1007/s10295-020-02265-9

FERMENTATION, CELL CULTURE AND BIOENGINEERING - ORIGINAL PAPER

Engineering a defined culture medium to grow *Piscirickettsia salmonis* for its use in vaccine formulations

Pablo Fuentealba[1,2] · Yesenia Latorre[1,2] · Ernesto González[1] · Irene Martínez[1,4] · Carmen Soto[3] · Claudia Altamirano[1,3]

Received: 6 December 2019 / Accepted: 10 February 2020 / Published online: 21 February 2020

Abstract

Piscirickettsia salmonis is a facultative Gram-negative intracellular bacterium that produces piscirickettsiosis, disease that causes a high negative impact in salmonid cultures. The so-far-unidentified nutritional requirements have hindered its axenic culture at laboratory and industrial scales for the formulation of vaccines. The present study describes the development of a defined culture medium for *P. salmonis*. The culture medium was formulated through rational design involving auxotrophy test and statistical designs of experiments, considering the genome-scale metabolic reconstruction of *P. salmonis* reported by our group. The whole optimization process allowed for a twofold increase in biomass and a reduction of about 50% of the amino acids added to the culture medium. The final culture medium contains twelve amino acids, where glutamic acid, threonine and arginine were the main carbon and energy sources, supporting 1.65 g/L of biomass using 6.5 g/L of amino acids in the formulation. These results will contribute significantly to the development of new operational strategies to culture this bacterium for the production of vaccines.

Keywords *Piscirickettsia salmonis* · Nutritional requirement · Amino acid · Defined medium · Vaccines

Introduction

Piscirickettsia salmonis is a facultative Gram-negative intracellular bacterium that produces the salmonid rickettsial septicemia (SRS, also known as piscirickettsiosis), reported for the first time in Chile in 1983 [8]. This pathogen causes severe economic losses to Chilean salmon production, since 69–86% of salmonid mortality (Atlantic salmon and Rainbow trout) is produced by this microorganism [49]. *P. salmonis* is also present in other salmon-producing countries as Ireland, Norway, Canada, United States and Scotland, but Chilean strains have higher virulence than others [47]. The fact that *P. salmonis* has been detected in other fish species has alerted the aquaculture sector for the spreading of this pathogen and its harmful effects [2, 3, 10].

This situation has encouraged research and development of new prophylactic methods to avoid or reduce the impact of this pathogen, among which improved bacterin (inactivated bacteria)-based vaccines stand out [32]. The current method to produce bacterin-based vaccines against *P. salmonis* involves salmon [17, 18] or insect [6] cell lines infection with the pathogen, followed by the recovery of the microorganisms from the spent culture medium. The

Pablo Fuentealba and Yesenia Latorre contributed equally to this work.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10295-020-02265-9) contains supplementary material, which is available to authorized users.

✉ Claudia Altamirano
claudia.altamirano@pucv.cl

1 Laboratorio de Cultivos Celulares, Escuela de Ingeniería Bioquímica, Pontificia Universidad Católica de Valparaíso, Avenida Brasil 2085 Valparaiso, Chile

2 Doctorado en Biotecnología, Pontificia Universidad Católica de Valparaíso-Universidad Técnica Federico Santa María, Valparaiso, Chile

3 CREAS CONICYT Regional GORE Valparaíso R0GI1004, Av. Universidad, Curauma, Chile

4 Present Address: Department of Chemical Engineering, Biotechnology and Materials, Centre for Biotechnology and Bioengineering (CeBiB), Universidad de Chile, Santiago, Chile

formulated vaccine is then delivered to the fish by injection. Mikalsen et al. [37] and Mauel et al. [35] reported by first time in 2008 the axenic culture of *P. salmonis* in an agar-solid medium which included: hemoglobin (Mikalsen's medium), sheep blood, fetal bovine serum, cysteine and glucose. Since then, several efforts have been advocated to develop new media for culturing this bacterium to decrease cultivation time and increase biomass, avoiding the use of fish or insect cell lines.

Gomez et al. reported in 2009 a liquid medium which had a composition similar to Mikalsen's and Mauel's media but changed sheep blood by blood fish lysate [22]. The first blood-free media were reported independently by Yañez et al. [54] and Marshall et al. [34], being them composed of vitamins (in the case of Yañez's medium), inorganic salts, peptones, some amino acids, one saccharide and fetal bovine serum. After that, Henríquez et al. [23], Yañez et al. [55] and Tandberg et al. [53] continued reporting both liquid and solid complex media for growing *P. salmonis*.

In a previous work, about the genome-scale metabolic reconstruction of *P. salmonis*, we showed that this bacterium has a greater capability to assimilate amino acids from the surrounding environment than to assimilate other carbon sources such as saccharides [20]. In fact, the only predicted saccharide transporter has the potential to incorporate arabinose, galactose and xylose. Reports about other bacteria such as *F. tularensis* [36] and *L. pneumophila* [33, 45] have also determined a greater diversity of transporters for amino acids as compared to saccharides. In addition, it has been determined that, although these microorganisms have the ability to assimilate glucose or glycerol, cell growth is usually not observed [33].

Chemically defined media have been widely used at laboratory scale for studying the specific requirements for growth and product formation under reproducible conditions. However, they also have advantages at the industrial scale, mainly for the preparation of biological products that require the compliance of good manufacturing practices [56]. For example, the use of fetal bovine serum should be avoided in vaccine production because it is variable in composition from lot-to-lot and may introduce prions and infectious agents [48]. Other constituents, such as yeast extract and peptone, also vary among producers and lots, possibly affecting the final fermentation products.

Generally, the design and improvement of culture media can be addressed using different approaches: random search, stoichiometry, genetic algorithms, neural networks and statistical methods (e.g., factorial design, Plackett–Burman design and surface response) [12, 19, 31, 39, 40, 42, 43, 51, 57].

Having in mind the relevance of chemically defined medium for animal pharmaceutical companies, the present investigation sought to formulate an optimized and balanced defined culture medium to grow *P. salmonis*. This goal was rationally addressed from basic knowledge, by means of a two-step procedure. First, an auxotrophy test was used to determine essential amino acids. Then, the concentration of these compounds was defined by means of a Plackett–Burman and Optimal Factorial designs. Then, this culture medium could be applied to the industrial production of *P. salmonis* (patent application WO2016082050A1) for the formulation of vaccines for salmons and salmons-like fish. On the other hand, having a chemically defined medium will contribute to deepening the understanding of the metabolism and pathogenesis of *P. salmonis* as a complement to available work on metabolic reconstruction [11, 20].

Materials and methods

P. salmonis culture

Piscirickettsia salmonis Chilean strain was kindly donated by Dr. Sergio H. Marshall (Laboratorio de Genética e Inmunología Molecular, Instituto de Biología, Pontificia Universidad Católica de Valparaíso, Chile). Cells were stored at − 80 °C in a solution of 0.5 M sucrose and glycerol (3:7, v/v). Cells were grown in shake flasks using the modified complex medium (CM medium) developed by our group (yeast extract 4 g/L, peptone 4 g/L, NaCl 9 g/L, K_2HPO_4 6.3 g/L, $CaCl_2{\cdot}2H_2O$ 0.08 g/L, $MgSO_4{\cdot}7H_2O$ 0.1 g/L, ferric citrate 0.022 g/L) [23]. The culture was performed at 23 °C, pH 6.0 and 100 rpm for 96 h [23].

Defined culture medium design

The first approximation to a defined medium (DM0 medium, Table 1) included twenty pure amino acids. The concentration of amino acids was set to achieve 680 mg/L of biomass, assuming each amino acid as the sole carbon and energy source. Additionally, the DM0 medium also contained inorganic salts included in the CM medium and MEM vitamin solution (Sigma-Aldrich). The culture was performed in shake flasks at 23 °C and 100 rpm. Samples were periodically withdrawn to measure pH and biomass concentration by optical density at 600 nm (OD_{600}).

The auxotrophy test for amino acids was carried out using twenty defined media named DM0-X, being X the amino acid that was not included in the medium formulation. The cultures were performed using 24-well flat-bottom plates filled with 1.5 mL in quadruplicate. Plates were incubated in an orbital shaker at 23 °C and 100 rpm. Periodically, one plate was removed from the incubator and uncovered to read the OD_{600}. A drop of each culture broth was placed on a microscope slide and observed to test the culture purity. Finally, the analyzed well plate was discarded.

Table 1 Composition of formulated defined media and their parameters

Component	Composition (g/L)				
	DM0 [20]	DM1	DM2	DM3	DM4
Histidine	0.56	0.56	0.56	0.14	0.14
Serine	0.77	–	–	–	–
Glycine	0.82	–	–	–	–
Cysteine	1.0	1.0	1.0	0.25	0.34
Tryptophan	0.41	–	–	–	–
Phenylalanine	0.41	0.41	0.41	0.10	0.10
Tyrosine	0.44	–	–	–	–
Alanine	0.65	–	–	–	–
Valine	0.52	0.52	0.52	0.13	0.13
Leucine	0.48	0.48	0.48	0.12	0.12
Isoleucine	0.48	0.48	0.48	0.12	0.12
Aspartic acid	0.73	–	–	–	–
Asparagine	0.73	–	–	–	–
Methionine	0.66	0.66	0.66	0.17	0.17
Threonine	0.65	0.65	0.65	2.20	1.73
Lysine	0.54	0.54	0.54	0.14	0.27
Glutamic acid	0.65	0.65	0.65	5.29	1.93
Glutamine	0.65	–	–	–	–
Proline	0.51	–	0.51	0.13	0.26
Arginine	0.65	0.65	0.65	2.87	1.19
K_2HPO_4	6.3	6.3	6.3	6.3	6.3
$MgSO_4 \cdot 7H_2O$	0.1	0.1	0.1	0.1	0.1
NaCl	9.0	9.0	9.0	9.0	9.0
$CaCl_2 \cdot 2H_2O$	0.08	0.08	0.08	0.08	0.08
Ferric citrate	0.032	0.032	0.032	0.032	0.032
MEM vitamin solution 100×	2×	2×	2×	2×	2×
Parameters	Results				
Maximum OD_{600}	2.7	2.0	2.0	6.0	5.3
Maximum biomass (g/L)	0.84	0.62	0.62	1.9	1.65
Maximum no. cell/mL	4.2×10^9	3.1×10^9	3.1×10^9	$9.6 \cdot 10^9$	8.4×10^9
Maximum volumetric productivity (g//L/h)	0.0175	0.0066	0.0081	0.025	0.022
Biomass yield (biomass/total amino acids consumed, g/g)	0.068	0.094	0.088	0.16	0.26

Defined culture medium improvement

The auxotrophy tests allowed to select twelve amino acids as important for *P. salmonis* growth. The effect of these amino acids on cell growth was studied through a Plackett–Burman design [44], which considered two levels for each tested amino acid: 100% (high, + 1) and 25% (low, − 1) of their concentration in the DM0 medium (Table 1).

Based on the number of variables (12 amino acids), at least 13 runs were required. However, since (1) the number of dummy variables should be one-third of all variables [4] and (2) Plackett–Burman designs are available in multiple of four runs [30], the software Design-Expert 8.0 was used to generate the conditions for the runs (Table 2). Thus, the obtained design had 20 runs including 7 dummy factors, which introduce the redundancy required by the statistical procedure and allow to estimate the variance [25]. The cultures were performed using 24-well flat-bottom plates filled with 1.5 mL. The plates were incubated in an orbital shaker at 23 °C and 100 rpm. Periodically, one plate was removed from the incubator, uncovered to read the OD_{600} and discarded. Analysis of variance (ANOVA) test was applied to the data. Before the statistical analysis, normality (Chi-squared) and homogeneity of variance (Bartlett) were verified for all data. When the data met the parametric requirements, the ANOVA test was applied, followed by a Tukey honestly significant difference test for multiple comparisons.

The ANOVA analysis showed that three amino acids contribute significantly to *P. salmonis* growth. The software Design-Expert was used to study the effect of these

Table 2 Plackett–Burman design to study the effect of twelve selected amino acids and biomass obtained at 96 h

Run	Amino acids[a]												Biomass (g/L)
	His	Cys	Fen	Val	Leu	Ile	Met	Thr	Lis	Glu	Pro	Arg	
PB-01	+1	+1	−1	−1	+1	+1	+1	+1	−1	−1	−1	−1	0.267 ± 0.009
PB-02	−1	+1	+1	−1	−1	+1	+1	−1	+1	−1	−1	+1	0.154 ± 0.017
PB-03	+1	−1	+1	+1	−1	−1	+1	+1	−1	−1	−1	+1	0.233 ± 0.047
PB-04	+1	+1	−1	+1	+1	−1	−1	−1	+1	+1	−1	−1	0.298 ± 0.005
PB-05	−1	+1	+1	−1	+1	+1	−1	+1	−1	−1	+1	−1	0.161 ± 0.004
PB-06	−1	−1	+1	+1	−1	+1	+1	+1	+1	+1	−1	−1	0.469 ± 0.015
PB-07	−1	−1	−1	+1	+1	−1	+1	+1	+1	−1	+1	−1	0.253 ± 0.013
PB-08	−1	−1	−1	−1	+1	+1	−1	+1	+1	+1	−1	+1	0.629 ± 0.051
PB-09	+1	−1	−1	−1	−1	+1	+1	−1	+1	+1	+1	−1	0.257 ± 0.008
PB-10	−1	+1	−1	−1	−1	−1	+1	−1	−1	+1	+1	+1	0.272 ± 0.025
PB-11	+1	−1	+1	−1	−1	−1	−1	+1	−1	+1	+1	−1	0.453 ± 0.016
PB-12	−1	+1	−1	+1	−1	−1	−1	+1	+1	−1	+1	+1	0.525 ± 0.021
PB-13	+1	−1	+1	−1	+1	−1	−1	−1	+1	−1	−1	+1	0.186 ± 0.003
PB-14	+1	+1	−1	+1	−1	+1	−1	+1	−1	+1	−1	+1	0.643 ± 0.050
PB-15	+1	+1	+1	−1	+1	−1	+1	+1	+1	+1	+1	+1	0.649 ± 0.025
PB-16	+1	+1	+1	+1	−1	+1	−1	−1	+1	−1	+1	−1	0.189 ± 0.009
PB-17	−1	+1	+1	+1	+1	−1	+1	−1	−1	+1	−1	−1	0.301 ± 0.011
PB-18	−1	−1	+1	+1	+1	+1	−1	−1	−1	+1	+1	+1	0.266 ± 0.024
PB-19	+1	−1	−1	+1	+1	+1	+1	−1	−1	−1	+1	+1	0.183 ± 0.008
PB-20	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	0.153 ± 0.011

[a]Levels of amino acids where high (+1) and low (−1) are 100% and 25% of the DM0 concentration, respectively

amino acids by means of an Optimal Factorial design (OFD) which used a two-factor interaction model. The design considered three-levels for three factors (tested amino acids), and a two-factor interaction model (19 points) with five lack-of-fit points (Table 3). The experiment was performed using 100 mL Erlenmeyer flasks filled with 20 mL of medium and pH 6.0. The cultures were performed in an orbital shaker at 23 °C and 100 rpm [23]. Samples were periodically withdrawn to measure OD_{600}.

Microscopy observation

Broth samples were periodically observed with an optical microscope to confirm the culture purity. The identity of *P. salmonis* was tested by a commercial kit of immunofluorescence, specific against this bacterium (SRS Fluorotest kit; GruposBios, Chile), while the formed biofilm was observed through confocal microscopy [34]. Briefly, this method involved the preparation of a permeabilized smear which was differentially stained with propidium iodide and lectin concanavalin A labeled with FITC for detection of cells and exopolysaccharides, respectively.

Analytical methods

The biomass was measured in a spectrophotometer at 600 nm (OD_{600}). A calibration curve was constructed to obtain the dry cell weight from the OD_{600} measurements ($OD_{600} = 3.16 \times$ dry cell weight (g/L) + 0.031) [29]. Additionally, the number of cells per milliliter was determined by flow cytometry (FC500-Becton Dickson). The cell counting was performed using the LIVE/DEAD™ BacLight™ kit according to the manufacturer's specifications. A calibration curve was constructed between OD_{600} and cell number ($OD_{600} = 6.19 \times 10^{-10} \times$ No. cell/mL + 0.069). The amino acid composition was measured by HPLC (Perkin Elmer 200 Series fluorescence detector) as described in Fuentealba et al. [20]. Briefly, samples were filtered (0.22-µm PDVF membrane), derivatized (AccQ-Fluor Reagent Kit) and ran (Waters AccQ-Tag column) using acetate buffer as mobile phase (19.05-g sodium acetate trihydrate, 2.37-mL triethylamine and 1-mg EDTA in 800 mL of miliQ water, pH 5.02 with H_3PO_4, diluted to 1 L) with a elution gradient of acetonitrile:water (60:40 v/v) at 37 °C and flow rate of 1 mL/min. The species were identified by fluorescence by exciting the sample to 250 nm and emitting at 395 nm.

Table 3 Optimal factorial design (OFD) to study the effect of three amino acids and biomass at 96 h

Run	Amino acids			Biomass (g/L)
	Thr[a]	Glu[b]	Arg[c]	
F-01	−1	−1	−1	0.350
F-02	−1	0	−1	0.331
F-03	−1	+1	−1	0.314
F-04	0	−1	−1	0.505
F-05	0	0	−1	0.750
F-06	+1	−1	−1	0.402
F-07	+1	0	−1	0.899
F-08	+1	+1	−1	1.012
F-09	−1	−1	0	0.305
F-10	−1	0	0	0.347
F-11	−1	+1	0	0.298
F-12	0	−1	0	0.554
F-13	0	0	0	1.157
F-14	0	+1	0	1.651
F-15	+1	0	0	1.906
F-16	+1	+1	0	1.720
F-17	−1	−1	+1	0.524
F-18	−1	+1	+1	0.881
F-19	0	−1	+1	1.054
F-20	0	0	+1	1.859
F-21	0	+1	+1	1.714
F-22	+1	−1	+1	1.158
F-23	+1	0	+1	1.853
F-24	+1	+1	+1	1.783

[a]Concentrations for low (−1), medium (0) and high (1) levels are 0.66, 2.2 and 4.4 g/L, respectively

[b]Concentrations for low (−1), medium (0) and high (1) levels are 0.65, 2.6 and 5.3 g/L, respectively

[c]Concentrations for low (−1), medium (0) and high (1) levels are 0.64, 1.4 and 2.9 g/L, respectively

Results and discussion

Nutritional composition of the culture medium

P. salmonis was able to grow in the DM0 defined medium [20], achieving a maximum biomass (2.7 ± 0.1 OD_{600}) at 48 h after inoculation (Fig. 1). During *P. salmonis* growth, the pH increased from 6.6 to 7.7, as previously described by Henríquez et al. [23], which is due to the accumulation of ammonium derived from amino acid degradation [20]. *P. salmonis* grew in DM0 medium forming bacterial aggregates (Fig. 2b–d); however, the culture in CM medium showed a planktonic phenotype (Fig. 2a). The fluorescence analysis showed that bacterial aggregates were formed by a variable number of cells (red) within an exopolysaccharide matrix (green), which is typically produced when the bacteria show a biofilm phenotype [24]. Marshall et al. [34] showed that LF-89 strain generates biofilm under stressing conditions, while Albornoz et al. [1] also describe this phenotype in other *P. salmonis* strains. Therefore, bacterial aggregates with a biofilm structure were induced by stressful conditions in DM0 medium caused by the replacement of complex protein-rich substrates in CM, i.e., yeast extract and peptone, by pure amino acids at high concentrations.

Piscirickettsia salmonis was able to grow in proline-, tyrosine-, alanine-, aspartic acid-, serine-, glycine-, tryptophan-, asparagine- and glutamine-free media (Fig. 3a), showing that these amino acids are non-essential for this microbe proliferation. Additionally, the fact that growth achieved at 78 h in these media was higher than that obtained in DM0 may be explained as substrate inhibition. The inhibition of microbial growth by some amino acids has been reported for several strains, such as *Escherichia coli* [13, 24], *Thiobacillus neapolitanus* [27], *Caulobacter crescentus* [16], *Neisseria gonorrhoeae* [5], *Chlamydia* sp. [7], and even for mammalian cells [15].

On the other hand, this bacterium was unable to grow in arginine-, valine-, isoleucine-, histidine-, cysteine-, phenylalanine-, methionine-, threonine- and lysine-free media (Fig. 3b), proving that they are essential for *P. salmonis* growth, under the conditions tested. In general, the present data are in agreement with the genome-scale metabolic reconstruction for *P. salmonis* performed by Fuentealba et al. [20] and Cortés et al. [11], although some discrepancies arise. Cortés et al. [11] report the ability for synthesizing phenylalanine, threonine and lysine de novo, while Fuentealba et al. [20] predict the biosynthesis of only the last two compounds. The fact that *P. salmonis* required these amino acids to grow may be explained considering that some genetic information remains phenotypically unexpressed.

Glutamic acid and leucine may be classified as pseudo-essential amino acids because *P. salmonis* could grow in glutamic acid- and leucine-free medium (Fig. 3c) but, compared to DM0 medium, the growth achieved in 78 h was 62.4 and 79.4% lower, respectively. Although, the genomic capacity for synthetizing glutamic acid was predicted by Fuentealba et al. [20] and Cortés et al. [11], the experimental data showed that its addition is required to achieve a significant growth over a reasonable cultivation time. On the other hand, despite that a leucine biosynthesis route was not previously reported [11, 20], the leucine-free medium allowed a slight cell growth (OD_{600} increased from 0.034 to 0.123; Fig. 3c).

Adaptive evolution explains why intracellular bacterial pathogens lack pathways for synthesizing some compounds that can be obtained from host cells [21]. Selective cultures and ^{13}C labeling experiments have shown that *Legionella pneumophila* requires nine essential amino acids [33]. On the other hand, Meibom and Charbit [36]

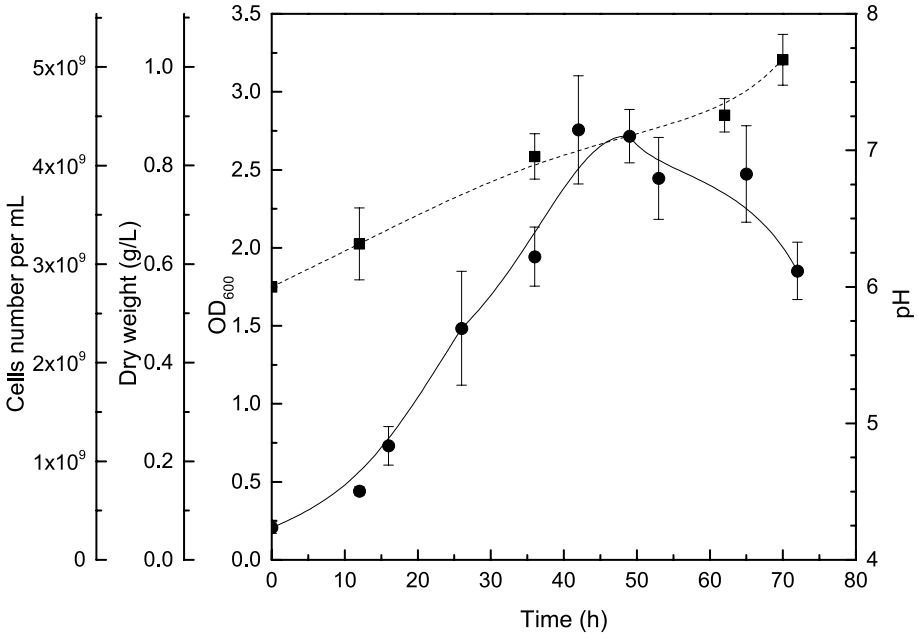

Fig. 1 Growth (filled circle) and pH (filled square) profiles for the culture of *P. salmonis* in shake flasks at 23 °C and 100 rpm using DM0 defined medium. Dry cell weight and cells number are related to optical density through calibration curves, which are valid over the growth period. Error bars represent the standard deviation of culture replicates ($n = 3$)

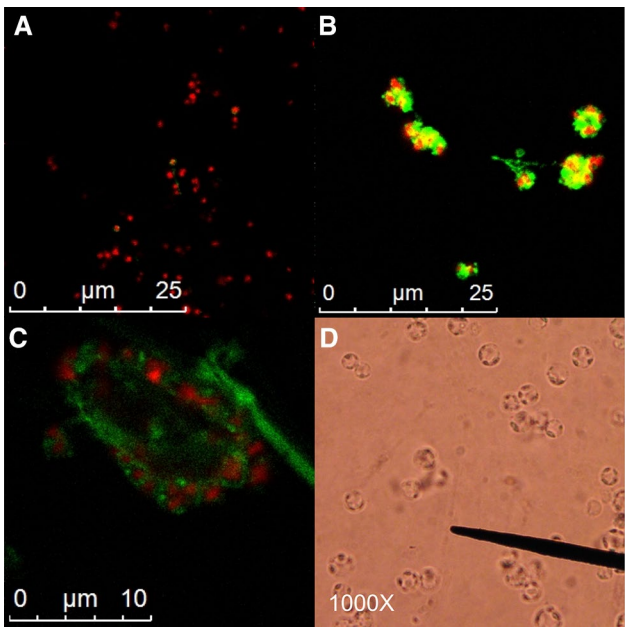

Fig. 2 Images of planktonic and bacterial aggregates (biofilm) phenotypes showed by *P. salmonis* culture in CM (**a**) and DM0 (**b**–**d**), respectively. Confocal microscopy (**a**–**c**) and optical microscopy (**d**). DNA in red (propidium iodide) and exopolysaccharides in green (concanavalin A + FITC), for **a**–**c**

show that essential amino acids for *Francisella tularensis* may go from 6 to 13. Additionally, *Chlamydia* spp. are auxotrophic for most amino acids and other molecules [41]. The information obtained in this work from the auxotrophy test may be used to improve available genome-scale models for *P. salmonis*.

Piscirickettsia salmonis phenotype obtained during the auxotrophy test was mostly planktonic until 31 h (Online Resource SD1), unlike the phenotype in DM0 and glutamic acid-free medium where bacterial aggregates predominated (Fig. 2b–d). After the 78 h of culture, the bacterial aggregates accounted for at least 50% of the cell population when glutamic acid-, serine-, tryptophan-, proline-, tyrosine-, aspartic acid-, glutamine-free and DM0 media were used. On the other hand, the bacterial aggregates in cultures using leucine-, asparagine-, alanine- and glycine-free media were less than or equal to 25%. The absence of some amino acids resulted in a lower production of bacterial aggregates, which may be related to a lower cellular stress by decreasing the osmolarity of culture medium [38, 46, 50].

The information obtained from the auxotrophy test allowed to design a defined medium lacking non-essential amino acids (DM1, Table 1), where the concentration of essential and pseudo-essential amino acids was obtained from DM0. Although, the maximal growth in DM1 medium (2.0 ± 0.1 OD_{600}, Fig. 4) was obtained 46 h later than in DM0, the new medium has two significant advantages: it induced a planktonic phenotype and the content of amino acids was reduced in 53.6% (to 6.6 g/L from 12.31 g/L in DM0). This result supports the previous observation that bacterial aggregates are related to changes in the concentration of amino acids. Since zwitterions do not contribute to the ionic strength of a solution [52], this behavior may be explained as a response to the environmental osmolarity, a variable that seems to be essential for intracellular pathogens [26].

Piscirickettsia salmonis was cultured in DM1-Glu, DM1-Leu and DM1 + Pro media (proline at its concentration in

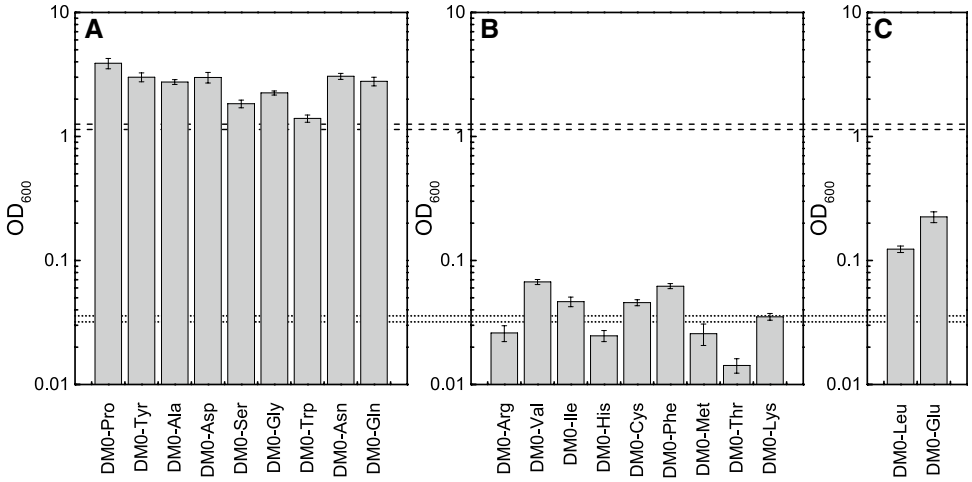

Fig. 3 Growth recorded at 78 h in the auxotrophy test for *P. salmonis* at 23 °C and 100 rpm in well plates. The medium lacking the amino acid X was labeled as DM0-X. The dashed lines (mean ± standard deviation) show the biomass achieved in the control with DM0, while dotted lines (mean ± standard deviation) show the initial biomass for all media. Amino acids were classified in three groups: non-essential (**a**), essential (**b**) and pseudo-essential (**c**). Error bars represent the standard deviation of culture replicates ($n = 4$)

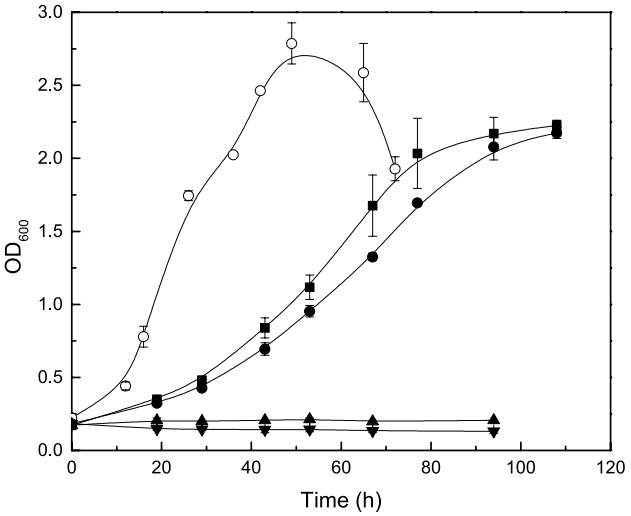

Fig. 4 Growth kinetics of *P. salmonis* in shake flasks at 23 °C and 100 rpm using defined media DM0 (open circle), DM1 (filled circle), DM1 lacking glutamic acid (DM1-Glu, filled upward triangle), DM1 lacking leucine (DM1-Leu, filled downward triangle) and DM1 amended with proline 0.51 g/L (DM1 + Pro, renamed as DM2, filled square). Error bars represent the standard deviation of culture replicates ($n = 2$)

DM0) to re-evaluate the effect of pseudo-essential amino acids and proline in a medium lacking non-essential amino acids. Figure 4 (as Fig. 3c) shows that both glutamic acid and leucine are required to achieve a significant growth of this bacterium. On the other hand, proline was re-evaluated because: (1) it belongs to the alpha-ketoglutarate biosynthesis family along with arginine and glutamate, and *P. salmonis* requires the last two amino acids to grow, and (2) it has a complex role in pathogen–host interaction, which may include carbon, nitrogen and energy source, cell signaling and stress protection [9]. Thus, Fig. 4 shows that, although proline is not an essential amino acid for *P. salmonis*, its presence reduces in 18% the time to achieve maximum growth (2.0 ± 0.1 OD_{600}) allowing a planktonic phenotype (data not shown). Hence, the medium DM1 + Pro was renamed as DM2, maintaining proline in the medium formulation.

Adjustment of nutritional supplementation

Since the medium DM2 included twelve amino acids important to culture *P. salmonis*, this section is focused on the rational dosage of these compounds. This goal was addressed using a Plackett–Burman design (Table 2) [44] to know which amino acids were highly related to microbial growth. Then, an OFD was used to evaluate three different levels of these compounds (Table 3) and to define the best levels of each one.

Piscirickettsia salmonis grew in Plackett–Burman runs achieving a biomass of 0.153 and 0.649 g/L for PB-20 and PB-15, respectively (Table 2). The ANOVA analysis allowed to classify amino acids in two groups (Online Resource SD2). Threonine, glutamic acid and arginine in the Group I presented significant contributions (higher than dummy runs) and positive estimates, which shows that high concentrations stimulate the proliferation of *P. salmonis*. Conversely, the Group II includes amino acids with contributions in the order of dummy runs with positive and negative estimates, showing that low concentrations of these amino acids (i.e. 25% of their concentration in DM2) are enough to obtain a significant growth. Hence, the OFD was performed to study the three amino acids of the Group I using the low concentration for amino acids belonging to the Group II.

The OFD, through the software Design-Expert, allowed to study the effect of threonine, glutamic acid and arginine on the microbial growth by running 24 cultures (F-1 to F-24, Table 3). The biomass achieved behaved as a three-modal distribution (Online Resource SD3), with values between 0.30 and 1.91 g/L. The runs that achieved the highest biomasses (from 1.65 to 1.91 g/L) had in common two

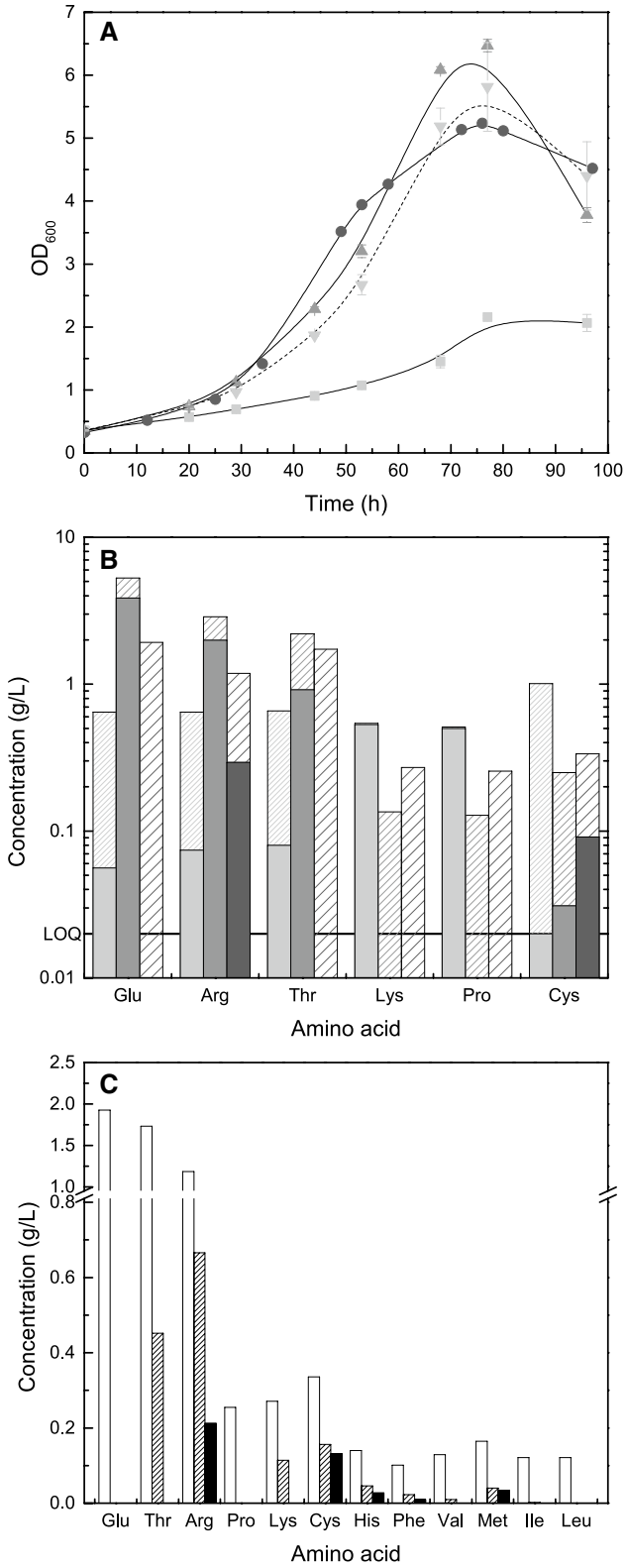

Fig. 5 Growth profiles (**a**) and amino acid consumption (**b**, **c**) of *P. salmonis* in formulated media at 23 °C and 100 rpm. **a** Growth profiles in media DM2 (filled square), F-15 (filled downward triangle), F-21 (renamed as DM3, filled upward triangle) and DM4 (filled circle), while **b** displays the concentration of some amino acids at 80 h (solid bar) compared to its initial concentration (hatched bar) for DM2 (light gray), DM3 (gray) and DM4 (dark gray). Panel C depicts the concentration of amino acids at 0 (open bar), 53 (hatched bar) and 72 h (solid bar) in DM4 medium. The line across the bars in **b** (LOQ) shows the limit of quantification. Error bars in **a** represent the standard deviation of culture replicates ($n = 2$)

characteristics: (1) at least one of the evaluated amino acids was in the high condition and (2) none of the other amino acids evaluated was in the low condition.

The first ANOVA analysis showed that Glu–Arg interaction was negligible (p value > 0.1). Thus, Glu–Arg interaction term was removed from the model to carry out a new ANOVA analysis (Online Resource SD4). The new ANOVA analysis showed a p value < 0.0001 for the model and p values < 0.052 for all effects. Additionally, since both (1) the predicted R^2 (0.7627) was in reasonable agreement with the adjusted R^2 (0.9218) (i.e. the difference was less than 0.2) and (2) the signal–noise ratio (Adeq precision = 12.36) was greater than 4, the Eq. 1 could be used to explore the space design where R is the response variable (biomass at 96 h, g/L) while x_1, x_2 and x_3 are threonine, glutamic acid and arginine, respectively (coded equation).

$$\begin{aligned} R = {} & 0.9782 - 0.5255 \cdot x_1 + 0.1771 \cdot x_1^2 - 0.3051 \cdot x_2 \\ & + 0.1136 \cdot x_2^2 - 0.3430 \cdot x_3 + 0.0378 \cdot x_3^2 + 0.2453 \cdot x_1 x_2 \\ & - 0.1459 \cdot x_1^2 x_2 - 0.0988 \cdot x_1 x_2^2 - 0.0136 \cdot x_1^2 x_2^2 \\ & + 0.2219 \cdot x_1 x_3 - 0.0093 \cdot x_1^2 x_3 - 0.1739 \cdot x_1 x_3^2 - 0.0725 \cdot x_1^2 x_3^2. \end{aligned} \quad (1)$$

The optimization for response variable yield the maximum biomass (1.9 g/L) at the coordinates $(0, +1, +1)$, i.e., predicting the maximum biomass at the central level of threonine and high levels of glutamic acid and arginine. This point was experimentally tested in run F-21 (biomass 1.7 g/L), which belongs to the group that achieved the highest biomasses. The fact that the maximum biomass (1.91 g/L) was obtained for a different run (F-15) may be attributed to limitations in both experimental design and mathematical model.

Figure 5a shows growth profiles obtained after re-evaluating runs F-15 and F-21 in shake flasks. Microbial growth obtained in F-21 (1.9 g/L) was slightly higher than the biomass in F-15 (1.8 g/L), which is agreement with the optimization using Eq. 1. Hence, Plackett–Burman and OFD lead to the medium F-21, renamed as DM3, which allowed to increase threefold the biomass obtained in DM2 (Fig. 5a). This medium showed scarce bacterial aggregates in the stationary phase, a condition that did not compromise biomass and cell quantification, since the parameters (optical density, cell population and biomass) were consistent with a planktonic phenotype. The presence of bacterial aggregates in the last phase of the

culture could be explained by environmental starvation, as described below.

The concentration of amino acids in DM2 and DM3 media was measured at 0 and 80 h The consumption of histidine, phenylalanine, valine, methionine, isoleucine and leucine was lower than 5% in DM2 and lower than 30% in DM3 (data not shown), demonstrating that these compounds did not limited growth in both media. Figure 5b shows that threonine, glutamic acid and arginine were almost depleted in DM2 at 80 h, which may have limited the growth in this medium. On the other hand, the higher biomass obtained in DM3 caused that proline and lysine levels were negligible (lower than the limit of quantification) at 80 h (Fig. 5b). Additionally, cysteine was significantly consumed in both cultures (Fig. 5b).

The composition of DM3 (Table 1) and the amino acid consumption recorded in this medium (Fig. 5b) were used to formulate the DM4 medium (Table 1). In this medium, the concentrations of the amino acids that are a source of carbon and energy were adjusted according to the consumption presented in DM3. The concentration of threonine, glutamic acid and arginine in this new medium was set 35% higher than the recorded consumption (Fig. 5b). The concentration of cysteine, lysine and proline was set to 55%, 100% and 100% higher than their respective consumption (Fig. 5b) to avoid nutritional limitations. The concentration of other compounds was maintained from DM3 (Table 1).

The growth profile obtained for *P. salmonis* in DM4 (Fig. 5a) showed a slight decrease in biomass production compared to DM3, but the biomass yield (biomass/total amino acids consumed) increased by 1.6 times (Table 1). The growth profile in DM4 shows an inflection at 53 h which coincides with the depletion of glutamic acid, proline, isoleucine and leucine (Fig. 5c). Subsequently, the start of the stationary phase at 72 h corresponds to the depletion of threonine, lysine and valine (Fig. 5c). The other amino acids (arginine, cysteine, histidine, phenylalanine and methionine) remain in a small proportion at 72 h (Fig. 5c).

A biomass of 1.65 g/L (equivalent to 5.3 OD_{600}) at 75 h of culture is greater than or equal to the biomass previously reported based on the cultivation of *P. salmonis* in complex media. For example, Yañez et al. [54] reached 1.7 OD_{620} after 6 days (144 h) of culture using AUSTRAL-SRS medium; Henríquez et al. [23] obtained 2.0 OD_{600} after 58 h of culture using a medium with 8 g/L peptone and 8 g/L yeast extract; Eliassen et al. [14] reported a maximum biomass of 5.3 OD_{600}, after 144 h, in a commercial culture medium (SF900II) normally used for the cultivation of insect cells. In addition, the volumetric productivity obtained in the DM4 medium (0.022 g/L/h) is the highest reported and it is adequate for the development of a process for the production of *P. salmonis* biomass at industrial level.

The data showed that DM4 medium has a balanced formulation, adjusted to the nutritional demand of *P. salmonis* and does not induce bacterial aggregates. Thus, the scarce aggregates observed in DM3 may be the result of stress caused by the depletion of some amino acids, combined with high concentrations of others. This phenotype can be presumably related with a persistence state to avoid environmental starvation [28, 50]. Finally, it was possible to improve the growth of *P. salmonis* in a chemically defined medium with a planktonic phenotype, more suitable for a scalable fermentation process than cultures with biofilm phenotype.

Conclusion

This study focused on developing a chemically defined culture medium to support the axenic growth of *P. salmonis*. Firstly, we showed that nine essential and two pseudo-essential amino acids are required for cell growth. Secondly, a rational strategy allowed us to obtain an optimized culture medium (DM4) that promotes a planktonic phenotype. Third, the role of glutamic acid, arginine and threonine as carbon and energy sources was confirmed. This work makes a systematic assessment of *P. salmonis* nutritional requirements which can be used to improve control and consistency of fermentations for vaccine production.

Acknowledgements This work was supported by the FONDEF project [Grant Number D10I1185] and PhD scholarship CONICYT of Ministry of Education, Chile government.

Compliance with ethical standards

Obtained data supported the patent application WO2016082050A1.

References

1. Albornoz R, Valenzuela K, Pontigo JP et al (2017) Identification of chemotaxis operon cheYZA and cheA gene expression under stressful conditions in *Piscirickettsia salmonis*. Microb Pathog 107:436–441
2. Arkush KD, McBride AM, Mendonca HL et al (2005) Genetic characterization and experimental pathogenesis of *Piscirickettsia salmonis* isolated from white seabass *Atractoscion nobilis*. Dis Aquat Organ 63:139–149
3. Athanassopoulou F, Groman D, Prapas T, Sabatakou O (2004) Pathological and epidemiological observations on rickettsiosis in cultured sea bass (*Dicentrarchus labrax* L.) from Greece. J Appl Ichthyol 20:525–529
4. Bartolucci AA, Singh KP, Bae S (2015) Robustness and Ruggedness. In: Bartolucci AA, Singh KP, Bae S (eds) Introduction to statistical analysis of laboratory data. Wiley, New York, pp 213–234

5. Bhatnagar RK, Berry A, Hendry AT, Jensen RA (1989) The biochemical basis for growth inhibition by L-phenylalanine in *Neisseria gonorrhoeae*. Mol Microbiol 3:429–435
6. Birkbeck TH, Griffen AA, Reid HI et al (2004) Growth of *Piscirickettsia salmonis* to high titers in insect tissue culture cells. Infect Immun 72:3693–3694. https://doi.org/10.1128/IAI.72.6.3693
7. Braun PR, Al-Younes H, Gussmann J et al (2008) Competitive inhibition of amino acid uptake suppresses chlamydial growth: involvement of the chlamydial amino acid transporter BrnQ. J Bacteriol 190:1822–1830
8. Bravo S, Campos M (1989) Coho salmon syndrome in Chile. FHS/AFS Newsl 17:3
9. Christgen SL, Becker DF (2019) Role of proline in pathogen and host interactions. Antioxid Redox Signal 30:683–709. https://doi.org/10.1089/ars.2017.7335
10. Contreras-Lynch S, Olmos P, Vargas A et al (2015) Identification and genetic characterization of *Piscirickettsia salmonis* in native fish from southern Chile. Dis Aquat Organ 115:233–244
11. Cortés MP, Mendoza SN, Travisany D et al (2017) Analysis of *Piscirickettsia salmonis* metabolism using genome-scale reconstruction, modeling, and testing. Front Microbiol 8:2462
12. Das M, Grover A (2018) Fermentation optimization and mathematical modeling of glycerol-based microbial poly(3-hydroxybutyrate) production. Process Biochem 71:1–11. https://doi.org/10.1016/j.procbio.2018.05.017
13. De Felice M, Levinthal M, Iaccarino M, Guardiola J (1979) Growth inhibition as a consequence of antagonism between related amino acids: effect of valine in *Escherichia coli* K-12. Microbiol Rev 43:42
14. Eliassen TM, Solbakk IT, Haugseth KT et al (2007) Process for culturing bacteria of the *Piscirickettsia* genus. Patent Application WO 2008/002152 A8. https://www.lens.org/lens/patent/WO_2008_002152_A8_20090723
15. Englesberg E, Bass R, Heiser W (1976) Inhibition of the growth of mammalian cells in culture by amino acids and the isolation and characterization of L-phenylalanine-resistant mutants modifying L-phenylalanine transport. Somat Cell Genet 2:411–428
16. Ferber DM, Ely B (1982) Resistance to amino acid inhibition in *Caulobacter crescentus*. Mol Gen Genet MGG 187:446–452
17. Fryer JL, Lannan CN, Garcés LH et al (1990) Isolation of a rickettsiales-like organism from diseased coho salmon (*Oncorhynchus kisutch*) in Chile. Fish Pathol 25:107–114
18. Fryer JL, Lannan CN, Giovannoni SJ, Wood ND (1992) *Piscirickettsia salmonis* gen. nov., sp. nov., the causative agent of an epizootic disease in salmonid fishes. Int J Syst Evol Microbiol 42:120–126
19. Fu XT, Lin H, Kim SM (2009) Optimization of medium composition and culture conditions for agarase production by *Agarivorans albus* YKW-34. Process Biochem 44:1158–1163. https://doi.org/10.1016/j.procbio.2009.06.012
20. Fuentealba P, Aros C, Latorre Y et al (2017) Genome-scale metabolic reconstruction for the insidious bacterium in aquaculture *Piscirickettsia salmonis*. Bioresour Technol 223:105–114
21. Gesbert G, Ramond E, Tros F et al (2015) Importance of branched-chain amino acid utilization in *Francisella* intracellular adaptation. Infect Immun 83:173–183
22. Gómez F, Henríquez V, Marshall S (2009) Additional evidence of the facultative intracellular nature of the fish bacterial pathogen *Piscirickettsia salmonis*. Arch Med Vet 41:261–267
23. Henríquez M, González E, Marshall SH et al (2013) A novel liquid medium for the efficient growth of the salmonid pathogen *Piscirickettsia salmonis* and optimization of culture conditions. PLoS One 8:e71830. https://doi.org/10.1371/journal.pone.0071830
24. Hobley L, Harkins C, MacPhee CE, Stanley-Wall NR (2015) Giving structure to the biofilm matrix: an overview of individual strategies and emerging common themes. FEMS Microbiol Rev 39:649–669
25. Jain SP, Singh PP, Javeer S, Amin PD (2010) Use of Placket–Burman statistical design to study effect of formulation variables on the release of drug from hot melt sustained release extrudates. AAPS PharmSciTech 11:936–944. https://doi.org/10.1208/s12249-010-9444-6
26. Janakiraman A, Lesser CF (2017) How to manage stress: lessons from an intracellular pathogen. Virulence 8:359–361. https://doi.org/10.1080/21505594.2016.1256538
27. Johnson CL, Vishniac W (1970) Growth inhibition in *Thiobacillus neapolitanus* by histidine, methionine, phenylalanine, and threonine. J Bacteriol 104:1145–1150
28. Jolivet-Gougeon A, Bonnaure-Mallet M (2014) Biofilms as a mechanism of bacterial resistance. Drug Discov Today Technol 11:49–56
29. Koch AL (2007) Growth Measurement. In: Reddy CA, Beveridge TJ, Breznak JA et al (eds) Methods for general and molecular microbiology, 3rd edn. ASM Press, Washington D.C., pp 172–199
30. Lawson J, Erjavec J (2017) Screening designs. In: Lawson J, Erjavec J (eds) Basic experimental strategies and data analysis for science and engineering. CRC Press, Boca Raton, pp 163–201
31. Link H, Weuster-Botz D (2011) 2.11—medium formulation and development. In: Moo-Young M (ed) Comprehensive biotechnology, 2nd edn. Academic, Burlington, pp 119–134
32. Maisey K, Montero R, Christodoulides M (2017) Vaccines for piscirickettsiosis (salmonid rickettsial septicaemia, SRS): the Chile perspective. Expert Rev Vaccines 16:215–228. https://doi.org/10.1080/14760584.2017.1244483
33. Manske C, Hilbi H (2014) Metabolism of the vacuolar pathogen *Legionella* and implications for virulence. Front Cell Infect Microbiol 4:125
34. Marshall SH, Gómez FA, Ramírez R et al (2012) Biofilm generation by *Piscirickettsia salmonis* under growth stress conditions: a putative in vivo survival/persistence strategy in marine environments. Res Microbiol 163:557–566
35. Mauel MJ, Ware C, Smith P (2008) Culture of *Piscirickettsia salmonis* on enriched blood agar. J Vet Diagn Investig 20:213–214. https://doi.org/10.1177/104063870802000211
36. Meibom KL, Charbit A (2010) *Francisella tularensis* metabolism and its relation to virulence. Front Microbiol. https://doi.org/10.3389/fmicb.2010.00140
37. Mikalsen J, Skjaervik O, Wiik-Nielsen J et al (2008) Agar culture of *Piscirickettsia salmonis*, a serious pathogen of farmed salmonid and marine fish. FEMS Microbiol Lett 278:43–47. https://doi.org/10.1111/j.1574-6968.2007.00977.x
38. Monds RD, O'Toole GA (2009) The developmental model of microbial biofilms: ten years of a paradigm up for review. Trends Microbiol 17:73–87. https://doi.org/10.1016/j.tim.2008.11.001
39. Nagata Y, Chu KH (2003) Optimization of a fermentation medium using neural networks and genetic algorithms. Biotechnol Lett 25:1837–1842. https://doi.org/10.1023/a:1026225526558
40. Navarrete-Bolaños JL, Téllez-Martínez MG, Miranda-López R, Jiménez-Islas H (2017) An experimental strategy validated to design cost-effective culture media based on response surface methodology. Prep Biochem Biotechnol 47:578–588. https://doi.org/10.1080/10826068.2017.1280825
41. Omsland A, Sixt BS, Horn M, Hackstadt T (2014) Chlamydial metabolism revisited: interspecies metabolic variability and developmental stage-specific physiologic activities. FEMS Microbiol Rev 38:779–801
42. Patil S, Nikam M, Patil H et al (2017) Bioactive pigment production by *Pseudomonas* spp. MCC 3145: statistical media optimization, biochemical characterization, fungicidal and DNA intercalation-based cytostatic activity. Process Biochem 58:298–305. https://doi.org/10.1016/j.procbio.2017.05.003

43. Pitol LO, Finkler ATJ, Dias GS et al (2017) Optimization studies to develop a low-cost medium for production of the lipases of *Rhizopus microsporus* by solid-state fermentation and scale-up of the process to a pilot packed-bed bioreactor. Process Biochem 62:37–47. https://doi.org/10.1016/j.procbio.2017.07.019
44. Plackett RL, Burman JP (1946) The design of optimum multifactorial experiments. Biometrika 33:305–325
45. Price CTD, Richards AM, Abu Kwaik Y (2014) Nutrient generation and retrieval from the host cell cytosol by intra-vacuolar *Legionella pneumophila*. Front Cell Infect Microbiol 4:111
46. Rinaudi LV, Giordano W (2010) An integrated view of biofilm formation in rhizobia. FEMS Microbiol Lett 304:1–11
47. Rozas M, Enríquez R (2014) Piscirickettsiosis and *Piscirickettsia salmonis* in fish: a review. J Fish Dis 37:163–188. https://doi.org/10.1111/jfd.12211
48. Sargent B (2011) The need for defined, animal-free, components in vaccine production media. http://cellculturedish.com/the-need-for-defined-animal-free-components-in-vaccine-production-media/
49. Sernapesca (2018) Informe Sanitario de Salmonicultura en Centros Marinos—Año 2017. http://www.sernapesca.cl/sites/default/files/informe_sanitario_2017_0.pdf
50. Seth AK, Geringer MR, Hong SJ et al (2012) In vivo modeling of biofilm-infected wounds: a review. J Surg Res 178:330–338
51. Spaargaren DH (1996) The design of culture media based on the elemental composition of biological material. J Biotechnol 45:97–102. https://doi.org/10.1016/0168-1656(95)00152-2
52. Stellwagen E, Prantner JD, Stellwagen NC (2008) Do zwitterions contribute to the ionic strength of a solution? Anal Biochem 373:407–409. https://doi.org/10.1016/j.ab.2007.10.038
53. Tandberg JI, Lagos LX, Langlete P et al (2016) Comparative analysis of membrane vesicles from three *Piscirickettsia salmonis* isolates reveals differences in vesicle characteristics. PLoS One 11:e0165099. https://doi.org/10.1371/journal.pone.0165099
54. Yañez AJ, Valenzuela K, Silva H et al (2012) Broth medium for the successful culture of the fish pathogen *Piscirickettsia salmonis*. Dis Aquat Organ 97:197–205. https://doi.org/10.3354/dao02403
55. Yañez AJ, Silva H, Valenzuela K et al (2013) Two novel blood-free solid media for the culture of the salmonid pathogen Piscirickettsia salmonis. J Fish Dis 36:587–591
56. Zhang J, Greasham R (1999) Chemically defined media for commercial fermentations. Appl Microbiol Biotechnol 51:407–421. https://doi.org/10.1007/s002530051411
57. Zuo S, Xiao J, Zhang Y et al (2018) Rational design and medium optimization for shikimate production in recombinant *Bacillus licheniformis* strains. Process Biochem 66:19–27. https://doi.org/10.1016/j.procbio.2017.12.012