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Partial Evaluation of Autochthonous Probiotic Potential of the Gut Microbiota of *Seriola lalandi*

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Abstract

Seriola lalandi is an economically important species that is globally distributed in temperate and subtropical marine waters. Aquaculture production of this species has had problems associated with intensive fish farming, such as disease outbreaks or nutritional deficiencies causing high mortality. Intestinal microbiota are involved in many processes that benefit a host, such as disease control, stimulation of the immune response, and the promotion of nutrient metabolism. The aim of this study is to evaluate the in vitro probiotic properties of bacteria isolated from the intestinal content of wild *Seriola lalandi*. The probiotic potential was evaluated in terms of (i) the antimicrobial activity against vibrios causing outbreaks in farmed fish; (ii) the ability to stimulate genes related to an innate immune response in fish; and (iii) antibiotic resistance. Nineteen isolates identified as *Pseudomonas*, *Shewanella*, *Psychrobacter*, and *Acinetobacter* showed antimicrobial activity and significant relative expression of cytokines, serum amyloid A protein (SAA), hepcidin, and lysozyme. A positive correlation was observed between the levels of expression and the bacterial load after 24 h of exposure. *Pseudomonas* isolates showed a level of antibiotic resistance. In conclusion, isolates of the genera *Shewanella*, *Psychrobacter*, and *Acinetobacter* could serve as potential probiotics in *S. lalandi* culture.

Keywords Probiotic · Microbiota · Antimicrobial activity · Immune-related genes · *Seriola lalandi*

Introduction

Seriola lalandi (yellowtail kingfish) is a marine, pelagic, and carnivorous fish found globally in subtropical and temperate waters of the Pacific and Indian Oceans [1]. This species is mainly cultivated in Japan [2], Australia [1], and New Zealand [3]. In Chile, its cultivation is considered within the aquaculture diversification program [4]. This species has excellent attributes that promote its cultivation, including high rates of growth and market acceptance [5]. A problem associated with the cultivation of this species is the high mortality, up to 90%, in the stage of larvae and fry [6]. The disease outbreaks have increased with the intensification of aquaculture, especially during the early stages of fish development; an example of this principal problem in larval culture is vibriosis [7].

The control and prevention of diseases in aquaculture are antimicrobials; however, the excessive use of these has been questioned given the selection of bacteria resistant to antimicrobials [8, 9]. It has been described that the use of antimicrobials in farmed fish causes an imbalance in the intestinal microbiota known as dysbiosis [10–13]. This imbalance in the composition of the microbiota could cause an increase in the colonization of pathogenic bacteria, increasing host mortality [13]. In this sense, the use of probiotics is proposed as an alternative to overcome the problems associated with the excessive use of antimicrobials in the control of bacterial diseases in intensive culture systems. Among the reported benefits of the use of probiotics in aquaculture species are inhibition of colonization by pathogens [14–16]; the improvement in the immune response of the host [17–20]; enzymatic contribution, improving the digestion process, given by the extracellular production of enzymes, such as proteases, lipases, chitinases, phytases, and amylases [21]; and contribution with

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nutrients such as vitamins, amino acids, and fatty acids [22]. Desirable characteristics for the selection of probiotic potentials include (i) not causing host damage; (ii) having the ability to colonize and adhere to host epithelial surfaces; (iii) having the ability to reach target organs and exercise their function; (iv) lacking antimicrobial resistance genes [23, 24]; (v) bile and pH tolerance testing; and (vi) antagonistic activity. Previous studies have reported the use of autochthonous bacteria as an alternative to controlling bacterial diseases in fish culture [18, 25–31]. As Nayak [32] and Mills et al. [33] described, compared to the benefits provided by bacteria isolated from other sources, beneficial bacteria isolated from a host could be more beneficial if administered again in the same species or similar species. Currently, there is increasing attention on the composition of the gut microbiota of fish for their contribution to processes such as nutrition and defense of the host [32, 34].

Microbiota have been defined as the community of microorganisms present in most individuals of a population or a species that, despite continuous contact with different tissues, do not cause damage to their host [35]. Most of these microorganisms reside in the digestive tract, where they influence a wide range of biological processes that generate beneficial effects for the host. These microorganisms contribute to nutrition, digestion processes, and the provision of vitamins; in defense, microbiota play a protective role in preventing colonization by pathogens through competition for nutrients, competition for exclusion, and production of antimicrobial substances, and these microorganisms can modulate the host's immune system [23, 24, 36, 37]. Many studies suggest that microbial communities have co-evolved with their respective hosts establishing mutualistic interactions for many physiological aspects, such as the synthesis of enzymes, vitamins, metabolites, and antimicrobial compounds and the development of the intestinal mucosa of the host and the immune system [32, 36, 38–40]. A recent study of Rosshart et al. [41] highlights the importance of the establishment of the microbiota in the response of the host to pathogenic microorganisms. These authors showed that wild and laboratory mice have different survival against a pathogen, 90% in wild mice and less than 20% in laboratory mice, attributing this response to the composition of the organism's microbiota depending on the origin. This reflects that the animals in captivity, kept under controlled conditions, present a microbiota different from that of wild animals, which have been exposed to various environmental factors, pathogens, stress, etc. and that this microbiota selected by the host through natural selection confers benefits in the response to pathogens. Intensive culture systems maintain controlled conditions of various parameters, such as water quality, diet, and population density, which can lead to the establishment of a different intestinal microbiota compared to wild fish of the same species [4, 42–45]. A previous study by Ramírez and Romero [4] describes significant differences in the components of the intestinal microbiota of wild *S. lalandi* and aquaculture. At the genus level, those differentially represented in wild *S. lalandi* from that study are *Psychrobacter*, *Shewanella*, *Pseudomonas*, and *Acinetobacter*. The aim of this work was to study the probiotic potential of autochthonous bacteria isolated from wild *S. lalandi* by evaluating the antagonist activity against *Vibrio* sp. and the in vitro stimulation of genes related to the immune response.

Materials and Methods

Isolation of Bacterial Strains

A total of 12 fish of *S. lalandi* were collected from the Region of Coquimbo, Chile, specifically from latitude S 30.104; longitude W 71.377 to latitude S 30.302; longitude W 70.608 in February 2015. After capture, the fish were weighed, and the intestinal contents were aseptically removed, stored in ice, and transported to the laboratory. The intestinal contents were homogenized in a 10 mL sterile phosphate-buffered saline solution (PBS 1×) pH 7.2. The homogenates were disseminated in trypticase soy agar (Merck) supplemented with 2% NaCl (Oxoid) (TSA2), Luria–Bertani (LB), de MAN, ROGOSA, and SHARPE (MRS, Merck) and yeast extract-peptone-dextrose growth medium (YEPD) dishes supplemented with 2% NaCl. After incubation at 28 °C for 2 days, the predominant colonies were isolated in fresh media and examined for different morphologies. The isolates were identified based on 16S rRNA gene sequencing. Isolates from the bacterial genera *Pseudomonas*, *Shewanella*, *Psychrobacter*, and *Acinetobacter* were evaluated in this study. These isolates were selected according to previous results by Ramírez and Romero [4] in which these genera were found to be dominant in the gut microbiota of wild *S. lalandi.*

Antimicrobial Activity

The antimicrobial activity of the isolated strains was studied against *Vibrio* sp., a common pathogen in aquaculture, by the double-layer method of Dopazo et al. [46] modified according to Leyton and Riquelme [47]. On a plate count agar plate (prepared with 75% sea water and 25% distilled water) (PCAsw), 10 μL of an 18-h culture of the strain of interest grown in trypticase soy broth (TSB, Merck) supplemented with 2% NaCl (TSB2) was inoculated. The inoculum was incubated for a period of 36 h at 20 °C. Once the bacterial growth was verified, the macrocolony was inactivated by exposure to chloroform vapors for a period of 45 min. Subsequently, 4 mL of TSB2 soft agar media supplemented with 0.7% bacteriological agar (Oxoid) was used, which was inoculated with 100 μL of an 18-h culture of the three

pathogenic bacteria of genus *Vibrio* cultivated in TSB2. Then, the medium was shaken gently and briefly by pouring on the plate with the macrocolony of the antagonist strain. Plates were incubated at 20 °C for 24 h. Subsequently, inhibition or growth of the pathogenic strain around the antagonist strain was verified.

Stimulation of Immune-Related Genes

Infection Kinetics in Cell Line

To determine the transcriptional activity of genes associated with the innate immune system during the infection process with probiotic potentials, an infection kinetic assay was performed using qRT-PCR in the SHK-1 cell line [48]. The cell line was maintained in L-15 medium (Leibovitz, Gibco) supplemented with 5% fetal bovine serum (FBS, Gibco), 4 mM L-glutamine, 50 μg mL^{-1} gentamicin sulfate, and 40 μM β-mercaptoethanol and was cultured at 18 °C in 75 cm^2 tissue culture–treated flasks (Orange Scientific). The kinetic infection was measured at earlier stages of the infection (12 and 24 h. The cells used in this study corresponded to passages 45 and 48.

A single colony grown in TSA2 plates was used to inoculate 4 mL of TSB2 medium, incubated at 28 °C and 100 rpm for 24 h. Then, 1 mL of the medium of each isolate was used to quantify the bacterial concentration by means of a Petroff–Hausser chamber. First, it was centrifuged, and the pellet was washed twice with PBS 1×, to eliminate traces of culture medium. The volume corresponding to 1×10^5 bacteria was used to infect with a multiplicity of infection (MOI) of 1 in every 1.92 cm^2 well with cells for every kinetic time point, including biological triplicates. SHK-1 cells were grown to 90% confluency in 24-well tissue culture plates under the conditions described above, but this time without addition of antibiotic. After infection, followed by an incubation period of 2 h at 18 °C, the cells were washed once with 1× PBS, and fresh L-15 medium was added to eliminate excessive bacterial growth [49]. Finally, the infected cells were incubated at 18 °C during the times described above.

RNA Isolation and cDNA Synthesis

Total RNA was isolated from cells using the TRIzol isolation reagent according to the manufacturer's procedure (Roche, Canada). The concentration of extracted RNA was calculated at a wavelength of 260 nm using NanoQuant spectrophotometry (Tecan). To detect the purity of RNA, the optical density (OD) absorption ratio at 260/280 nm was determined, and only samples with a ratio of more than 1.8 were used for cDNA synthesis. cDNA was synthesized from 1 μg of RNA pre-treated with RNase-Free DNase RQ1 (Promega) for 1 h at 37 °C, using a kit ImProm-II™Reverse Transcription System (Promega) according to the manufacturer's procedure.

Relative Expression of Immune-Related Genes

Triplicate qPCR reactions were carried out for each sample analyzed. The PCRs were performed with the SYBR green method. Arrays were run on a Corbett Rotor Gene 6000 instrument. The reactions were set for each sample with 1 μL of cDNA, 5 μL of 2× concentrated SYBR Green PCR Master Mix (Roche) containing SYBR Green as a fluorescent intercalating agent, 0.5 μM forward primer, and 0.5 μM of reverse primer (Table 1). The thermal profile for all reactions was 5 min at 95 °C, and then 40 cycles of 15 s at 95 °C, 10 s at 60 °C, and 10 s at 72 °C, except for the V3 region of the 16S rRNA, which had a profile of 40 cycles of 5 s at 95 °C and 2 s at 60 °C. Fluorescence monitoring occurred at the end of each cycle. Additional dissociation curve analysis was performed and showed in all cases one single peak. Relative quantification of cDNA was made using Elongation Factor 1a (EF-1a) as a reference gene (housekeeping gene). Modification of gene expression is represented with respect to the control sampled at the same time as the treatment. The graphics for the relative expression were performed as a heatmap in the R environment using the ggplot package.

Enzymatic Analysis

The enzymatic activities of the isolates were determined by using the API ZYM system (bioMérieux, Marcy-l'Etoile, France) according to the manufacturer's guidelines. Briefly,

Table 1 Primers for real-time quantitative PCR assay

Gene	Primer sequence (5′ → 3′)	Amplicon size
EF-1α	F: GTGGAGACTGGAACCCTGAA R: CTTGACGGACACGTTCTTGA	180
β-actin	F: CAACTGGGACGACATGGAGA R: AGTGAGCAGGACTGGGTGCT	80
IL-1β	F: CGTCACATTGCCAACCTCAT R: ACTGTGATGTACTGCTGAAC	200
TNFα	F: GGCGAGCATACCACTCCTCT R: TCGGACTCAGCATCACCGTA	150
IL-8	F: GAATGTCAGCCAGCCTTGTC R: TCCAGACAAATCTCCTGACCG	220
SAA	F: TTGTTCTGACCCTCGTTGTAGGAGC R: CATGTCGCCATATGCACGCC	110
Lys	F: GTGGCTGGCTGCGGGGTCT R: GCAGATGCTGATGGATAGT	140
Hep	F: GAGGAGGTTGGAAGCATTGA R: TGACGCTTGAACCTGAAATG	120
V3	F: CCTACGGGAGGCAGCAG R: GTATTACCGCGGCTGCTGG	177

F, forward; *R*, reverse

isolated colonies grown on TSA plates were used to inoculate and were resuspended in a NaCl 0.85% solution (bioMérieux, Marcy-l'Etoile, France) to obtain a turbidity of 5–6 McFarland ($1.5–1.8 \times 10^9$ bacteria mL^{-1}), and 65 μL of this suspension was added to each cupule. Test strips were incubated for 4 h at 30 °C, and following incubation, 1 drop of ZYM A (API; Tris-hydroxymethyl-aminomethane, hydrochloric acid, sodium laurel sulfate, H_2O) and ZYM B (API; fast blue BB, 2-methoxyethanol) were added to each cupule. Test strips were read after 5 min, and the results were scored using the following classification: 0 negative reaction; 1–2 weak activity; 3–5 strong activity.

Antimicrobial Resistance Patterns

The resistance/susceptibility profiles of the different isolates to five antimicrobial agents were determined by an agar disk diffusion method as described in the Clinical and Laboratory Standards Institute (CLSI) guideline M42-A [50], using Müeller–Hinton agar (Difco) supplemented with NaCl (2%). The antibacterial susceptibility patterns of resistant isolates were performed using disks containing the following antibacterial agents: florfenicol (FFC, 30 μg), oxytetracycline (OT, 30 μg), oxolinic acid (OA, 2 μg), flumequine (UB, 30 μg), and sulfamethoxazole–trimethoprim (SXT, 1.25 and 23.75 μg). All disks were obtained from Oxoid Ltd. (Basingstoke, Hampshire, England). Bacterial strains were suspended in sterile 0.85% saline to a turbidity to match a McFarland No. 2 standard (bioMerieux S.A.), diluted 1:20, and streaked on the used media. Plates were incubated for 24–48 h at 22 °C and *E. coli* ATCC 25922 was used as the control strain. Characterization of isolates as resistant was stated according to standards suggested by Miranda and Rojas [51].

Statistical Analysis

The analysis of the relative gene expression was conducted following the method described by Pfaffl [52]. A 5% level of significance was used for all statistical tests ($P < 0.05$). Statistical analysis was conducted using the Relative Expression Software Tool (REST©). The Pearson correlation test was used to find any correlation between load bacteria and the respective immune gene expression.

Results

Isolated Intestinal Microbiota of *Seriola lalandi*

From the intestinal content samples of 12 specimens of *S. lalandi*, 388 bacterial isolates were obtained, of which 59% corresponded to bacteria of phylum *Proteobacteria*, 16% to *Firmicutes*, 13% to *Actinobacteria*, and 10% to *Bacteroidetes*. The genera with the highest number of isolates were *Alcaligenes* (18%), *Myroides* (9%), *Microbacterium* (6%), and *Pseudochrobactrum* (5%). Among the genera of interest, *Pseudomonas*, *Shewanella*, *Psychrobacter*, and *Actinobacteria*, 39 isolates were obtained.

Antimicrobial Activity

Direct antimicrobial activity of the selected isolates was carried out against three isolates obtained from outbreaks of mortality of fish in culture; these isolates were confirmed as *Vibrio* sp. Of the 39 isolates evaluated, nearly 50%, i.e., 19 isolates, registered growth inhibition of *Vibrio* sp. as shown in Table 2.

Relative Expression of Immune-Related Genes

First, the cytopathic effect of the 19 bacterial isolates selected above was evaluated for their antimicrobial activity against strains of *Vibrio* sp. using a multiplicity of infection (MOI) equal to 1. After 24 h post-infection (hpi), no cytopathic effect was observed in the monolayer on the part of the isolates evaluated. Subsequently, stimulation tests were performed up to 12 and 24 hpi. The results show that the expression of the evaluated genes is higher at 24 hpi than at 12 hpi. For example, at 24 hpi, citokynes (IL-8, TNFα, and IL-lβ) increased almost 30 times the expression observed at 12 hpi; the same tendency was observed in effectors such as (SAA), hepcidin and lysozyme, reaching 3 or 5 more expression (Fig. 1; Supplementary material). Different gene expression was also observed among bacterial genera. The isolates of the genera *Pseudomonas* and *Shewanella* present higher levels of expression at 12 and 24 hpi, reaching expression levels over 200–500 times the control, especially in citokynes. Isolates with higher expression levels were P21, P19, P14, P11, and P25 in the case of *Pseudomonas* (Fig. 1a), like those shown by the two isolates of *Shewanella* (Fig. 1b; Supplementary material), with expression levels over 100 times the control. The expression levels generated by the isolates of *Psychrobacter* and *Acinetobacter* (Fig. 1c, d) are lower than those previously described, with the exception of P29, P16, and P17, which were all significant with respect to the control, with the exception of P15 for the IL-1b and Hep genes (Fig. 1c). In addition, it was determined by Pearson correlation that the expression of the evaluated genes, although not significant, is associated with the bacterial load (Table 3) and has a positive correlation after 12 h of exposure (r 0.3–0.4). After 24 hpi, the correlation is positive (r 0.7–0.85) and significant ($P < 0.001$).

Table 2 Antimicrobial activity of bacteria isolated from wild *S. lalandi* against pathogenic vibrios in aquaculture fish

Isolate code	Molecular identification	*Vibrio* 25LT1	*Vibrio* T25H1	*Vibrio* LS1
P15	*Psychrobacter* sp.	++	++	++
P16	*Psychrobacter* sp.	++	++	+
P17	*Psychrobacter* sp.	++	++	+
P20	*Psychrobacter* sp.	++	–	–
P29	*Psychrobacter* sp.	++	++	–
P30	*Psychrobacter* sp.	–	–	+
P18	*Pseudomonas* sp.	++	++	++
P19	*Pseudomonas* sp.	+	+	–
P14	*Pseudomonas* sp.	++++	++	–
P21	*Pseudomonas* sp.	++	–	–
P11	*Pseudomonas fragi*	+++	–	–
P25	*Pseudomonas* sp.	++	–	–
P35	*Pseudomonas* sp.	++	–	–
P36	*Pseudomonas* sp.	+	–	–
P33	*Acinetobacter* sp.	++	–	–
P34	*Acinetobacter* sp.	++	–	–
P27	*Acinetobacter* sp.	++	–	–
P13	*Shewanella baltica*	++	+++	–
P24	*Shewanella* sp.	++++	–	–

Plus sign (+) indicates presence of inhibition halo; (+) inhibition halo of 7–10 mm; (++) 11–20 mm; (+++) 21–30 mm, (++++) greater than 30 mm, and (−) absence of inhibition halo

Enzymatic Activity

Nineteen enzymatic activities were tested with the API ZYM system. Some activities were shown by all or most strains and at high levels of esterase (C4), esterase lipase (C8), Leu-arylamidase, and naphthol-AS-BI-phosphohydrolase, highlighting the isolates of genus *Shewanella* that also showed high levels of activity of alkaline phosphatase, α-chymotrypsin, and acid phosphatase. In contrast, other activities such as those of α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mamnosidase, and α-fucosidase were detected at low levels and only in the case of isolates of *Shewanella* showed high to weak activity of α-chymotrypsin and *N*-acetyl-β-glucosaminidase. These results are shown in Table S1 (supplementary material).

Resistance of Antimicrobial Agents

The evaluated isolates belonging to the genera *Shewanella*, *Psychrobacter*, and *Acinetobacter* were susceptible to all the antimicrobial agents evaluated. On the other hand, among the isolates of the genus *Pseudomonas*, only two were susceptible to all the antimicrobials evaluated. Isolates P19, P14, P21, P11, P25, and P35 showed resistance to florfenicol; in addition, P21 and P11 showed resistance to sulfamethoxazole–trimethoprim as shown in Table 4.

Discussion

The microbiota plays an important role in the maintenance of the intestinal mucosal barrier, avoiding colonization by pathogenic microorganisms and contributing to immune homeostasis, this given by a close relationship between the microbiota and the host. In this way, the microbiota of the same species is presented as a source of probiotic potentials in aquaculture species. In the present study, 19 of 39 isolates evaluated showed antimicrobial activity against strains of *Vibrio* sp. derived from mortality outbreaks in aquaculture fish. It has been previously described that isolates of the genus *Psychrobacter* have an in vitro antagonistic effect against several pathogenic species, such as *Staphylococcus aureus*, *Vibrio harveyi*, *V. metschnikovii*, and *V. alginolyticus* [53]; *V. anguillarum*; and *Aeromonas salmonicida* [26]. The literature also describes a similar behaviour for some strains of *Pseudomonas* against *V. anguillarum* [14, 15]. On the other hand, strains of *Acinetobacter* have been described with antagonistic activity against pathogens such as *Vibrio cholera*, *Flavobacterium* sp., and *A. hydrophila* [54]. In the case of *Shewanella*, activity has been described against *V. anguillarum*, *A. salmonicida* [26], and *Vibrio harveyi* [37]. The antimicrobial activity produced by these strains could be attributed to the production of antimicrobial substances such as organic acids, bacteriocins, siderophores, and/or hydrogen peroxide, which have been described as mechanisms of pathogen inhibition [55]. For example, the

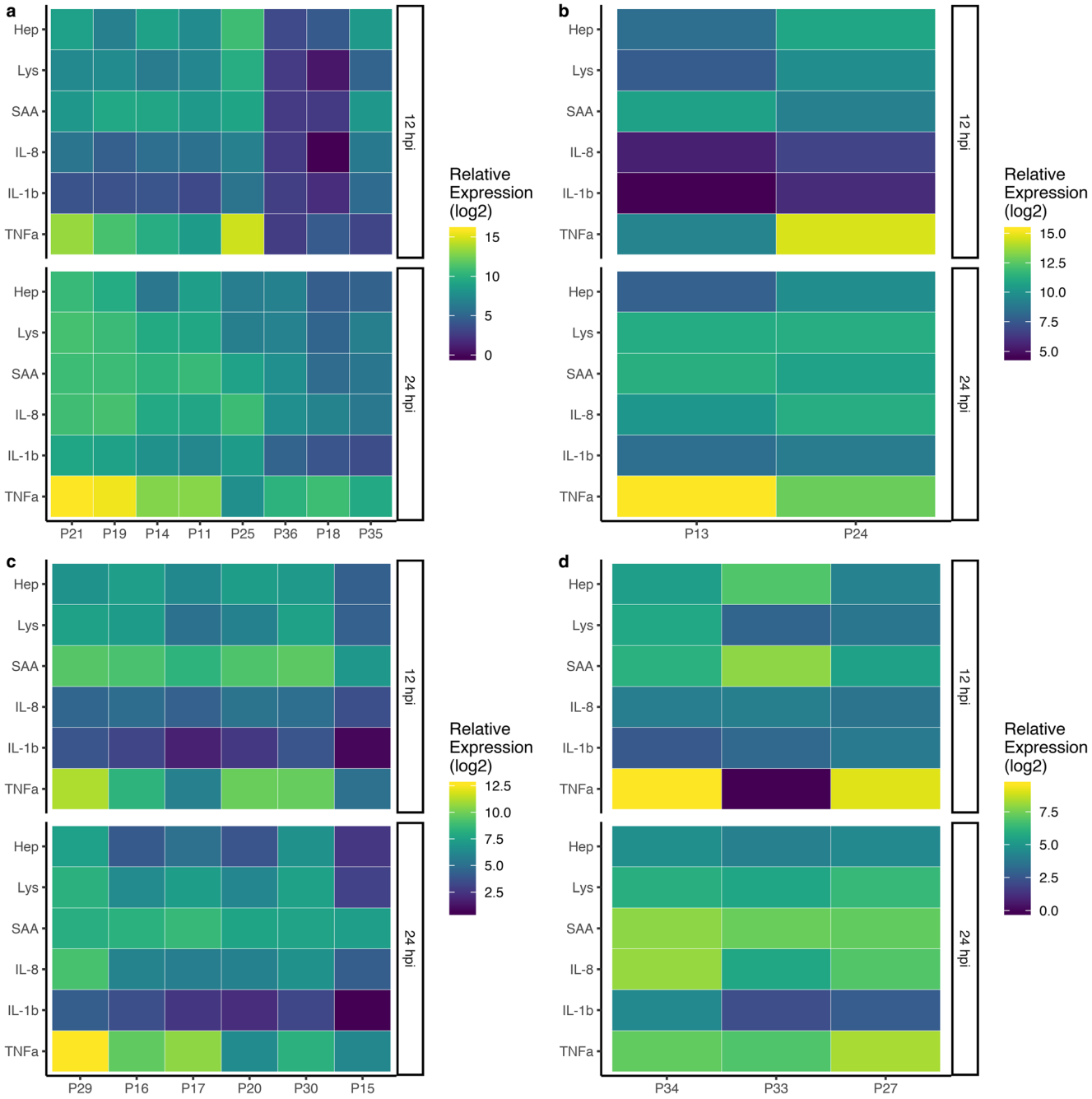

Fig. 1 Relative expression of genes associated with the innate immune system after 12 and 24 h of exposure to wild *S. lalandi* isolates. **a** *Pseudomonas* isolates; **b** *Shewanella* isolates; **c** *Psychrobacter* isolates; and **d** *Acinetobacter* isolates. EF-1a was used as the reference gene

study by Gram et al. [14] showed that the antimicrobial activity of *Pseudomonas fluorescens* against *V. anguillarum* is associated with the production of siderophores, similar to that described by Spanggaard et al. [15]. The antimicrobial activity of these strains against *Vibrio* can be considered relevant since vibriosis has been reported to generate significant mortality in marine fish cultures [7].

In this study, the levels of relative expression of genes associated with the innate immune system, by the isolates, revealed a positive tendency with respect to the bacterial load. This was corroborated by performing the Pearson correlation analysis, showing a positive (r between 0.70 and 0.85) and significant correlation ($P < 0.001$) after 24 hpi, demonstrating that the higher the bacterial load, the greater the relative expression of the genes. This reflects the need to evaluate the administered dose in the potential use as probiotics. Among the genes analyzed in this study, cytokines have been considered by various authors as a criterion for evaluating probiotic potential in fish [18, 19, 56–58]. The stimulation of cytokines initiates the response cascade of the immune system, where

Table 3 Pearson correlations (*P* value) between relative expression of genes associated with the innate immune system and the bacterial load

Gene	Time post-infection	
	12 h	24 h
TNFα	0.40 (0.09)	0.75 ($P < 0.001$)
IL-1β	0.37 (0.12)	0.73 ($P < 0.001$)
IL-8	0.30 (0.26)	0.70 ($P < 0.001$)
SAA	0.37 (0.12)	0.85 ($P < 0.001$)
Lys	0.38 (0.18)	0.81 ($P < 0.001$)
Hep	0.32 (0.11)	0.70 ($P < 0.001$)

TNFα and IL-lβ play an essential role in the activation of prostaglandin, leukotriene, nitric oxide, and other cytokines, such as IL-6 and IL-8 [59]. IL-8 is produced mainly by macrophages, epithelial cells, and endothelial cells and acts as a neutrophil chemotactic factor that induces mostly the migration of neutrophils and other granulocytes to the site of an infection, thereby inducing phagocytosis [60]. In this study, the isolates evaluated showed significant overexpression compared to the control group after 24 h of exposure of the genes associated with cytokines and chemokine, with the exception of an isolate of the genus *Psychrobacter* (P15). Previously, Beck et al. [18] reported that the administration of a mix of autochthonous bacteria exerted significant stimulation of IL-8 and TNFα in addition to the substantial increase in survival of *Paralichthys olivaceus* challenged with *Streptococcus iniae*. On the other hand, Giri et al. [61] used heat-killed whole-cell products (HKWCP) from a probiotic strain of *Pseudomonas aeruginosa* (VSG2), demonstrating that it stimulates cytokine responses in head kidney macrophages (HK) of *Labeo rohita*. This report proposes a form of administration of beneficial bacteria that could be evaluated for the improvement of the *Seriola* culture. Previously, Lazado et al. [17] reported that strains of *Pseudomonas* (GP21) and *Psychrobacter* (GP12) stimulate the expression of IL-1β and IL-8 in head kidney leukocytes (HK) of *Gadus morhua*. Recent reports of Mohammadian et al. [19, 20] show that the administration of autochthonous probiotics generates significant stimulation of cytokines in the head kidney of *Tor grypus.* Also, it shows that probiotic strains generate significant decrease in mortality associated with infection with *Aeromonas hydrophila* [19].

In the early response of the innate immune system, an essential component is the serum amyloid A protein (SAA). It binds to gram-negative bacteria; acts as an opsonin; and, therefore, improves phagocytosis [62]. In addition, Bayne and Gerwick [59] describe that their function could help repair tissue damage. In this study, the 19 isolates evaluated showed significant stimulation of the SAA gene after 24 h of exposure. Given the described functionality of this protein, the stimulation of this gene by the evaluated isolates could be beneficial when faced with an infection with pathogenic bacteria. Previously, it has been described that SAA is strongly

Table 4 Resistance to antimicrobial agents of wild *S. lalandi* isolates

Isolated code	Molecular identification	FFC	OTC	OA	UB	STX
P15	*Psychrobacter* sp.	S	S	S	S	S
P16	*Psychrobacter* sp.	S	S	S	S	S
P17	*Psychrobacter* sp.	S	S	S	S	S
P20	*Psychrobacter* sp.	S	S	S	S	S
P29	*Psychrobacter* sp.	S	S	S	S	S
P30	*Psychrobacter* sp.	S	S	S	S	S
P18	*Pseudomonas* sp.	S	S	S	S	S
P19	*Pseudomonas* sp.	R (12)	S	S	S	S
P14	*Pseudomonas* sp.	R (14)	S	S	S	S
P21	*Pseudomonas* sp.	R (11)	S	S	S	R (10)
P11	*Pseudomonas fragi*	R (10)	S	S	S	R (0)
P25	*Pseudomonas* sp.	R (8)	S	S	S	S
P35	*Pseudomonas* sp.	R (0)	S	S	S	S
P36	*Pseudomonas* sp.	S	S	S	S	S
P33	*Acinetobacter* sp.	S	S	S	S	S
P34	*Acinetobacter* sp.	S	S	S	S	S
P27	*Acinetobacter* sp.	S	S	S	S	S
P13	*Shewanella baltica*	S	S	S	S	S
P24	*Shewanella* sp.	S	S	S	S	S

FFC, florfenicol; *OTC*, oxytetracycline; *OA*, oxolinic acid; *UB*, flumequine; and *STX*, sulfamethoxazole–trimethoprim; *S*, susceptible; *R*, resistant

regulated during septicemia in *Salvelinus alpinus* [63]. Similar to that described by Kania et al. [64] demonstrating that the increase in the expression of the SAA gene in *O. mykiss* is directly related to the bacterial load post-infection, showing a positive Pearson correlation ($r = 0.974$; $P < 0.01$), similar to the results obtained in this study.

Another important component of host defense is the production of antimicrobial peptides (AMP). Recently, Muncaster et al. [65] characterized two antimicrobial peptides in *S. lalandi*, piscidin and hepcidin, describing that the latter has higher expression in the liver, with respect to spleen and gills. Álvarez et al. [66] describe the effect of synthetic hepcidin as a protector against a challenge with *V. anguillarum* in *Dicentrarchus labrax*. The cumulative mortality of *D. labrax* infected with *V. anguillarum* was 72.5% until day 7 post-injection; for its part in fish previously injected with hepcidin (Hep1), the cumulative mortality was 23.5%. Previously, Álvarez et al. [67] described that synthetic hepcidin presents antimicrobial activity against *Piscirickettsia salmonis*. On the other hand, Jiang et al. [68] showed that stimulation in hepcidin production prior to a challenge with pathogenic bacteria such as *A. hydrophila* and *V. alginolyticus*, increases the survival of challenged fish, from 46.7 to 76%. These authors propose a cooperative effect between the antibacterial and regulatory activities of iron, by hepcidin in the innate immune defense against bacterial infection. Given that the increase in hepcidin decreases the availability of iron in the serum, this is a major element required for bacterial growth. Among the isolates of *Seriola* evaluated, the isolates of the genera *Pseudomonas* and *Shewanella* emerged due to the robust induction in the expression of hepcidin, and therefore, they have advantages as candidates for probiotics for *S. lalandi*.

Several studies evaluating probiotic potential in fish have investigated the activity of serum lysozyme. Meidong et al. [69] reported a significant increase in serum lysozyme activity in *Pangasius bocourti* after administration of a *Bacillus* strain in the diet. Similar results have been published in different species such as *Catla catla* [70, 71], *Labeo rohita* [72, 73], *Cyprinus carpio* [74], *Oreochromis niloticus* [75], and *O. mykiss* [76]. The importance of the increase in serum lysozyme has to do with the bactericidal activity. Meidong et al. [69] reported this activity against *A. hydrophila* to be approximately 50% after 60 days of administration with a *Bacillus* strain. Notably, in this study, the activity of serum lysozyme was not evaluated, but the relative expression of this gene was evaluated, and the relative expression values were significant with respect to the control by the 19 isolates evaluated. In this sense, Caipang et al. [26] observed that the expression of lysozyme in the head kidney of *Gadus morhua* increased in the presence of a strain of *Psychrobacter* (GP11). Subsequently, Muñoz-Atienza et al. [57] described that the administration of a *Weissella* strain significantly regulates the expression of the lysozyme gene in the skin and intestine in addition to substantially decreasing mortality accumulated in *Scophthalmus maximus* larvae. In this study, the isolates that generated higher levels of lysozyme expression were those of the genus *Pseudomonas* and *Shewanella*, and those that generated lower levels belonged to *Psychrobacter* and *Acinetobacter*.

Our results show that the isolates evaluated, especially those of the genera *Pseudomonas* and *Shewanella*, could be beneficial for the host, helping in the response to *Vibrio* sp. infections. However, considering another judgment in this evaluation, such as resistance to antibiotics, it is observed that six of the eight isolates belonging to the genus *Pseudomonas* have resistance to florfenicol and/or sulfamethoxazole-trimethoprim. It has been described that *Pseudomonas* species harbor multiple intrinsic and acquired resistance genes [77, 78]. Intrinsic resistance is mainly conferred by mechanisms such as low permeability of the outer membrane, synthesis of β-lactamases and efflux systems. In addition, due to the plasticity of the genome, it is believed that members of *Pseudomonas* sp. can acquire almost all known mechanisms of resistance to antimicrobials [79]. Although this study was not evaluated at the level of genes associated with antimicrobial resistance, it has been described that resistance could be associated with mobile elements such as integrons, plasmids, and transposons, so that these would have the ability to transfer this resistance to other bacteria of the microbiota; therefore, isolates of this genus would be discarded as candidates for probiotics. In the case of sulfamethoxazole–trimethoprim resistance, Shin et al. [80] describe three genes associated with resistance to sulfonamides, *sul1*, *sul2*, and *sul3*. Of which, the *sul1* gene is linked to other resistance genes in class 1 integrons and in large conjugative plasmids. On the other hand, *sul2* is generally located in small plasmids, also in large transmissible multiresistant plasmids or through a common region of insertion elements (ISCR2) and *sul3*, a plasmid-borne sulfonamide resistance gene. For its part, resistance to trimethoprim is associated with the *dfr* gene, of which 30 different genes have been described, which are generally found in gene cassettes within integrons. In the case of resistance associated with florfenicol, three genes associated with the resistance of florfenicol, *cfr*, *fexA*, and *floR*, have been described, of which *floR* has been reported in several gram-negative bacteria [81]. The presence of these genes has been described in mobile elements such as the *floR* gene in plasmids [82] and transposons [81], similar to that described for the *fexA* and *cfr* genes [83].

According to the results obtained, 10 of the isolates of the genera *Shewanella*, *Psychrobacter*, and *Acinetobacter* are proposed as potential probiotics in cultures of *S. lalandi*; notably, the two isolates of *Shewanella* have higher levels of enzymatic activity of alkaline phosphatase, Leu-arylamidase, α-chymotrypsin, and *N*-acetyl-β-glucosaminidase. The

presence of alkaline phosphatase is desired in probiotic candidates because it could potentially inhibit an inflammatory response in the intestine and give an immunomodulation benefit to the host [84]. The review by Ray et al. [85] also describes the importance of the contribution to nutrition in fish of enzymes of the microbiota, including proteases, lipases, amylase, chitinase, and cellulase.

Conclusion

The present study reveals that 11 isolates of the genera Shewanella, Psychrobacter, and Acinetobacter could serve as probiotic candidates in the culture of Seriola lalandi. However, more studies should be done to investigate the adhesion properties of these potential probiotic bacteria using animal models and to corroborate these results through in vivo challenges.

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Author Contributions CR performed microbial isolation, DNA and RNA extraction, and data analysis and interpretation and wrote the original draft. RR performed the antimicrobial activity and antibiotic resistance studies and reviewed and edited. JR designed the work and reviewed and edited manuscript. All authors have made intellectual contributions to the work and approved it for publication.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

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